Ultraviolet Resonance Raman Study of Drug Binding in Dihydrofolate Reductase, Gyrase, and Catechol *O*-Methyltransferase

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ABSTRACT This paper presents a study of the use of ultraviolet resonance Raman (UVRR) spectroscopic methods as a means of elucidating aspects of drug-protein interactions. Some of the RR vibrational bands of the aromatic amino acids tyrosine and tryptophan are sensitive to the microenvironment, and the use of UV excitation radiation allows selective enhancement of the spectral features of the aromatic amino acids, enabling observation specifically of their change in microenvironment upon drug binding. The three drug-protein systems investigated in this study are dihydrofolate reductase with its inhibitor trimethoprim, gyrase with novobiocin, and catechol *O*-methyltransferase with dinitrocatechol. It is demonstrated that UVRR spectroscopy has adequate sensitivity to be a useful means of detecting drug-protein interactions in those systems for which the electronic absorption of the aromatic amino acids changes because of hydrogen bonding and/or possible dipole-dipole and dipole-polarizability interactions with the ligand.

INTRODUCTION

A key process in the development of new drugs is elucidation of the nature of the interaction between the drug molecule and the target protein. Such knowledge then makes it possible to make systematic structural modifications of the drug molecule to optimize the interaction. There are a variety of techniques currently available for obtaining information about drug-protein interactions, such as the measurement of kinetics and binding affinities. The two methods that have traditionally been employed to obtain structural information of proteins and drug-protein complexes are x-ray diffraction and NMR spectroscopy. Both of these methods have disadvantages: x-ray diffraction requires the preparation of a crystal, which can be time consuming or even impossible; and NMR spectroscopy is not easily applied to larger proteins of more than a few hundred amino acids.

Other analytical techniques that can be applied to proteins in solution are circular dichroism, ultraviolet absorption, and fluorescence spectroscopy, but these all have limitations. Circular dichroism is useful mainly in probing the global structure of proteins; however, far UVCD can yield information about protein secondary structure and near UVCD can probe the local environment of aromatic residues, especially tryptophan (Woody and Dunker, 1996). Although ultraviolet absorption spectroscopy is widely employed to study the phenolic hydrogen bonding (H-bonding) of tyrosine (Tyr, Y) (Cantor and Schimmel, 1980; Demchenko, 1983), this approach requires one to differentiate the effect of Tyr H-bonding from the contribution due to all of the other amino acids (Hildebrandt et al., 1988). With fluorescence spectroscopy, if any tryptophan (Trp, W) amino acids are present, they will tend to dominate the spectrum, the signals from other residues often being completely swamped. Furthermore, it is difficult to quantitatively relate changes in fluorescence yields to structural changes in proteins.

Resonance Raman (RR) spectroscopy with ultraviolet (UV) excitation radiation has been demonstrated to be a valuable new method for the study of biological molecules (Spiro, 1987; Asher, 1993a,b). Recent advances in UV lasers (Asher et al., 1993), especially the extension of laser sources into the deep UV region, have led to the establishment of UVRR spectroscopy as an active area of research. This is evidenced by recent papers in the literature, which document the use of UVRR in the study of the structures of hemoglobin (Copeland et al., 1985), cytochrome *c* (Copeland and Spiro, 1985), insulin (Rava and Spiro, 1985a), lactalbumin (Kronman et al., 1981), angiotension (Cho and Asher, 1996), enkephalins (Takeuchi et al., 1992), human serum albumin (including its ligand-binding modes) (Hashimoto et al., 1995), the extracellular domain of the human tumor necrosis factor receptor (Tuma et al., 1995), as well as a variety of DNA structures (Takeuchi and Sasamon, 1995). In this paper we explore the feasibility of using UVRR spectroscopic methods to help elucidate aspects of drug-protein interactions.

The advantages of UVRR as compared to normal Raman spectroscopy are much improved selectivity and sensitivity. Both advantages arise because of the large enhancement of the cross section of Raman scattering that occurs when the excitation laser frequency closely matches the electronic absorption frequency of a particular chromophore. The increased sensitivity of UVRR means that spectra can be obtained at lower sample concentrations, typically on the order of 1 mg ml^{-1}, which obviates the problem of protein aggregation, which can occur in solutions at higher concen-

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trations. The increased selectivity means that only the resonantly enhanced chromophores will be featured, yielding greatly simplified spectra of proteins. The extent and selectivity of the enhancement depend on the excitation wavelength used, and this can be exploited to good effect, because in the UV range it is the aromatic amino acids of a protein, namely Tyr, Trp, and phenylalanine (Phe, F), which are enhanced, and not the aliphatic amino acids. As the vast majority of amino acids in proteins are generally aliphatic, the efficacy of using UV excitation radiation is immediately apparent.

It is well known that some of the vibrational bands of Tyr and Trp are sensitive to the microenvironment. Indeed, detailed information about the microenvironment of these aromatic side chains can be obtained from, for example, variations in intensity of the vibrational modes, or changes in the intensity ratio of Fermi resonance doublets. A specific example is the Fermi resonance doublet of Tyr, which is sensitive to the extent of H-bonding of the phenolic hydroxyl (Chen et al., 1973; Siamwiza et al., 1975; Rava and Spiro, 1985b; Johnson et al., 1986). Now drug binding is characterized by local changes around the binding site where H-bonds are formed or broken, and where dipole-dipole and dipole-polarizability interactions possibly occur; and in some cases it is characterized by additional conformational changes of the protein. If the aromatic amino acids are located near the binding site, or if they are exposed to a different environment because of conformational changes in the protein upon binding, it is anticipated that this will be reflected in the UVRR spectrum. Hence these changes have the potential both to allow qualitative detection of drug binding, and to enable quantitative study of the nature of the interaction between the drug and its target.

The fact that changes in environment and extent of H-bonding can occur simultaneously and can both produce changes in the observed UVRR spectra is a complicating factor, because the two effects cannot be definitively decoupled without the knowledge of further structural information. It is generally difficult to perform the relevant calibration experiments required to isolate one effect from the other, because dissolving the aromatics in a range of solvents of varying polarity in itself creates a change in both the environment and the extent of H-bonding. Furthermore, it is impossible to reproduce exactly the environment of the aromatic in the protein by dissolving it in a bulk solution. And so, until the relationships between the different contributing factors have been elucidated, interpretation of observed spectral changes necessarily relies on additional structural information.

There has been one previous UVRR study in the area of drug-protein interaction, undertaken by Hashimoto et al. (1995). They have studied the interaction of human serum albumin with warfrin, ibuprofen, and palmitate, and have obtained evidence for a direct interaction between warfrin and the Trp at position 214 (Trp^{214}), and between ibuprofen and the phenolic hydroxyl of Tyr^{411}. Human serum albumin is readily available, and these experiments were performed using 2-ml samples at albumin concentrations of 120 mM. The sample was recirculated with a spinning quartz cell.

Three different drug-protein systems were chosen for the work reported here, all of which are of current pharmaceutical interest. For some of the systems there is additional structural information from other techniques (particularly from x-ray diffraction data) with which to compare the UVRR data. The drug-protein systems used in our study are dihydrofolate reductase (DHFR) with its inhibitor trimethoprim (TMP), gyrase with novobiocin, and catechol *O*-methyltransferase (COMT) with dinitrocatechol (DNC).

EXPERIMENTAL

Materials

All of the samples and drugs were generous gifts from F. Hoffman-La Roche and were used as supplied. Each of the three systems is described in turn.

DHFR/TMP

The DHFR used in this study was isolated from *Staphylococcus aureus*, and is an 18.1-kDa enzyme of 159 residues (including 2 Trp, 3 Tyr, and 11 Phe); its amino acid sequence is given in Table 1. The enzyme was overproduced in *Escherichia coli*; the recombinant protein was isolated and purified to homogeneity as described by Dale et al. (1994). In addition to the wild type, the mutant DHFR(F98Y), with point mutation F98Y, has also been isolated; this mutant shows resistance to TMP. The TMP was purchased from Sigma; its structure is depicted in Fig. 1 *a*. The DHFR and DHFR/TMP complex samples were made up to concentrations of ~1.3 mg ml^{-1} in phosphate buffer.

Gyrase/novobiocin

F. Hoffmann-La Roche have isolated a 25.9-kDa fragment of the B subunit of gyrase, which incorporates the binding pocket for the coumarin drug novobiocin. The gyrase was prepared by the method described by Lewis et al. (1994, and references therein). This 233-amino-acid fragment has a total of 2 Trp, 11 Tyr, and 5 Phe; the complete amino acid sequence is listed in Table 2. The x-ray crystal structure of the *S. aureus* protein fragment has been solved by F. Hoffmann-La Roche (unpublished results) and has been found to be comparable to the *E. coli* structure (Ali et al., 1993), especially with respect to the novobiocin binding pocket. The novobiocin was purchased from Fluka; its structure is shown in Fig. 1 *b*. UVRR spectra of

TABLE 1 Amino acid sequence of *S. aureus* dehydrofolate reductase

1	TLSILVAHDL	QRVIGFENQL	PWHLPNDLKH	VKKLSTGHTL	VMGRKTFESI
51	GKPLPNRRNV	VLTSDTSFNV	EGVDVIHSIE	DIYQLPGHVF	IFGGQTLFEE
101	MIDKVDDMYI	TVIEGKFRGD	TFFPPYTFED	WEVASSVEGK	LDEKNTIPHT
151	FLHLIRKKV				

The mutant has the point mutation F98Y.

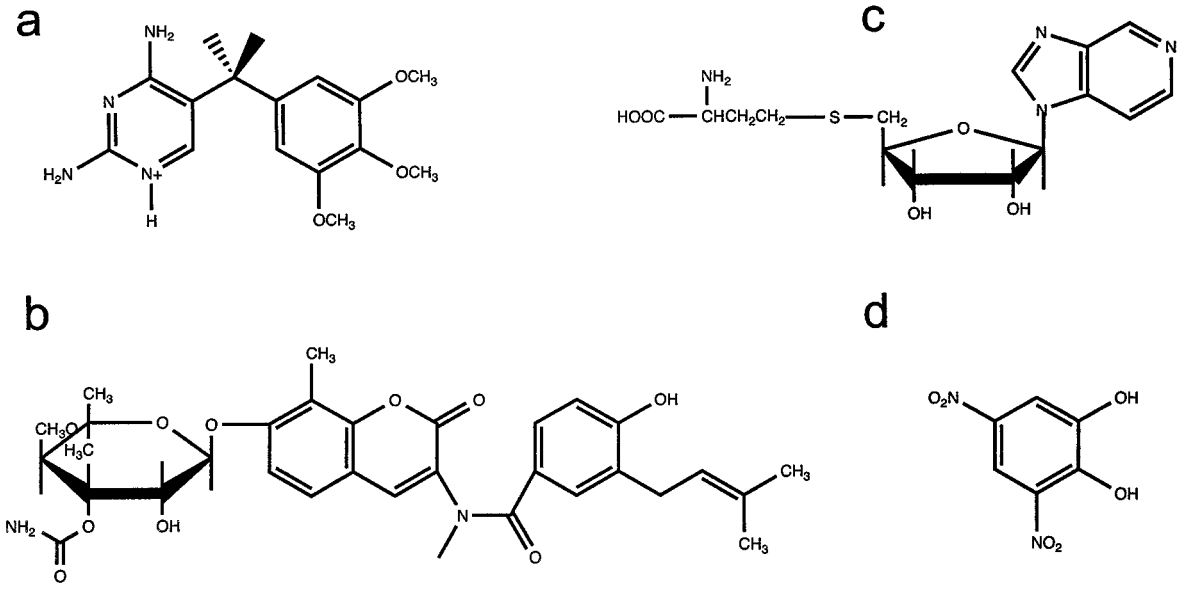

FIGURE 1 Chemical structures of (*a*) trimethoprim (TMP), (*b*) novobiocin, (*c*) dinitrocatechol (DNC), and (*d*) sinefungine.

gyrase were measured using samples of concentration ~1.0 mg ml^{-1} made up in Tris buffer at pH 8.5.

COMT/DNC

The rat liver COMT used in this work is a 24.7-kDa protein of 221 residues (including 2 Trp and 10 Tyr) (see Table 3 for the amino acid sequence). The preparation of COMT has been described by Vilbois et al. (1994). The DNC was synthesized by F. Hoffmann-La Roche, and the sinefungine was purchased from Sigma; their structures are shown in Fig. 1, *c* and *d*, respectively. UVRR spectra were recorded for COMT made up in Tris buffer (pH 7.5) at concentrations of ~2.7 mg ml^{-1}, and for COMT complexes with sinefungine and DNC at equivalent concentrations.

Method

The UVRR apparatus has previously been described in detail (Couling et al., 1997), and only the salient features are discussed here. Continuous-wave UV radiation of wavelength 244 nm is obtained from an intracavity frequency-doubled argon-ion laser (Spectra-Physics model 2020). Optics are UV optimized throughout, and collecting optics consist of a parabolic mirror and a Dilor model XY triple-grating spectrometer with a double foremonochromator. Detection is by means of a Dilor multichannel diode array. All samples were held at 25°C in a 100-μl glass cuvette with a sapphire window, and were continuously stirred to minimize photodegradation. The sapphire has only one sharp RR vibrational peak, which is near 750 cm^{-1} (Watson et al., 1981). The power of the beam at the cuvette was ~9 mW. Whenever a buffer was used, a background measurement of neat buffer was recorded to verify that its spectrum did not interfere with the spectra from the protein-ligand systems. Spectra were recorded with the spectrometer entrance slit set at 80 μm, which corresponds to a spectral resolution of 8.6 cm^{-1}. Adequate signal-to-noise ratios were obtained by accumulating 30 spectra of 30-s duration each, together with dark background subtraction. Data collection was performed with an IBM-compatible personal computer; the spectra were subsequently analyzed and manipulated using the GRAMS/386 scientific package of Galactic Industries Corporation.

The solutions in this study have not been spiked with an internal marker, because the salts commonly used for this purpose are not Raman enhanced, which means that they have to be used at high concentrations. Extensive experiments would have to be undertaken to see whether, and if so then to what extent, the internal marker affects a protein's structure. A consequence of the absence of an internal standard is that absolute changes in intensity of vibrational bands could not be quantitatively measured from spectra.

To obtain the various peak ratios quoted in the Results and Discussion that follow, the baseline and peak heights were measured using GRAMS. The major contribution to the uncertainty in the ratios arises from the estimate of the baseline position. The quoted uncertainty limits were obtained by calculating these ratios from the highest and lowest possible baselines, whereas the numerical values were calculated using the average of these extremes. When the spectra were remeasured, the ratios were generally found to be within these error margins. Where successive spectra were recorded to allow spectral subtractions (via GRAMS) to reveal small changes in vibrational features, care was taken to ensure reproducibility of experimental conditions. If, for example, the UVRR spectrum of a protein was to be subtracted from its drug-protein complex, care was taken to maintain equal solution concentrations.

RESULTS AND DISCUSSION

The UVRR spectra of the various proteins and drug-protein complexes investigated in this study will be systematically presented and discussed in this section. Each of the three

TABLE 2 Amino acid sequence of the 25.9-kDa fragment of the B subunit of *S. aureus* gyrase

1	VTALSDVNNT	DNYGAGQIQV	LEGLEAVRKR	PGMYIGSTSE	RGLHHLVWEI
51	VDNSIDEALA	GYANQIEVVI	EKDNWIKVTD	NGRGIPVDIQ	EKMGRPAVEV
101	ILTVLHAGGK	FGGGGYKVSG	GLHGVGSSVV	NALSQDLEVY	VHRNETIYHQ
151	AYKKGVPQFD	LKEVGTTDKT	GTVIRFKADG	EIPTETTVYN	YETLQQRIRE
201	LAFLNKGIQI	TLRDERDEEN	VREDSYHYEG	GIK	

TABLE 3 Amino acid sequence of rat liver catechol-*O*-methyltransferase

1	MGDTKEQRIL	RYVQQNAKPG	DPQSVLEAID	TYCTQKEWAM	NVGDAKGQIM
51	DAVIREYSPS	LVLELGAYCG	YSAVRMARLL	QPGARLLTME	MNPDYAAITQ
101	QMLNFAGLQD	KVTILNGASQ	DLIPQLKKKY	DVDTLDMVFL	DHWKDRYLPD
151	TLLLEKCGLL	RKGTVLLADN	VIVPGTPDFL	AYVRGSSSFE	CTHYSSYLEY
201	MKVVDGLEKA	IYQGPSSPDK	S		

systems will be examined separately, but it is perhaps best to begin with a general discussion of the interpretation of UVRR spectra.

As has already been mentioned, a number of Tyr and Trp vibrational bands show sensitivity to their environment. The well-known Tyr Fermi resonance doublet, which is seen at 830/850 cm^{-1}, has been ascribed to the interaction of the ν_1 ring-breathing mode with the overtone of the out-of-plane deformation mode ν_{16a} (Siamwiza et al., 1975; Rava and Spiro, 1985b). All work published to date indicates that the major factor affecting this Tyr doublet ratio is the extent of H-bonding of the phenolic hydroxyl (-OH) (Chen et al., 1973; Siamwiza et al., 1975; Rava and Spiro, 1985b; Johnson et al., 1986), which can serve as either a proton donor or an acceptor, or as both simultaneously (Baker and Hubbard, 1984). A certain correlation exists between the intensity ratio I_{850}/I_{830} of the doublet peaks and the nature of the H-bonding. In general, the ratio is small for a Tyr whose -OH acts as a hydrogen donor, and is conversely large for a Tyr whose -OH acts as a hydrogen acceptor (Harada and Takeuchi, 1986b). In a previous study we have determined I_{850}/I_{830} for Tyr by using the model compound *p*-cresol (the vibrational pattern of which is similar to that of Tyr) dissolved in a range of solvents of varying polarity and known H-bond enthalpies of formation (Couling et al., 1997). These UVRR spectra were obtained by using an excitation radiation of wavelength 244 nm. For those Tyr amino acids in a protein that are exposed to aqueous solution, hence acting as weak proton donors or acceptors, one expects $I_{850}/I_{830} > 1$, whereas for those Tyr that are buried in hydrophobic pockets, a ratio $I_{850}/I_{830} < 1$ is expected (Siamwiza et al., 1975). In proteins the tyrosyl residue is known to exist as a strong hydrogen donor to groups such as CO_2^- and NH_2, a medium-to-weak hydrogen donor and/or acceptor, or a strong hydrogen acceptor from groups like NH_3^+ and CO_2H (Harada and Takeuchi, 1986b).

Among the tryptophyl bands in the Raman spectra of proteins, the region around 1350 cm^{-1}, which comprises two or three peaks, has been found to be sensitive to its environment (Chen et al., 1973, 1974; Harada et al., 1986). The relationship between the intensity ratio of the Fermi resonance doublet peaks at ~1340 cm^{-1} and ~1360 cm^{-1}, I_{1360}/I_{1340}, has been studied for indole, which is a model compound for Trp, having the same chromophore. I_{1360}/I_{1340} for a Trp residue in a protein whose environment is similar to that of indole in solution was found to be less than 1 if exposed to aqueous solution, ~1 if in contact with aromatic amino acid residues, and greater than 1 if in contact with aliphatic side chains (Harada and Takeuchi, 1986). However, care must be taken in applying the relationship of the Fermi resonance doublet obtained from off-resonance Raman studies to resonance Raman spectra (Rava and Spiro, 1985a). We nevertheless expect that the general trend of the spectral change and its interpretation to hold equally in the resonance Raman case (Harada et al., 1986). A complete assignment of the Trp bands at ~1350 cm^{-1} and their interpretation are not possible at present for any but the simplest of systems. For example, the behavior of this doublet in UVRR spectra of horse cytochrome *c* (Copeland and Spiro, 1985) cannot be explained by the indole study. A quantitative analysis is further complicated by the possible overlap of the CH bending band that occurs around 1320–1340 cm^{-1}.

There is evidence from previous work that the UV Raman cross section for 244-nm excitation radiation increases for Tyr and Trp going from a hydrophilic to a hydrophobic environment (Hashimoto et al., 1995). This can be explained by a red shift in the electronic absorption as a result of dipole-dipole, dipole-polarization, and/or H-bonding interactions (Cho and Asher, 1996). By the use of an internal intensity standard, the enhancement in intensity has been estimated to be a factor of 2 to 4 at this wavelength (Hashimoto et al., 1995). Although it was not possible to spike our samples with an internal marker in this study, we nevertheless anticipate that the signals from these aromatic amino acids should dominate the UVRR spectra.

The results for the DHFR/TMP, gyrase/novobiocin, and COMT/DNC systems are now discussed in this order.

The dehydrofolate reductase/trimethoprim system

DHFR, which catalyzes the NADPH-dependent reduction of 7,8-dihydrofolate to 5,6,7,8-tetrahydrofolate, is an intracellular receptor for a number of drug molecules. Inhibition of this DHFR-catalyzed reaction attenuates the biosynthesis of certain nucleotides and amino acids. Some drugs display selective inhibition of DHFR from different species; for example, the folate analog TMP binds 10^5-fold less tightly to mammalian DHFR than it does to bacterial DHFR (Champness et al., 1986; Stryer, 1995), making it a potent antibacterial drug. A point mutation on bacterial DHFR, F98Y, has been shown to be responsible for determining the TMP resistance (Dale et al., 1997).

A large amount of kinetic and spectroscopic evidence from solution studies has pointed to the mechanistic importance of conformational changes of DHFR upon ligand

binding (Kraut et al., 1987). DHFR has three Tyr and two Trp residues that are close to the binding site, and which should prove to be good UVRR spectroscopic indicators of drug binding. Trp^{22} is highly conserved in DHFR proteins produced from various strains and is directly involved in the TMP binding. It is this Trp residue that forms, via the NH group, an H-bond with a water molecule in the binding pocket. The water molecule, in turn, forms H-bonds with Asp^{27}, which itself interacts with the 2 amino group and the NH bond of TMP. This structural feature is present in all known DHFR/TMP bindings (W. Huber, F. Hoffmann-La Roche, private communication).

Because TMP is not resonantly enhanced at our excitation wavelength of 244 nm, it was not possible in this study to use UVRR spectroscopy to detect structural changes in the drug upon binding.

The UVRR spectra of DHFR and the DHFR/TMP complex are shown together in Fig. 2, *a* and *b*, respectively. A number of vibrational bands can be identified (mode numbering follows that of Rava and Spiro, 1985b) in both spectra, among which is the strong peak at 1619 cm^{-1}, which is due to the ν_{8a} vibrations of Trp at 1622 cm^{-1} and Tyr at 1617 cm^{-1}. There is also a contribution from the amide I band at 1654 cm^{-1}. The Trp doublet peaks occur at 1354 cm^{-1} and 1340 cm^{-1}. Weak bands at 1208 cm^{-1} and 1180 cm^{-1} arise from the C_6H_5-C stretching of phenylalanine and the Tyr ν_{9a} mode, respectively. The peak occurring around 1000 cm^{-1} is the Phe ν_1 ring-breathing mode.

Because there are no resonantly enhanced spectral features of TMP at our experimental wavelength, only changes in the UVRR spectrum of the enzyme can be observed upon binding of the drug. In particular, the environment-sensitive ratio I_{1356}/I_{1340} of the Trp doublet at ~1350 cm^{-1} is seen to change from 1.02 ± 0.05 for DHFR (see Fig. 2 *a*) to 1.3 ± 0.1 for the DHFR:TMP (1:1) complex (see Fig. 2 *b*). A Trp residue is located next to the binding pocket, and it is expected that its environment will change significantly once the drug binds. It is not possible from our measurements of I_{1356}/I_{1340} to make quantitative deductions about the exact environment of the Trp residues, because the doublet ratio has thus far only been studied for simple proteins and model compounds such as indole (Harada et al., 1986). An important observation is that no changes in this doublet ratio were discernible in the UVRR spectra of mutant DHFR (F98Y) and DHFR(F98Y):TMP (1:1) complex. The RR spectra indicate that wild-type DHFR undergoes binding of TMP, leading to substantial environmental and/or structural changes, whereas the mutant, as expected, does not bind TMP. The Tyr doublet at 850/830 cm^{-1} could not be seen in any of these spectra.

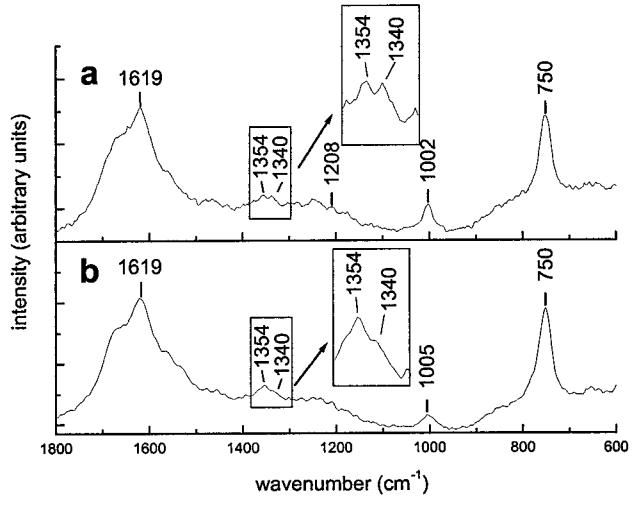

FIGURE 2 UV Raman spectra of the DHFR/TMP system with (*a*) free DHFR and (*b*) DHFR:TMP (1:1). For clarity, the tryptophan doublet regions are shown enlarged.

The gyrase/novobiocin system

The bacterial enzyme DNA gyrase is a type II topoisomerase that catalyzes the introduction of negative supercoils into closed circular DNA (Gellert et al., 1976a; Wang, 1985; Reece and Maxwell, 1991; Ali et al., 1993). The enzyme from *E. coli* consists of two proteins, A and B, of molecular masses 97 kDa and 90 kDa, respectively. The active enzyme is an A_2B_2 heterodimeric complex (Ali et al., 1993). A great deal of attention has been focused on the enzyme's structure, mechanism of action, and interaction with antibacterial agents. DNA gyrase is the target of two groups of antibacterial agents, the quinolines and the coumarins, both of which inhibit the DNA supercoiling reaction (Gellert et al., 1976b; Lewis et al., 1996). The coumarins, such as novobiocin, inhibit the ATPase reaction of gyrase and bind to the B subunit (Ali et al., 1993). F. Hoffman-La Roche have isolated a 25.9-kDa fragment of the *S. aureus* B subunit, which incorporates the binding pocket for novobiocin. This fragment contains 2 Trp, 11 Tyr, and 5 Phe, and its crystal structure (W. Huber, F. Hoffmann-La Roche, unbublished results) closely resembles that of the *E. coli* fragment (Lewis et al., 1996).

Novobiocin has a highly flexible molecular frame, and it is expected that the drug molecule is forced into a preferred conformation when it binds to the protein. This conformational change should be reflected in the vibrational spectrum of novobiocin. The drug molecule has extended ring conjugation, and is itself resonantly enhanced at 244 nm. The UVRR spectra of the enzyme and the novobiocin overlap, and so to distinguish between spectral changes due to the enzyme and those arising from the drug, the novobiocin spectrum had to be subtracted from spectra of the drug-target complex by the use of GRAMS. Similarly, the gyrase spectrum was subtracted when the effect on novobiocin upon binding was to be examined.

The spectrum of unbound gyrase can be seen in Fig. 3 *a*, and the spectrum of the gyrase:novobiocin (1:1) complex is shown in Fig. 3 *c*. The binding pocket of gyrase contains neither Tyr nor Trp residues, the nearest Tyr being ~10 Å

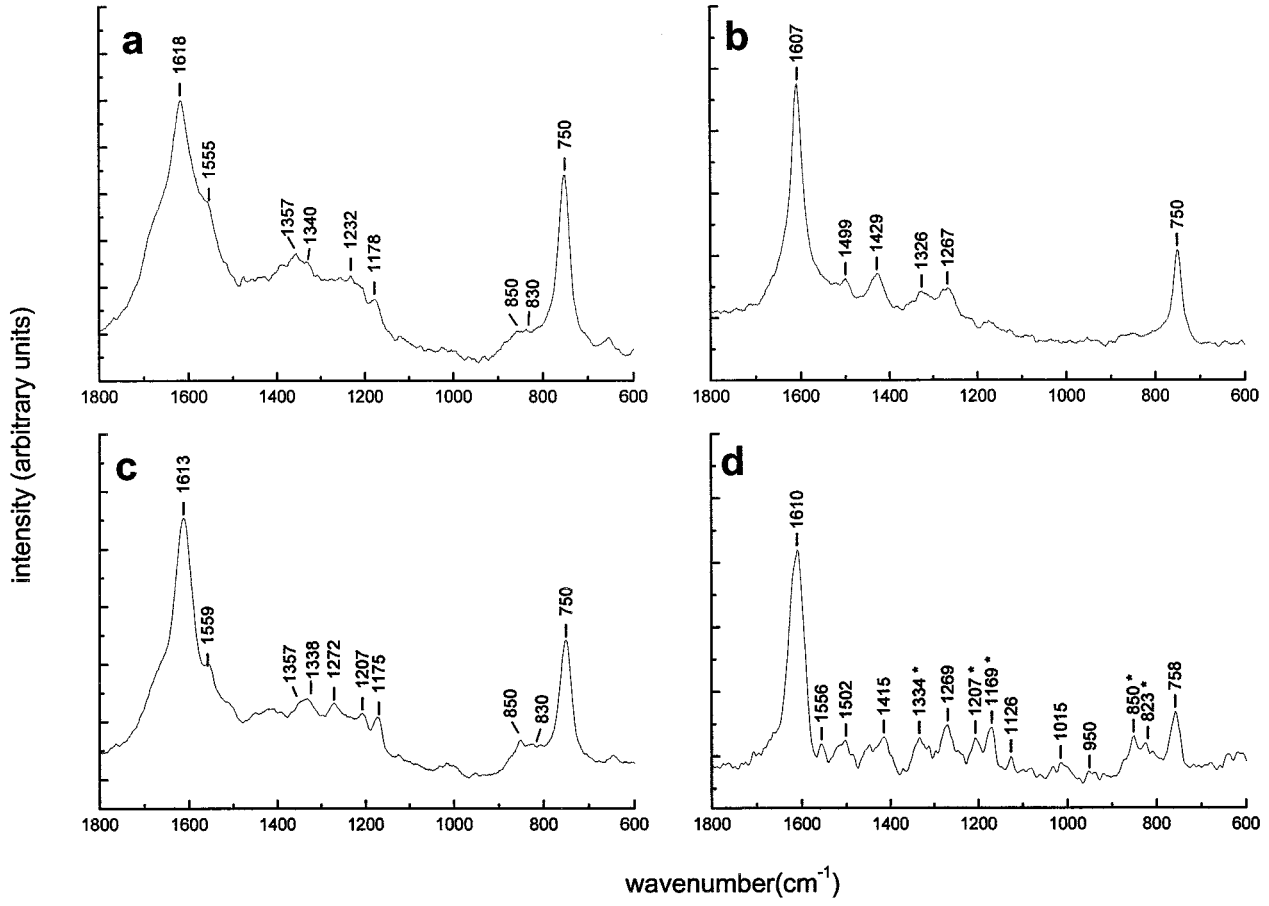

FIGURE 3 UV Raman spectra of the gyrase/novobiocin system with (*a*) free gyrase, (*b*) free novobiocin, (*c*) gyrase:novobiocin (1:1) complex, (*d*) gyrase:novobiocin (1:1) complex minus free gyrase.

away, and the nearest Trp ~20 Å away from the binding site (W. Huber, F. Hoffmann-La Roche, private communication). Nevertheless, both the Trp doublet at ~1350 cm^{-1} and the Tyr Fermi resonance doublet at ~840 cm^{-1} are observed to undergo changes in their intensity ratios when the drug binds. A frequency upshift of the Trp ν_3 band from 1555 cm^{-1} (see Fig. 3 *a*) to 1559 cm^{-1} (see Fig. 3 *c*) upon complexation corresponds to a change in the torsion angle $|\chi^{2,1}|$ for the C_2-C_3-C-C linkage from 115° to over 120° (Miura et al., 1989). The Tyr Fermi resonance doublet for free gyrase has an intensity ratio $I_{850}/I_{830} = 1.0 \pm 0.2$, whereas the gyrase:novobiocin (1:1) complex has an intensity ratio $I_{850}/I_{830} = 1.7 \pm 0.1$. The Trp doublet changes its ratio from $I_{1357}/I_{1340} = 1.36 \pm 0.08$ to $I_{1357}/I_{1338} = 0.94 \pm 0.02$ upon binding of the drug. A new band that appears at 1272 cm^{-1} is a novobiocin vibrational feature. The ν_{8b} vibration of Tyr occurs at 1618 cm^{-1} for the free enzyme, but undergoes a frequency downshift to 1613 cm^{-1} when the novobiocin binds. The relatively weak peak around ~1175 cm^{-1} in the spectra of gyrase and the gyrase:novobiocin (1:1) complex is the Tyr ν_{9a} band.

The frequency downshifts of the Tyr ν_{9a} vibration from 1178 cm^{-1} to 1175 cm^{-1} and the ν_{8b} mode from 1618 cm^{-1} to 1613 cm^{-1}, when novobiocin binds gyrase, together with the increase in the strength of the Tyr ν_{9a} mode, are consistent with the changes observed in the tyrosine and tryptophan doublet ratios. The change would correspond to an alteration of the tyrosine and tryptophan local environment from hydrophobic to hydrophilic. However, because these amino acids are not close to the binding pocket, the change in environment has to be ascribed to dipole-dipole and dipole-polarizability interactions between the novobiocin and the aromatic amino acids (Kamlet et al., 1981; Cho and Asher, 1996).

In addition to the substantial changes that occur in the UVRR spectrum of gyrase when novobiocin binds, the spectrum of the drug itself undergoes several changes. When the gyrase-subtracted UVRR spectrum of the bound drug, given in Fig. 3 *d*, is compared with the spectrum of free novobiocin at the same concentration (0.5 mg in 0.05 ml Tris buffer; Fig. 3 *b*), it is evident that the spectrum of the bound drug features both shifted bands and new bands. The coumarin-hydroxybenzoate part of novobiocin is structurally similar to warfarin (chemical structure not shown). A UVRR study of warfarin (Hashimoto et al., 1995) ascribed most of the observed bands at 1603, 1511, 1485, 1448, 1419, and 1333 cm^{-1} to the hydroxycoumarin part of the drug.

The spectrum of the gyrase:novobiocin (1:1) complex minus the spectrum of gyrase contains changes due to both the novobiocin and the gyrase. The spectral changes due to gyrase are marked (*) in Fig. 3 *d*. Similar spectral features were observed by Takeuchi and Sasamon (1995) when binding warfarin to human serum albumin. These features were assigned to the hydroxycoumarin part of the drug, although a detailed vibrational assignment has not been performed. An increase in the intensity of the bands at 1605 cm^{-1} and 1560 cm^{-1}, and a decrease in the intensity of those at 1484 cm^{-1} and 1419 cm^{-1} was observed in the UVRR spectra once warfarin bound to the albumin, and these features were attributed to the binding of warfarin to a hydrophobic region. In contrast, we observe substantial changes in the novobiocin spectrum that differ from the changes we observe for novobiocin in a nonaqueous solvent or as a function of pH, as seen in Fig. 4. The evidence strongly suggests that novobiocin undergoes substantial structural changes upon binding to gyrase.

Because the crystal structures of the Apo enzyme and that of the complex were found to be similar (W. Huber, F. Hoffmann-La Roche, private communication), we must conclude that the spectral changes in the Tyr and Trp observed upon ligand binding are associated with dipole-dipole and dipole-polarizability interactions (Kamlet et al., 1981; Cho and Asher, 1996) between the novobiocin chromophore and the aromatic amino acids.

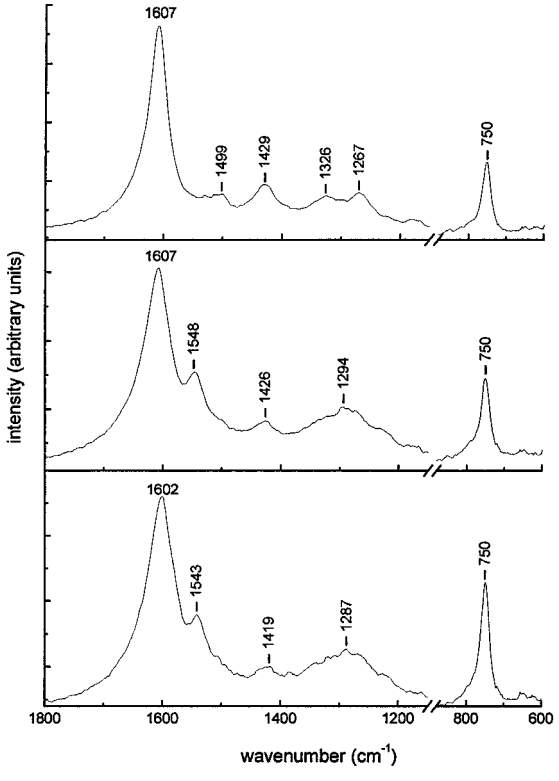

FIGURE 4 UV Raman spectra of novobiocin (*a*) at pH 7, (*b*) at pH 11, and (*c*) in methanol.

It should be noted that Lewis et al. (1996) do not state whether changes in the novobiocin structure upon binding were observed.

The catechol *O*-methyltransferase/ dinitrocatechol system

Catechol *O*-methyltransferase (COMT) is an enzyme distributed within the brain and its peripheral organs. It is important for the central nervous system, as it metabolizes catecholamine neurotransmitters such as dopamine. The enzyme catalyzes the transfer of the methyl group from *S*-adenosyl-L-methionine (AdoMet) to catechol substrates such as 3,5-dinitrocatechol (DNC) (Vidgren et al., 1994). This transfer occurs in a ternary complex of the protein with the coenzyme (AdoMet) and the catechol derivative. The formation of the ternary complex is dependent on the presence of Mg^{2+}, this ion being complexed by amino acids such as Asp and Asn in the binding pocket of the cofactor. The binding site for DNC is in the shallow groove on the surface of the protein. No protruding part of the protein encloses the inhibitor. Sinefungine differs only slightly from AdoMet, and acts as the substrate in this study.

The rat liver COMT used in this study has 221 amino acids and a molecular mass of 24.7 kDa. The crystal structures of both the COMT and the ternary complex have been solved (Vidgren et al., 1994), and it is known that the complex formation causes significant conformational changes on the protein. One of the protein's two Trp residues, namely Trp^{143}, is located near the binding site, and is involved in the conformational change. Trp^{143} has a favorable van der Waals interaction with the 3-nitro group of the inhibitor, and so it is anticipated that the binding event should be detectable in the vibrational spectrum of Trp. The other Trp, Trp^{38}, is located edge to face with the planar structure of DNC, allowing for an ideal hydrophobic contact (Vidgren et al., 1994).

Because the UVRR spectrum of sinefungine has peaks in the Tyr region of interest, spectral subtractions of sinefungine are required if changes in the UVRR spectra of COMT are to be discerned upon binding of this ligand. The DNC does not show any RR spectral features at our excitation wavelength.

The UVRR spectra for this system are displayed in Fig. 5 and are now discussed in detail.

To compare the various spectra and to quantify possible changes, we quote peak ratios with their respective uncertainties in the region of the Trp doublet. In the spectrum of pure COMT (see Fig. 5 *a*), the peaks in this doublet region occur at 1370 cm^{-1}, 1361 cm^{-1}, 1345 cm^{-1}, and 1334 cm^{-1}, the latter being the dominant peak. The peak intensity ratio, $I_{1361}/I_{1335} = 0.66 \pm 0.04$, was measured with GRAMS.

For the COMT:DNC (1:1) mixture, the 1327 cm^{-1} peak still dominates, with $I_{1366}/I_{1332} = 0.62 \pm 0.06$. In the signal-to-noise resolution no other spectral features occur,

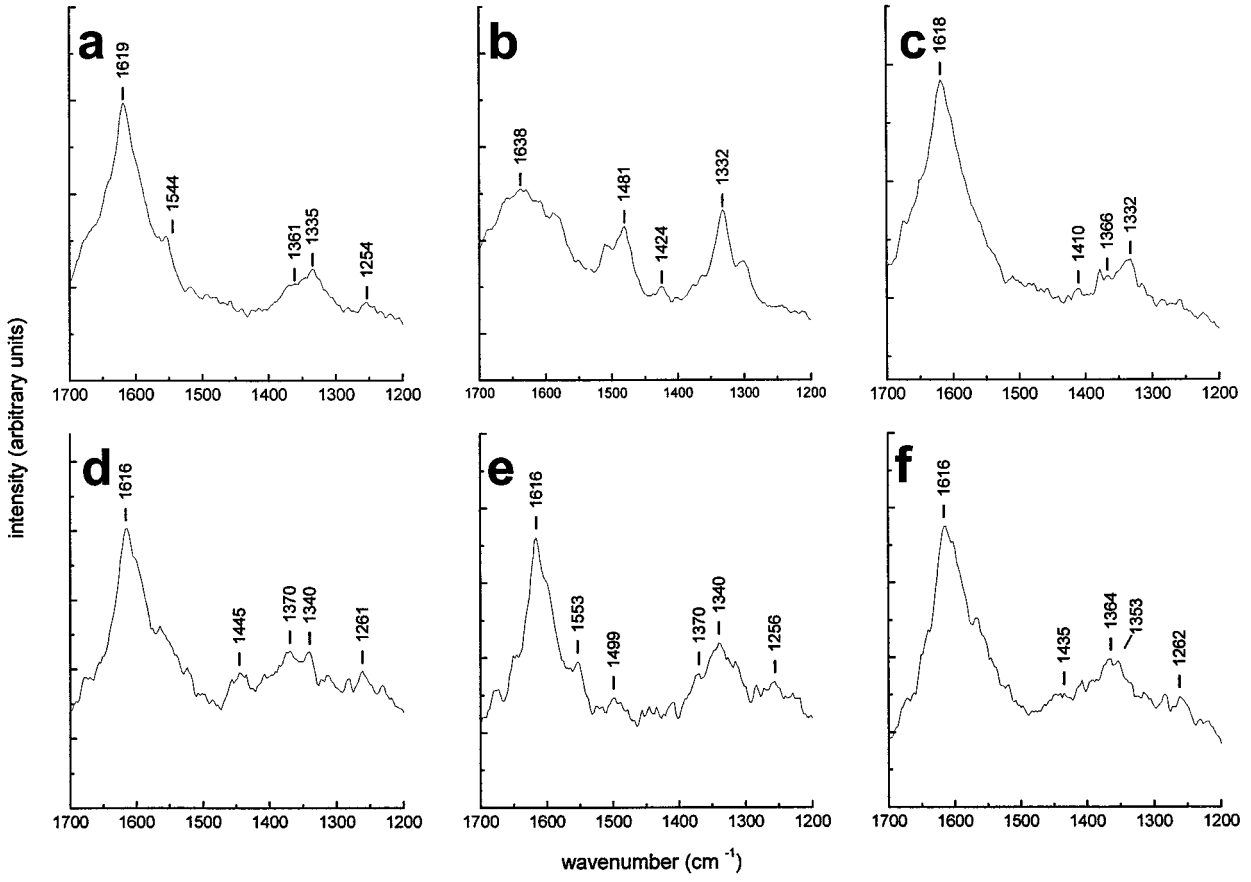

FIGURE 5 UV Raman spectra of the COMT system with (*a*) free COMT, (*b*) sinefungine, (*c*) COMT:DNC (1:1) complex, (*d*) COMT:sinefungine (1:1) minus sinefungine, (*e*) COMT:DNC:sinefungine minus sinefungine, and (*f*) COMT:sinefungine:DNC minus sinefugine.

indicating that no major structural changes have taken place. And indeed, the subtraction of the COMT spectrum from that of the COMT/DNC complex is featureless. However, the spectrum of the COMT:sinefungine (1:1) system indicates structural changes of COMT upon subtraction of the sinefungine spectrum. The 1370 cm^{-1} Trp peak becomes prominent, whereas the 1340 cm^{-1} peak is reduced in intensity, yielding $I_{1370}/I_{1340} = 0.93 \pm 0.05$.

In the COMT:DNC:sinefungine (1:1:1) system (added in this order), after subtraction of the sinefungine, the spectrum resembles that of pure COMT, which is reflected in the ratio $I_{1370}/I_{1340} = 0.62 \pm 0.11$. If one infers from this that the structure of COMT has not changed, then this is indicative of DNC's role as an inhibitor. Contrast this with the COMT:Sinefungine:DNC (1:1:1) system (added in this order), which after subtraction of the sinefungine, yields a spectrum bearing a close resemblance to the COMT:sinefungine complex, the ratio I_{1364}/I_{1353} being 0.99 ± 0.08. Because sinefungine has already bound, the DNC, which is a competitive inhibitor, can no longer inhibit sinefungine from binding. We would like to stress that the only difference between the COMT:DNC:sinefungine and the COMT:sinefungine:DNC spectra is the order in which the ligands were added. It follows that any spurious features that may arise because of peak alignment, subtraction, and ratioing are ruled out, and hence give us confidence in our analysis of the COMT system.

The crystal structure of the COMT/AdoMet complex has been determined (Vidgren et al., 1994), and Trp^{143} has been shown to have a favorable edge-to-face interaction with AdoMet. We expect Trp^{143} to display similar behavior when COMT binds sinefungine. From this crystal structure it is clear that the binding of the planar 3,5-dinitrocatechol takes place in a shallow groove on the outside of the protein, and involves an edge-to-face interaction with Trp^{38} that allows for a hydrophobic contact (Vidgren et al., 1994). Now the observed Trp doublet is the intensity-averaged signal from both Trp residues. The doublet ratio changes are more pronounced when COMT binds sinefungine than when it binds DNC. For free COMT, the ratio suggests that both Trp residues are in an aqueous environment. Once DNC has bound, a small change in the ratio is observed because of the edge-to-face interaction, although the environment is still largely aqueous. In contrast, the binding of singefungine results in a larger ratio change, which is probably due both to an intensity enhancement of the signal from Trp^{143}, so that it makes a relatively larger contribution to the observed signal of the two Trp residues, and to an interaction with singefungine, which results in a hydrophobic environment.

CONCLUSION

We have demonstrated that UVRR spectroscopy has adequate sensitivity to be a useful means of detecting drug binding. In this study we see that the technique indicates that DHFR binds TMP, whereas the mutant DHFR(F98Y) does not. It also indicates that the environment of one or more Trp residues change from being in contact with an aliphatic side chain to becoming in contact with an aromatic side chain. The intensity enhancement that occurs when going from a hydrophilic to a hydrophobic environment means that the observed change in the Trp Fermi resonance doublet ratio arises from the Trp located in the binding pocket, which comes into contact with the aromatic TMP upon binding. Even in the gyrase/novobiocin system, where no Tyr or Trp residues are located directly in the binding pocket, we have been able to detect changes in both the Trp and the Tyr Fermi resonance doublet ratios, which are clear indicators of binding. The observed spectral changes have been attributed to dipole-dipole and dipole-polarizability interactions. UV resonance enhancement of the novobiocin means that we have been able to show that the drug itself undergoes conformational changes upon binding. Finally, UVRR spectroscopy is able to correctly identify the role of DNC as an inhibitor in the COMT system, in which sinefungine, being similar to AdoMet, acts as the substrate. The changes were monitored using the doublet ratio of two Trp residues: one located in the shallow groove that binds DNC, and the other in the pocket where sinefungine binds. The changes indicate that the Trp residues are initially exposed to the aqueous solvent, and subsequently find themselves in a hydrophobic environment.

The environment inside a protein is unique, and it is not possible to undertake calibration experiments by simply placing an aromatic amino acid in different solvents to alter the extent of H-bonding or hydrophobicity. This problem makes definitive structural interpretation of the observed changes in the doublets difficult, and the analysis necessarily still relies on x-ray diffraction data. However, the UVRR technique has the strength that it can provide information about Tyr residues in the protein that would not be available from fluorescence spectroscopy data, where the fluorescence is dominated by tryptophans. It has the additional advantage over fluorescence spectroscopy methods, where the signal can be dominated by a particular Trp residue because of differences in quenching efficiency. UVRR spectroscopy would therefore be complementary to fluorescence techniques, and in fact both measurements could be performed in the same experiment. This is clearly a future direction that could be profitably explored. Further possibilities of future experiments include following doublet band ratios as a function of drug concentration to deduce binding constants. Whenever the drug is resonantly enhanced, such as for novobiocin, the spectral changes in the drug upon complexation can be used to investigate competitive binding. Our present study on COMT indicates that it would also be possible to observe agonist-antagonist interactions with the protein.

At present there is scope to improve our instrumentation, to make it possible to obtain spectra at even lower concentrations, or from smaller volumes, by improved throughput of the Raman spectrometer. This would also decrease the integration time. Further work is clearly required, but this study conclusively indicates that UVRR spectroscopy has potential as a new means of probing protein-drug interactions, and shows promise as an investigative technique in drug development.

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