Probing Enzyme-Substrate Interactions Using Fiber-Optic Ultraviolet Resonance Raman Spectroscopy

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1. Introduction

1.1 Raman Spectroscopy

Raman spectroscopy is an analytical methodology in which an intense source of monochromatic light interacts with molecules in a sample such that some of the light is shifted in frequency, a shift that can be correlated with the molecular structure of the molecules. It is a valuable tool for the study of biological systems because the measured spectra can provide a wealth of information about the structure and dynamic behavior of molecules. In addition, the sample preparation requirements are flexible, and spectra may be acquired easily from aqueous solutions due to the weak Raman scattering of water. However, several factors can hamper the utility of Raman spectroscopy. The Raman effect is inherently weak, limiting the sensitivity of the technique. Moreover, sample fluorescence can sometimes mask Raman signals. Finally, complex biological systems can produce spectra containing highly overlapping features, making data analysis and interpretation difficult.

1.2 UV Resonance Raman Spectroscopy (UVRRS)

Utilizing incident radiation in the ultraviolet (UV) region of the spectrum can help to alleviate some of the above limitations of Raman spectroscopy. The sensitivity of the technique is improved in the UV since Raman scattering intensity is proportional to the fourth power of the frequency of incident radiation. Thus, a marked increase in the UV Raman signal is observed when compared to near IR or visible excitation. Complex spectra can be simplified by further tuning the frequency of the incident radiation to coincide with an electronic transition of the chromophore of interest. In this case, only vibrations which are strongly coupled to the chromophore's electronic excited state are "resonantly enhanced." Resonance enhancement is typically three to five orders of magnitude and thus further increases the sensitivity of Raman spectroscopy. Finally, although fluorescence is not eliminated, it is usually Stokes-shifted more than the Raman signal and thus does not interfere with the latter [1].

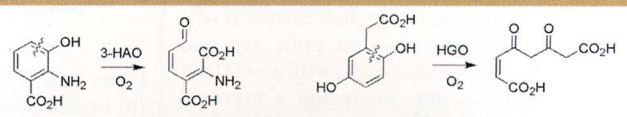
1.3 Fiber-Optic UV Resonance Raman Spectroscopy (FO-UVRRS)

Certain biomolecules, such as proteins, are susceptible to UV photodamage. This consideration has traditionally limited the use of UV light in RRS, as incident laser power densities must be kept low. To achieve higher laser powers (to maximize the Raman signal) while maintaining low laser power densities in the sample (to minimize photodamage), we designed a specialized fiber optic (FO) probe assembly [2]. For this probe, a central FO emits defocused UV light and six surrounding fibers collect the signal. This power density advantage of FO-UVRRS facilitates the investigation of the interactions between UV-absorbing compounds (substrates, inhibitors) and photolabile proteins.

1.4 Case Study: Ring-cleaving Dioxygenases

Dioxygenases are enzymes that catalyze the transformation of substrates with the incorporation of both atoms of dioxygen into the reaction product [3]. These reactions involve the activation of molecular oxygen, often in ways that are without precedent in organic chemistry. Consequently, there is considerable interest in elucidating how these enzymes work. Such enzymes are exemplified by the catechol dioxygenases, which catalyze the oxidative ring-opening of catechols. This reaction is critical to the aerobic degradation of aromatic compounds by soil bacteria. Related human enzymes are involved in different diseases: 3-hydroxyanthranilic acid dioxygenase (3-HAO) has a role in neurological disorders [4], and defects in homogentisate dioxygenase (HGO) cause alkaptonuria (Figure 1) [5].

Figure 1.



(a) (b)

Figure 1. (a) The Reaction Catalyzed by 3-HAO, a Potential Drug Target [4], and (b) HGO, the Enzyme Causing Alkaptonuria [5]

Figure 2.

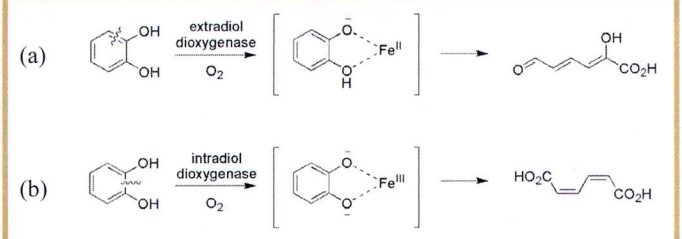


Figure 2. The Reactions Catalyzed by the Catechol Dioxygenases. (a) Extradiol Ring-opening is Proposed to Proceed Via a Monoanionic-bound Substrate. (b) Intradiol Ring-opening is Proposed to Proceed Via a Dianionic-bound Substrate

The catechol dioxygenases may be classified into two fundamentally different types of non-heme iron-dependent enzymes: intradiol dioxygenases utilize Fe(III) to catalyze ring-opening between the hydroxyls and extradiol dioxygenases utilize Fe(II) to catalyze ring-opening adjacent to the hydroxyls (Figure 2). This regiospecificity constitutes a longstanding puzzle for mechanistic enzymologists [6]. An important potential origin of this specificity lies in the iron coordination of monoanionic catechol in the extradiol dioxygenases (Figure 2a), contrasted with dianionic catechol in the intradiol enzymes (Figure 2b). Given its proposed significance in each mechanism, there is considerable interest in obtaining clear evidence for the substrate protonation state.

We set out to definitively assign the substrate protonation state in an extradiol enzyme: 2,3-dihydroxybiphenyl 1,2-dioxygenase (DHBD), an enzyme involved in biphenyl and PCB degradation and which cleaves 2,3-dihydroxybiphenyl (DHB), a substituted catechol. FO-UVRR and electronic absorption spectra of DHBD and the anaerobic DHBD:DHB complex were acquired. Difference spectra between the DHBD:DHB complex and free DHBD were calculated and employed to observe deuteration-dependent spectral shifts associated with bound DHB. This was complemented by comparison of the difference spectra to the spectra of free DHB substrate in different protonation states. [7].

2. Instrumentation and Sample Preparation

2.1 Fiber Optic Raman Spectroscopy

A schematic diagram of the fiber-optic Raman system is shown in Figure 3a. A frequency doubled Argon ion laser was used to produce 248 nm light with 20 mW power at the sample. Laser light was coupled into a 600 μm diameter fiber via a fiber-optic coupler. This "excitation fiber" along with 6 surrounding "collection fibers," each of 400 μm diameter, constitutes the complete fiber optic probe assembly (Figure 3b). The tip of this assembly was submerged in the sample solution. The tip of each collection fiber was polished to 45° with respect to the optic axis and each polished surface was coated with a ~600 nm layer of aluminum, thus producing a mirrored surface with which to deflect scattered light into the collection fiber. The Rayleigh light collected by the fibers was filtered using a dielectric stack interference filter. The Raman scattered light was dispersed with a 0.67 m monochromator and collected with a thermoelectrically cooled CCD camera. Data was manipulated using Grams software.

The fiber probe geometry shown in Figure 3b offers two main advantages over traditional UV Raman configurations and flush probe geometries [2]. As noted above, the light emerging from the excitation fiber is not focused. Hence, for a given laser power, the power densities are low compared with traditional, focused UV systems. This allows for an increase in laser power while maintaining power densities below the threshold for sample photodamage. Second, for absorbing samples, the cross section of collected Raman scattered light is higher than for flush probe geometries (Figure 3c).

2.2 Sample Preparation

Purified DHBD was exchanged into 20 mM Tris, pH 8.0, containing 100 mM Na_2SO_4 (in either H_2O or D_2O) by gel filtration chromatography under inert atmosphere. Enzyme-substrate complexes were prepared using a molar excess of DHBD. Samples were removed from the glovebox in appropriate airtight vials prior to spectral acquisition [7].

3. The Study of Enzyme-Substrate Binding

3.1 Electronic Absorption Spectroscopy (EAS)

Electronic absorption spectroscopy can provide useful information on the protonation state of the bound catechol. With the knowledge of the pK_a values of DHB, spectra of neutral, monoanionic, and dianionic DHB were obtained. Subtracting the spectrum of the free enzyme from the spectrum of the enzyme-substrate complex yielded the spectrum of DHBD-bound DHB. Comparison with spectra of DHB in different protonation states shows that this difference spectrum is most consistent with a monoanion (Figure 4).

3.2 FO-UVRRS

As in the case of the EAS experiments, FO-UVRRS spectra were acquired on each of the three ionization states of the free substrate (DHB), both in protonated and deuterated solvents. This established unique spectral markers for each state. The spectra of DHBD:DHB and free DHBD were acquired and the difference spectra (in H_2O and D_2O) were calculated. These latter are expected to contain spectral signatures of the bound substrate and were therefore compared with the spectra of free DHB. The assignment of the ionic state of the bound substrate was made in part on the basis of this comparison, as well as consideration of the D_2O-induced spectral shifts.

Figure 3.

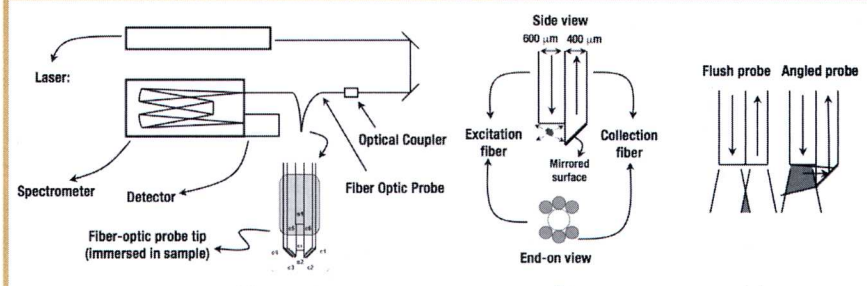


Figure 3. (a) The Fiber-Optic Raman System Used to Study Enzyme-Substrate Binding. (b) Detailed Side and End-on View of the Angled Fiber Geometry. Side View Shows Only One Collection Fiber for Clarity. Fiber Bundle is Dipped in Sample Solution. (c) The Angled Fiber Collection Advantage Over Flush Probe Geometries for Absorbing Samples. Grey Areas Indicate Areas of Collected Raman Signal.

3.3 FO-UVRRS of Free Substrate DHB

Figure 5 shows the UVRR spectra of free DHB in each of its ionization states, both in protonated and deuterated solvents. Although the UVRR spectra are expected to derive mainly from ring vibrations [8], these vibrations are coupled to motions of the hydroxyl groups and thus affected by the ionization state of the molecule.

Furthermore, in the case of neutral and monoanionic DHB, spectral shifts will be observed due to the isotopic substitution of the hydroxyl protons when performing the experiment in deuterated solvent. Hence, the absence of spectral shifts between protonated and deuterated solvents is used as an indicator of dianionic DHB.

The spectra of neutral, monoanionic or dianionic DHB are unique to each species. The major differences between spectra of neutral and monoanionic DHB in protonated solvent include: the shifting of the band at 1614 cm^{-1} (neutral) to 1602 cm^{-1} (monoanion); the disappearance of the 1511 cm^{-1} band; the appearance of bands at 1498 cm^{-1} and 1468 cm^{-1}; the decrease of the band at 1304 cm^{-1}; and the appearance of a Fermi doublet at 1169/1185 cm^{-1}. Major differences between monoanionic and dianionic species include: the replacement of the 1602 cm^{-1} band with a series of three resolved peaks between 1544 cm^{-1} and 1596 cm^{-1}; the disappearance of the 1468 cm^{-1} band; the shifting of the 1302 cm^{-1} band to 1316 cm^{-1}; the replacement of the Fermi doublet at 1169/1185 cm^{-1} with a band at 1164 cm^{-1}; and the increase in intensity of the 1070 cm^{-1} band.

As can be seen in Figure 5, the spectra of dianionic DHB in protonated and deuterated solvent are indistinguishable, as would be expected. Thus, D_2O-dependent band shifts indicate the presence of exchangeable hydroxyl protons.

Band assignments for neutral and monoanionic DHB were made [7] based on the similarity of the structure of DHB to that of biphenyl, a well characterized molecule. Assigning bands for dianionic DHB is more complicated, largely due to the paucity of reliable data for dianionic catecholic model compounds, and the fact that the electronic structure of dianionic DHB is sufficiently different that the same wavelength of excitation is in resonance with a different electronic state than the neutral and monoanionic DHB.

3.4 FO-UVRRS of DHB bound to DHBD

Figure 6 illustrates the spectra of enzyme (DHBD), enzyme-substrate complex (DHBD:DHB) (Figure 6a,b) and the difference spectrum (i.e. DHBD:DHB - DHBD) in protonated (Figure 6c) and deuterated (Figure 6d) solvent. Prior to subtraction, spectra were normalized to the 982 cm^{-1} peak of the sulfate internal standard. The difference spectra (Figure 6c,d) lack the 982 cm^{-1} band, indicating accurate spectral subtraction. The most prominent feature in Figure 6c (protonated form) is the 1603 cm^{-1} peak, which is also observed in the monoanionic free substrate (Figure 5). The characteristic set of three peaks between 1544 cm^{-1} and 1596 cm^{-1} observed in the dianionic spectrum of free DHB are not observed in the bound DHB spectrum (Figure 6c). Although the weaker bands in this difference spectrum cannot be conclusively assigned to either the monoanion or dianion, the 1603 cm^{-1} feature conclusively identifies the bound DHB as a monoanion.

Interestingly, D_2O did not affect the bound substrate in the same manner as it did the free substrate. The 1603 cm^{-1} band derives from a ring mode coupled to O-H bending [9]. The insensitivity of the

Figure 4.

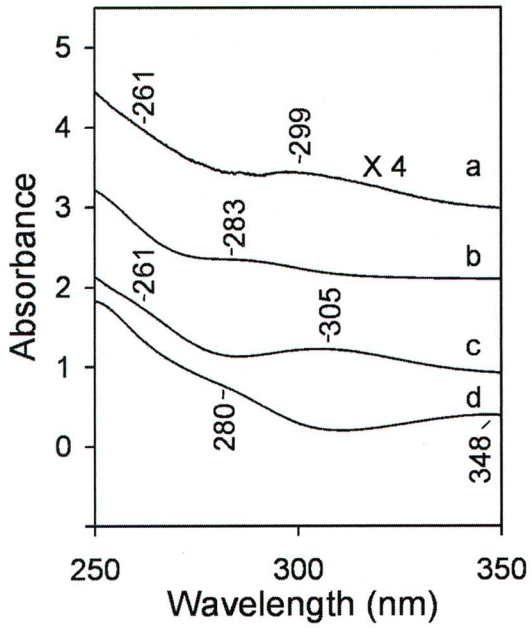


Figure 4. Electronic Absorption Spectra of (a) 25 μM DHB Bound to the Enzyme (Difference Spectrum); (b) 100 μM Neutral DHB in Tris pH 8.0; (c) 100 μM Monoanionic DHB in Potassium Phosphate Buffer pH 11; and (d) 100 μM Dianionic DHB in 250 mM Sodium tert-butoxide/tert-butyl Alcohol. Each Spectrum has been Vertically Offset for Clarity.

1603 cm^{-1} band to H/D substitution may be due to hydrogen bonding of the DHBD-bound DHB to amino acids in the enzyme, thereby inhibiting hydrogen atom motion and suppressing the involvement of the O-H bending in the 1603 cm^{-1} normal mode.

Figure 5.

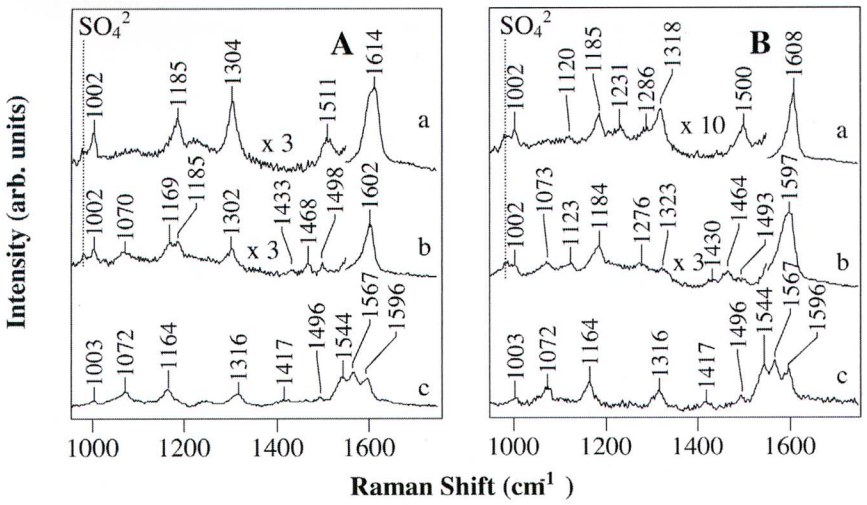


Figure 5. The Three Ionization States of Free DHB in H_2O (A) and D_2O (B). (a) 2 mM Neutral DHB; (b) 2 mM Monoanionic DHB; (c) 2 mM Dianionic DHB. All Solutions Also Contain 100 mM Na_2SO_4.

Figure 6.

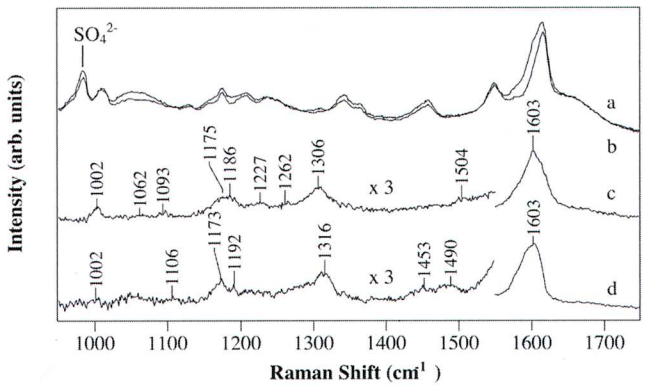


Figure 6. (a) FO-UVRRS Spectrum of Bound Enzyme (110 μm DHB Bound to 140 μM DHBD) (b) Spectrum of Unbound Enzyme (140 μM DHBD). (c) Difference Spectrum Showing Bound 110 μM DHB in Protonated Solvent. (d) Difference Spectrum Corresponding to 103 μM Bound DHB in Deuterated Solvent.

4. Conclusion

The specialized fiber optic probes developed in our lab have enabled the study of the ionization state of an enzyme-bound substrate. More particularly, FO-UVRRS coupled with EAS spectroscopy has provided conclusive evidence of the monoanionic binding of DHB to DHBD. We are currently investigating the ionization state of catechol bound to an intradiol dioxygenase. The case study summarized in this paper is an example of the general utility of FO-UVRRS as a useful tool to investigate interactions of molecular systems of biological and chemical interest.

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