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Kinetic Crystallography by Raman Microscopy

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Abstract

Raman spectra, obtained using a Raman microscope, offer an unique and incisive approach to follow interactions and reactions inside a single crystal under soak-in or soak-out conditions. The utility of this approach derives from the finding that the Raman spectra from single macromolecular crystals, under normal (non-resonance) conditions, are extremely stable, with a low “light background,” and provide ideal platforms for Raman difference spectroscopy. In turn, this allows the interrogation of sub-molecular changes in very large and complex macromolecular environments. There is often great synergy with X-ray crystallography, with the Raman spectroscopist providing crystallography colleagues with the best soak-in conditions to generate a targeted intermediate for flash freezing and X-ray analysis. On the other hand, X-ray structures at points along a reaction pathway provide invaluable benchmarks for interpreting the Raman data from populations seen by Raman to be changing in real-time. These principles will be illustrated by two reactions: The first involves a complex, branching reaction pathway underlying the inhibition of β -lactamases by clinically important pharmaceutical compounds, where different combinations of drug and enzyme function in different regions of the pathway. The second shows how temporal data can be derived for several events in the initiation step of RNA synthesis—more specifically, when one GTP molecule is joined to one ATP molecule to form a G•A dimer in the active site of a 115,000 Dalton crystalline RNA polymerase. Finally, we will summarize the extension of Raman microscopy to nucleic acid crystals and the information that has been obtained for RNA-based enzymes.

1. Introduction

In the past half-century, X-ray crystallography has revolutionized our understanding of the structures of the molecules of life. It has given an impressively detailed description *in crystallo* of many molecular assemblies of enormous complexity, such as the ribosome. In parallel, much effort has been expended using a variety of kinetic and spectroscopic methods to explore how these molecules interact and react together in solution. However, with a few exceptions, such as macromolecular NMR, the molecular details obtained in solution are lower in resolution compared to those from crystallographic studies. This has driven the quest to study reactions in single crystals that could yield high-resolution data on, for example, intermediates along a reaction pathway. In this way, some of the long-standing questions regarding the details of biochemical mechanisms might be resolved. Here, we advocate combining Raman spectroscopic and X-ray crystallographic studies on single crystals as an incisive route to achieve these goals. The approach is in its infancy but we

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hope to show sufficient signs of success, at least for favorable systems, that the reader might see potential applications in her or his own field of interest.

2. In the beginning

Soon after the birth of modern “bioRaman spectroscopy” in R. C. Lord’s laboratory in the 1960s [1,2], it was realized that Raman spectroscopy could be used to compare the conformations of proteins in solution with those in single crystals. Yu and coworkers compared the crystal and solution Raman spectra for proteins such as α -lactalbumin, insulin, and glucagon [3]. In these heroic studies, single protein crystals were mounted in a cuvette or glass vial and positioned “by eye” at the focal point of the fore-optics, that is directly at the front of a standard Raman instrument of the time. The work provided some of the earliest evidence that the main-chain structures of proteins in crystals are similar to those in solution and that crystallization has no detectable effect on the backbone conformation. Small differences observed between crystal and solution Raman data were interpreted in terms of changes in amino acid side-chain conformations. In a similar way, resonance Raman spectroscopy was used to study the porphyrin moieties in cytochrome P450 and myoglobin protein crystals [4,5]. The active site porphyrin structures were found to be identical for the crystal and solution phases. The 1980s and 1990s heralded new technologies permitting the development of Raman microscopy, where an optical microscope is coupled to a Raman spectrometer to collect Raman signals from sample regions as small as a few micrometers. This found applications in cell biology as well as in porphyrin protein crystal studies [6,7]. Most of the latter involved the peroxidase family, and since they were carried out under resonance Raman conditions, very low laser power had to be used in order to minimize photo-degradation induced by the laser beam [8]. Only a handful of non-resonance Raman microscopic studies of protein crystals were reported in the 1990s. For example, Thomas’s group found small differences in the Raman intensities of side-chains of subunits in the empty capsid of bean pod mottle virus in crystalline and solution states [9]. Finding a parallel to Yu’s earlier work, these differences were interpreted as the result of changes in a small number of side-chain environments between crystal and solution. In contrast, in a study of crystalline DNA-protein complexes, Peticolas and co-workers were able to detect major changes in the conformation of the oligonucleotide d(TCGCAATTCGCG) when it bound to the restriction endonuclease *EcoRI* [10]. Importantly, there were two technical advances in the 1990s: the introduction of red-sensitive charge-coupled photon detectors and holography-based notch filters that blocked unwanted elastically scattered photons. Together, these innovations greatly increased the sensitivity of Raman microscopes in the red region of the spectrum (600 to 800 nm). This was a critical breakthrough because the Raman microscope could now be operated using red laser excitation, which provided protein spectra free from the weak luminescence background that often accompanies blue/green laser excitation (near 500 nm).

3. Raman crystallography

We have coined the term Raman crystallography to describe the use of a Raman microscope to follow chemical changes within single crystals of macromolecules in a manner that is synergistic with X-ray crystallographic analysis [11,12]. In Raman crystallography, a crystal is usually placed in a hanging drop which then acts as the “laboratory” for carrying out soaking-in or soaking-out experiments (Figure 1). The crystal/drop is mounted atop a well of a commercially available crystallization tray, which rests on the stage of the microscope. The laser beam traverses the optical axis of the microscope and can be “seen” focused inside the crystal via a small CCD camera on the microscope and a computer monitor. This provides the essential element of control over the positioning of the focal volume. Back-scattered light from the crystal is collected by the microscope’s objective and makes its way

via fiber optics to a Raman spectrograph equipped with a CCD photon detector. This is shown in more detail in reference [11]. Raman spectra are collected for the “virgin” crystal and then a ligand or a substrate is injected into the hanging drop. Sequential spectra follow the soak-in and the subsequent chemical changes inside the crystal in real-time. These changes are observed by performing difference spectra [spectrum at time t after soak-in] minus [spectrum before soak-in]. In order to minimize spectral contributions from ligand that is non-specifically bound in, for example, solvent channels of the protein crystal, we use the lowest concentration that provides an acceptable signal-to-noise ratio. Much of the power of the approach derives from the finding that the crystal phase provides an ideal medium for Raman difference experiments:

- Very high signal-to-noise; the macromolecule is at its highest known concentration (typically 10s of millimolar). Non-resonance Raman conditions are adequate.
- Low extraneous light background; the contributions from luminescence, intrinsic or extrinsic (from impurities), are minimal in crystals.
- Very stable spectral profiles; they do not “drift”, which greatly facilitates accurate spectral subtractions.
- Fine control over the desired experiments inside the crystal by varying the soak-in and soak-out conditions.

4. Early examples with non-reacting systems

The first attempts at working in protein crystals involved “simple” soak-ins where no chemical reactions occurred. These have been reviewed elsewhere and here, to help the reader searching for applications, we will briefly give the kind of information that was obtained.

Crystal vs. solution conformation

The ability to compare properties in single crystals and in solution marks a powerful and almost unique advantage of the Raman approach, a fact that was realized in the pioneering studies of Nai-Teng Yu [3]. The intense pre-resonance Raman spectrum of the isalloxazine ring system of FAD in *para*-hydroxybenzoate hydroxylase enabled us to compare the properties of this protein-bound group in solution and single crystals [11,13]. They were found to be very similar. Of note, it has also been possible to make the comparison between protein-ligand interactions in solution and crystals for other systems under strictly non-resonance conditions [14,15].

Drug screening

In Dong et al. (2001), drug candidates were soaked into crystals of urokinase—a target for anti-cancer therapy [16]. Competitive binding studies showed that the tightest binding inhibitor could be identified in the Raman difference spectrum when 5 different compounds were soaked into the crystal at the same time. An added benefit was that some chemical detail on the interactions in the active site could be derived.

Co-operative and catastrophic conformational changes

The enzyme transcarboxylase (TC) is a 1.2 M Dalton machine involved in the metabolic transfer of CO₂. For the 360,000 Dalton hexameric subunit of TC, Zheng et al. (2002) showed that binding of substrate methylmalonyl-CoA in the crystal led to cooperative conformational changes in the protein structure [17]. During these studies, there was an additional unexpected finding: Single crystals from different protein sources, such as the 5S and 12S subunits of TC and insulin, can be made to undergo catastrophic conformational

changes by, for example, changing pH or reducing the proteins disulfide bonds [18]. The change involves the production of large amounts of “new” β -sheet structure and was irreversible. Interestingly, the crystals no longer diffracted X-rays although their morphology under a light microscope was unchanged.

5. Reaction between β -lactamases and clinical inhibitors

β -lactamase production is the most important mechanism by which Gram-negative bacteria develop resistance to β -lactam antibiotics, such as penicillins, cephalosporins, and carbapenems. These enzymes hydrolyze the β -lactam ring, which inactivates the antibacterial properties of the molecule (Figure 2, top) [19,20]. As a result, bacterial infections are often treated with two drugs: one such as a penicillin to inhibit bacterial cell wall synthesis and a second drug to inhibit the β -lactamases produced by the bacteria, thereby preventing the hydrolysis of penicillin. There are three β -lactamase inhibitors approved for clinical use: clavulanic acid, sulbactam, and tazobactam (Figure 2, bottom). Unlike β -lactam antibiotics, which are rapidly hydrolyzed by the β -lactamase, the acyl-enzyme formed by β -lactamase inhibitors chemically rearranges to form a transiently inhibited complex (Figure 3). Following acylation, the thiazolidinium ring of tazobactam or sulbactam or the oxazolidinium ring of clavulanic acid opens to give a reactive imine (species 1, Figure 3). The reactive imine can then participate in a number of transformations. The most likely process is hydrolytic deacylation of the imine acyl-enzyme, leading to free enzyme. The second most likely pathway is formation of a transiently inhibited enzyme in which the imine acyl-enzyme tautomerizes to yield a more stable *cis*- or *trans*-enamine (species 2 and 3, Figure 3). Finally, another form of inhibition arises from attack on the reactive imine by a second enzymatic nucleophile, S130, to form a cross-linked species in the active site that can degrade to an irreversibly-bound acrylate-like species (species 4, Figure 3).

In order to pursue structure-based inhibitor design, we sought to ‘trap’ the β -lactamase with an inhibitory intermediate. However, there was no consensus on which intermediate was responsible for inhibition. Moreover, early crystallographic snapshots of clavulanic acid and tazobactam bound to either a wild-type β -lactamase or an inhibitor resistant mutant featured a variety of reaction intermediates including the *trans*-enamine, the *cis*-enamine, the S130-bound acrylate, and an aldehyde (not shown in Figure 3) [21,22].

We used Raman crystallography to track reactions of the clinical inhibitors, clavulanic acid, sulbactam, and tazobactam, in single crystals in real time to provide guidance for X-ray crystallographic work. For the enzyme, we used SHV-1, a class A β -lactamase, or a variant. SHV-1 is most commonly found in *K. pneumoniae* and is responsible for up to 20% of the plasmid-mediated ampicillin resistance in this species. The spectroscopic analysis provided information about the chemical identity of the intermediates, their relative populations, and their rates of formation and disappearance. In addition, we used these parameters to explore the consequences of single amino acid substitutions, which often result in an inhibitor resistant phenotype, at the level of the acyl-enzymes. In a typical experiment, a single β -lactamase crystal is mounted in a four-microliter hanging drop of the appropriate mother liquor and several apo, or ‘enzyme-only,’ spectra are recorded (Figure 1). Then, one microliter of inhibitor is added to the hanging drop to give a final concentration of inhibitor between 5 and 20 millimolar. Following soak-in, spectra of the enzyme-inhibitor complex are recorded every two minutes for about one hour. For each time point, the subtraction [Raman spectrum of complex] minus [Raman spectrum of free enzyme] is carried out by a computer and yields the Raman spectrum of the bound ligand. Under these conditions, contributions from the free ligand is minimal and characteristic Raman bands, such as the C=O stretch of β -lactam carbonyl, are not observed.

In our initial studies, we used a deacylation-deficient β -lactamase, SHV E166A. The deacylation mechanism of class A β -lactamases relies on an invariant glutamic acid residue, E166, to activate a water molecule for nucleophilic attack on the acyl-enzyme. An aliphatic residue, such as alanine, cannot activate this water molecule and prevents regeneration of active enzyme by blocking hydrolysis of the acyl-enzyme. For the E166A mutant, the accumulation of reaction intermediates in the active site facilitates their detection by Raman difference spectroscopy and analysis by X-ray crystallography. Partial Raman difference spectra of tazobactam, sulbactam, and clavulanic acid bound to SHV E166A are shown in Figure 4. The Raman data showed that all three inhibitors formed a characteristic intermediate with a relatively intense stretching frequency near 1600 cm^{-1} . With support from spectral prediction software and isotope exchange experiments in D_2O , we tentatively assigned this band to the stretching motion of the *trans*-enamine's $\text{O}=\text{C}-\text{C}=\text{C}-\text{NH}$ moiety (species 3, Figure 3) [23]. We used the 1600 cm^{-1} feature to follow the kinetics of the *trans*-enamine buildup. The *trans*-enamine reached maximum populations at 10, 22, and 29 minutes for sulbactam, clavulanic acid, and tazobactam, respectively (Figure 5a). Interestingly, tazobactam formed more enamine intermediate as either sulbactam or clavulanic acid, which possibly correlates with its superior performance *in vivo*.

At the time of our study, the reported X-ray crystallographic snapshots of β -lactamase/ β -lactamase inhibitor complexes were obtained by using harsh soak-in techniques to 'trap' the inhibitor acyl-enzyme, such as high inhibitor concentrations, long soak-in times, and multiple soaks. As a result, none of these crystal structures featured the β -lactamase stoichiometrically bound to the *trans*-enamine—the intermediate that is thought to be responsible for inhibition of the enzyme. We communicated the time points for maximal *trans*-enamine populations to our X-ray crystallographic collaborators, which led to the first high-resolution crystal structures of tazobactam, sulbactam, and clavulanic acid stoichiometrically bound as *trans*-enamine intermediates in the E166A variant of SHV β -lactamase [24,25].

The synergy between spectroscopy and crystallography is further exemplified by comparing the band profiles of the enamine symmetric stretching frequency near 1600 cm^{-1} from the Raman difference data [25]. The bandwidths at half height are 18, 25, and 32 cm^{-1} for the intermediates formed from tazobactam, sulbactam, and clavulanic acid, respectively. Increasing bandwidth is interpreted in terms of increasing conformational heterogeneity—that is, although all of the inhibitors form a *trans*-enamine in the active site, there is conformational variability about this mean structure in either a static or a dynamic sense. These Raman measurements are reflected by the X-ray crystallographic results, which showed that, unlike tazobactam, the electron density for the intermediates formed from sulbactam and clavulanic were essentially invisible beyond the $-\text{NH}-$ moiety, i.e. the 'tail' of the enamine. Quantitatively, the X-ray data indicated an increase in thermal motion within the enamine at N4 as reflected by its B-factors for sulbactam and clavulanic acid. The correlation between these B-factors and the Raman data is shown in Figure 6. The observed trend of *trans*-enamine bandwidth as detected using Raman measurements of tazobactam < sulbactam < clavulanic acid was mirrored by both the B-factor at position N4 and atoms beyond this point. This showed that the level of agreement between Raman and X-ray crystallography reaches even beyond identifying the *trans*-enamine intermediate and that Raman can assess fine molecular details of bond rigidity and local disorder.

Raman crystallography can often provide detailed information about an enzyme-ligand complex when X-ray crystallography fails to produce a structure. For example, an intense effort by our collaborators to observe acyl-enzyme complexes between the clinical inhibitors and wild-type SHV-1 failed under similar 'soak-in' conditions to those used for the deacylation-deficient variant. This difference was attributed to the faster deacylation rate of

the wild-type enzyme and the flash freezing process, which is known to introduce perturbations to protein structure and perhaps further destabilize the acyl-enzyme of interest. On the contrary, Raman data at room temperature were obtained for the same complexes, which allowed us to examine the reactions of tazobactam, sulbactam, and clavulanic acid in single crystals of wild-type SHV-1 and to relate the identity of intermediates on the reaction pathway to the efficacy of the inhibitors [26]. These difference spectra are shown in Figure 7. Note that, unlike the deacylation-deficient mutant, E166A, only tazobactam formed a significant population of *trans*-enamine with wild-type SHV-1 (species 3, Figure 3). Again, the O=C-C=C-NH stretch of the *trans*-enamine, a chemically inert species, appears near 1600 cm^{-1} . In the difference spectra of clavulanic acid and sulbactam with SHV-1, we observed peaks corresponding to the C=NH⁺ stretch of the imine near 1660 cm^{-1} and the O=C-C=C-NH stretch of the *cis*-enamine near 1585 cm^{-1} , which are both hydrolytically labile (species 1 and 2, Figure 3). Thus, we were able to explain the superiority of tazobactam over sulbactam and clavulanic acid in *in vivo* and *in vitro* assays. Until this study, the majority of explanations for this observation relied on kinetic studies, which reported small differences among the ligands' apparent dissociation constants or number of turnovers before inactivation. Now, the Raman data added that tazobactam formed a predominant population of *trans*-enamine, an important inhibitory species on the reaction pathway, whereas clavulanate and sulbactam formed a mixture of *trans*-enamine and two labile species, the *cis*-enamine and imine.

As shown in Figure 3, the imine serves as a branch point for both a reversible and an irreversible pathway to β -lactamase inhibition. For reversible inhibition, the imine equilibrates with the *cis*- and *trans*-enamine. The latter species is responsible for transient inhibition of the enzyme and the duration of transient inhibition is determined, in part, by its stability. For irreversible inhibition, the imine species is captured by the hydroxyl group of S130, which leads to formation of a vinyl ether and permanent modification of this residue (species 4, Figure 3). The involvement of S130 in the irreversible inactivation of class A β -lactamases was initially based on molecular dynamics simulations and later demonstrated by mass spectrometry. Nonetheless, the contribution of this second, irreversible pathway to overall inhibition of the β -lactamase was unclear. In a preceding section, we used both Raman and X-ray crystallography to demonstrate that the *trans*-enamine intermediate was present within minutes in single crystals of SHV E166A and peaked in about 20 minutes. In order to elucidate the intermediates formed between the E166A variant and sulbactam on a longer time scale, we monitored the reaction in a single crystal for approximately 30 hours. After 5 hours, a new peak was identified at 1530 cm^{-1} , which increased in intensity until the termination of the experiment at 30 hours [27]. Based on quantum mechanical calculations and comparison of the difference spectrum to those of model compounds, the 1530 cm^{-1} feature was assigned to a complex vibration of the S130-bound acrylate (species 4, Figure 3). We performed the same experiments with another SHV mutant in which the proposed nucleophile, serine 130, was replaced by glycine [28]. The 1530 cm^{-1} band was not observed, which further confirmed our assignment of this feature to the S130-bound acrylate. More importantly, we showed that the acrylate species took hours to emerge in the E166A variant with sulbactam, as opposed to the *trans*-enamine that reached a maximum population within 30 minutes. In other words, the irreversible acrylate product is not a significant route to inhibition of SHV E166A.

When combined with X-ray crystallography, Raman microscopy is a powerful technique to investigate the effects of amino acid substitutions on catalysis and enzyme-ligand interactions. For example, many inhibitor-resistant β -lactamases differ from the parent enzymes by one amino acid substitution around the active site, such as M69V or S130G. In general, inhibitor-resistant β -lactamases have higher IC_{50} , K_i , and turnover numbers for β -lactamase inhibitors than those of the parent enzyme. We used both Raman and X-ray

crystallography to determine the molecular factors that lead to β -lactamase inhibitor resistance for the M69V variant of SHV by introducing this mutation to the deacylation deficient mutant, E166A [29]. Thus, by comparing the properties of the E166A and M69V/E166A forms, we could explore the consequences of the resistance mutation at the level of the acyl-enzyme forms for the β -lactamase inhibitors. Although one might expect the inhibitor-resistant mutant to form a greater population of the deacylation-prone imine, the Raman difference spectra for each of the inhibitors in the E166A and M69V/E166A variants were very similar. In particular, detailed analysis of the main enamine Raman vibration near 1595 cm^{-1} revealed that the structure and flexibility of the enamine fragments were essentially identical for each of the three inhibitors in E166A and in the M69V/E166A double mutant. X-ray structures of the *trans*-enamine intermediates formed by the three inhibitors in M69V/E166A confirmed this finding. However, a comparison of Raman results for M69V/E166A and E166A showed that the M69V mutation resulted in a 40% and 25% reductions in the enamine population when the β -lactamase crystals are soaked in 5 mM tazobactam and clavulanic acid, respectively (Figure 5b and c). No difference was observed in the *trans*-enamine populations of sulbactam between the two enzymes (Figure 5d). Analysis of the X-ray structures of the three intermediates in M69V/E166A demonstrated that, compared to the structures for the E166A form, the oxyanion hole becomes smaller, providing one explanation for why acylation is less efficient following the M69V substitution.

6. Reactions in an RNA polymerase—the next step in complexity

In the previous section, we saw how complex reaction pathways could be mapped in β -lactamases, which are small enzymes around 30,000 Daltons. We now turn to a much larger, multi-component system involved in the synthesis of RNA, the N4 mini-vRNAP transcription complex. It consists of a 115,000 Dalton protein enzyme and a 36 nucleotide hairpin DNA, which carries the code for the synthesis of a two nucleotide dimer at the start of a RNA chain [30]. Starting with crystals of the protein/DNA complex, called the binary complex (BC) [31], the group of Katsuhiko Murakami soaked in two GTPs (or one GTP and one ATP, depending on the complementary code of the DNA template) and Mg^{2+} or Mn^{2+} . This led to the X-ray crystallographic identification of two trapped substrate complexes, which are candidates for intermediates on the reaction pathway shown in Figure 8: substrate complex I (SCI), with two NTPs and one M^{2+} in the active site, and substrate complex II (SCII), with two NTPs and two M^{2+} s in the active site. Finally, they were able to show that they could run the complete reaction of transcription initiation in the crystal and detect electron density due to a G•G dimer and pyrophosphate products near the active site (manuscript in press).

Using these structures, the Murakami group was able to detect changes in protein and DNA conformation and the formation and dissolution of metal sites during and after formation of the covalent bond that linked the two GTPs. While these structures represent a *tour-de-force*, the temporal orchestration of these events could not be defined and remained controversial.

At this point, we considered undertaking Raman crystallographic studies on the reaction. Although these dealt with a system that is far more complex than those previously studied, four factors led to a successful conclusion:

- The system had been very well characterized by the Murakami group. In particular, the detailed structures of the four states (Figure 8), BC, SCI, SCII and the product complex (PC), provided unambiguous benchmarks that assisted in the interpretation of the Raman data.

- The reactions in the crystal were much slower (minutes) than the corresponding reactions in solution (sub-second). These allowed kinetic data to be amassed *in crystallo* via the signatures of Raman marker bands.
- In systems of great complexity, molecular information can be extracted when accurate Raman difference spectra can be obtained. Thus, here, accurate difference data could be obtained for SCI minus BC, SCII minus BC, and PC minus BC (Figure 8). The three sets of difference spectra provided detailed information on the relatively small regions of the system that changed between, for example, SCI and BC. Moreover, since each set of difference data changes as a function of time, the results provide kinetic data on the parts of the system that were changing during the soak in experiments producing SCI, SCII and PC.
- We used GTP and ATP substrates that were completely labeled with ^{13}C and ^{15}N [32]. For clarity, these are denoted G*TP and A*TP. This allowed the unambiguous assignment of the isotopically shifted substrate GTP and ATP modes during the reactions, uncomplicated by the corresponding modes of A and G residues on the DNA template.

Example: Assigning bands in the SCI minus BC spectrum and using them to generate kinetic data

The basis of these experiments was to record the spectrum from a single crystal of the BC complex, soak in G*TP and A*TP and Mg^{2+} under the conditions defined by the Murakami group, and record the spectra of the latter at 5 minute intervals. Then, SCI (at time t) minus BC gives the time dependency of the changes seen upon going from BC to SCI.

Figure 9 shows the SCI minus BC spectra at 51 minutes after soak-in commences. The assignments are based on the Raman spectra of ATP, GTP, A*TP, G*TP [32], studies on the interactions of metal cations with the oxygen atoms of non-bridging $-\text{PO}_2^-$ groups [33,34] and the extensive Raman literature on proteins and nucleic acids [35–37].

Based on the structural differences between the BC and SCI X-ray crystal structures and known Raman spectra, we assigned signals of nucleotide substrates (A*TP base: 703 cm^{-1} , A*TP; G*TP bases: 1519 cm^{-1} , nucleotide triphosphate group: 1123 cm^{-1}) and α -helix (945 cm^{-1} ; assigned to the so-called Fingers O-helix near the active site). The negative signal near 1400 cm^{-1} is due to the presence of the symmetric stretch from “ligand free” carboxylate groups ($-\text{COO}^-$), in particular aspartate side chains in BC that disappear in the SCI due to chelating Mg^{2+} . The “disappearance” is caused by the newly formed $-\text{COO}^- \cdots \text{Mg}^{2+}$ interaction, which shifts the $-\text{COO}^-$ symmetric stretch away from the 1400 cm^{-1} region. We used this signal as a marker for monitoring the interaction between the active site aspartate side chains D559/D951 and Mg^{2+} in the active site.

The kinetic data are shown in Figure 10. Upon addition of G*TP, A*TP and Mg^{2+} to the BC, the α -helix (Fingers O-helix) signal immediately appeared and remained constant from 5 to 180 min, indicating that the O-helix conformational change was completed at an early stage of the transition from BC to SCI (Figure 10a). Strong positive intensities of A*TP and G*TP bases and their triphosphate signals were also observed at the early stage (Figure 10b). However, their signals simultaneously decayed between 4- and 5-fold during the time course, reached a minimum value at 25 min and remained constant after that. The slowest change observed within the complex was the establishment of D559/D951 and Mg^{2+} interactions (Figure 10a). The negative signal near 1400 cm^{-1} increased steadily and reached a maximum between 30 and 40 min. after the soaking. The time-dependent Raman spectra measurements break down the motions observed by the X-ray crystallography snapshots: a 4- to 5-fold excess of the nucleotide substrates approach the active site of

RNAP immediately triggering the closing of Fingers O-helix with the completion of the Mg^{2+} ligation by D559/D951 occurring on a longer timescale, which is associated with the expulsion of non-specifically bound NTPs from the active site and SCI formation. These studies have been extended to SCII and PC and provide unique insights into the temporal development of these species. As was the case for some of the experiments involving β -lactamase reactions the Raman data are suggesting conditions for soaks that can reveal target species as yet uncharacterized by X-ray crystallography.

7. RNA crystals and other studies

RNA-based enzymes, ribozymes, provide very interesting comparisons and contrasts to protein enzymes making them a natural extension for Raman crystallographic analysis. Our work on ribozyme crystals has been reviewed recently and will not be repeated here [38]. However, Table 1, from Gong et al. (2009) summarizes the chemical information obtained using the HDV ribozyme as a paradigm. Studies providing unique data on the pK_a s of active site bases have continued for the hairpin [39] and GlmS ribozymes (unpublished work Gong, Carey and Ferre D'Amare). The information gained on peak assignments in general and the effects of metal binding to $-PO_2^-$ groups have proven valuable in the interpretation of the difference spectra from RNA polymerase experiments [33,34].

While we have focused on our own work, other groups are beginning to report *in crystallo* Raman studies on other systems [40–44]. These are summarized in Table 2.

8. Conclusion

Raman microscopy, usually combined with Raman difference spectroscopy, provides unique and important molecular information on chemical changes occurring in macromolecular crystals. Presently, for “slow” reactions occurring within a single crystal, it has been possible to identify and quantify key intermediates on complex reaction pathways. In turn, this information has been used to inform crystallographers of the conditions that are needed to generate intermediates for low-temperature trapping and subsequent high-resolution structural analysis. For expediency, the initial studies have used commercially available Raman microscopes. However, custom built “directly coupled” microscopes should be able to reduce data acquisition times by at least an order of magnitude to access a time domain of seconds rather than tens to hundreds of seconds. As the work on RNA polymerase has shown, isotope substitutions permit information to be obtained from single groups within a protein-DNA complex exceeding 120,00 Daltons in size.

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Abbreviations

BC	binary complex
CCD	charge-coupled device
E166A	a deacylation-deficient variant of SHV-1 β -lactamase
HDV	hepatitis D virus
M69V	an inhibitor resistant variant of SHV-1 β -lactamase
NTP	nucleoside triphosphate

N*TP	nucleoside triphosphate in which the nucleic acid is completely labeled with ^{13}C and ^{15}N
PC	product complex
RNAP	RNA polymerase
SCI	substrate complex I
SCII	substrate complex II
SHV	class A β -lactamase of <i>K. pneumoniae</i> initially thought to be a “sulfhydryl variant” of the TEM enzyme
TC	transcarboxylase
WT	wild-type

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Figure 1.

Figure 2.

Figure 3.

Figure 4.

Figure 5.

Figure 6.

Figure 7.

Figure 8.

Figure 9.

Figure 10.

Table 1

Summary of chemical information obtainable by Raman crystallography of RNA.

Chemical Information	Example
Proton-binding: identification of RNA base and determination of pK_a	pK_a of C75 in HDV ribozyme crystal near 6.5
Metal-binding interactions: identification of metal	Bound innersphere Mg^{2+} itself has peak at $\sim 322\text{ cm}^{-1}$.
Metal-binding: quantification of number of metals and affinity	Agreement between metals from Raman and from crystal structure.
Metal-binding interactions: identification of RNA ligands	Innersphere Mg^{2+} - PO_2^- interaction has a differential near 1100 cm^{-1} . Innersphere Mg^{2+} N7 of guanine interaction has a differential near 1490 cm^{-1} .
Isotope effects on metal binding	Determination of $H_2^{18}O$ and $2H_2O$ effects on bound innersphere Mg^{2+} ion.
RNA conformational changes	ribose, phosphate backbone
Compare crystal behavior to solution	Similar pK_a , metal binding found for HDV ribozyme.
Chemical reaction in crystal; identify reaction intermediates and determine optical conditions for trapping target species for X-ray analysis	Not demonstrated for RNA but shown for proteins

Table 2

Other Raman microscopy studies.

Reference	Summary
Adam et al. (2009)	Combined X-ray diffraction and <i>in crystallo</i> UV-vis absorption, fluorescence, and Raman data revealed that radical formation in the fluorescent protein IrisFP involved a reversible distortion of the chromophore (4-(<i>p</i> -hydroxybenzylidene)-5-imidazolinone), suggesting a transient loss of π conjugation. A molecular mechanism for transient ("blinking") and permanent ("bleaching") loss of fluorescence in fluorescent proteins was proposed.
Katona et al. (2009)	X-ray diffraction combined with <i>in crystallo</i> Raman spectroscopy revealed a 'trapped' iron(III)-(hydro)peroxo species with the (hydro)peroxo group bound 'end-on' in crystals of superoxide reductase, a non-heme mononuclear iron enzyme that scavenges superoxide radicals.
Violot et al. (2009)	Using the red fluorescent protein Keima, which exhibits a large Stokes shift, <i>in crystallo</i> , Raman spectroscopy was used to monitor the <i>cis/trans</i> isomerization of a protein-bound chromophore as a function of pH.
McGheehan et al. (2006)	X-ray radiation-induced damage in DNA was monitored by online Raman spectroscopy. The decrease in intensity of the C-Br non-symmetric bending vibration of 8-bromo-2'-deoxyguanosine positively correlated with the loss of bromide definition in the electron-density maps.
Pascal et al. (2005)	A spectroscopic analysis of LHCII, the major plant light-harvesting protein, compared the molecular configuration of protein-bound neoxanthin and chlorophyll <i>b</i> in crystals and solution by resonance Raman spectroscopy. Major differences in the pigment configuration and pigment-protein interactions were found between the crystal and solution states.