

Analytical Methods

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COMMUNICATION

Detection of Interaction between Protein Tryptophan Residues and Small or Macromolecular Ligands by Synchrotron Radiation Magnetic Circular Dichroism

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Presented herein is a novel method for the detection of tryptophan-ligand interactions utilising magnetic circular dichroism. Two examples are presented, one a small molecule and another, a large polysaccharide, heparan sulfate. These data are also the first reported example of a Trp:glycosaminoglycan interaction shown by magnetic circular dichroism.

Studying protein-ligand interactions is of interest in many areas of biological research as improved understanding of intercellular communication, disease states and drug design are sought. One challenge is to establish an interaction in solution and identify the location of the ligand on the protein surface. This is particularly challenging when the binding partner is another biological macromolecule, for example, a polysaccharide, whose complexes with proteins cannot be crystallised readily and are often too immobile for solution NMR studies. Examples of such complexes include proteins, such as lysozyme and the fibroblast growth factor (FGF) family, which both interact with mammalian cell-surface polysaccharides, although ligand-protein complexes can present other challenges, such as glycosylation, which may occur in several forms, as well as hydrophobicity. Substantial effort may be expended in attempting to discover the location of ligands binding their target proteins in solution but, in some cases, this may not even be possible with the techniques currently available. In the absence of crystallography studies, the few available techniques include fluorescence and circular dichroism (CD) spectroscopies.

Historically, CD has been employed to study two main aspects; the first is protein secondary structure using far UVCD (180-250 nm)¹ and the second is to study interactions with surface aromatic amino acids, Phe, Tyr and Trp, in the near UV CD region (250-350 nm)². Synchrotron radiation circular dichroism (SRCD), which provides high intensity UV radiation, is CD conducted on a synchrotron beam line, which provides high photon flux, lower wavelength range (which

assists secondary structure identification and quantification) and good reproducibility³.

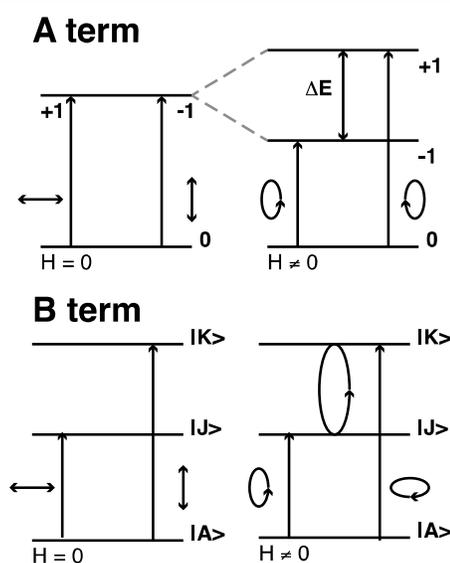


Figure 1. Schematic representation of the transitions involved in type-A and type-B components in MCD with a sample in a magnetic field (H), which is parallel to the direction of light propagation. **(Top)** Type-A components arise from Zeeman splitting of an excited state, leading to differentially absorbed left and right circularly polarised light, resulting in signals of equal intensity and opposite sign. **(Bottom)** Type-B components arise from states that are not necessarily degenerate but, whose mixing is facilitated by application of a magnetic field. Type-B components are most commonly observable in biological molecules, which generally exhibit low symmetry.

Recent applications of SRCD to study protein conformation in more challenging environments than the simple solutions usually examined, include the study of proteins in oil immersions⁴ and in dispersions of amphiphilic PEG derivatives⁵. There remain other instances, in which following interactions with proteins in solution remains a challenge.

These include the binding of proteins to small ligands and also to other large macromolecules, including polysaccharides. Here, the use of magnetic circular dichroism (MCD), in its synchrotron format, synchrotron radiation magnetic circular dichroism (SRMCD) is explored.

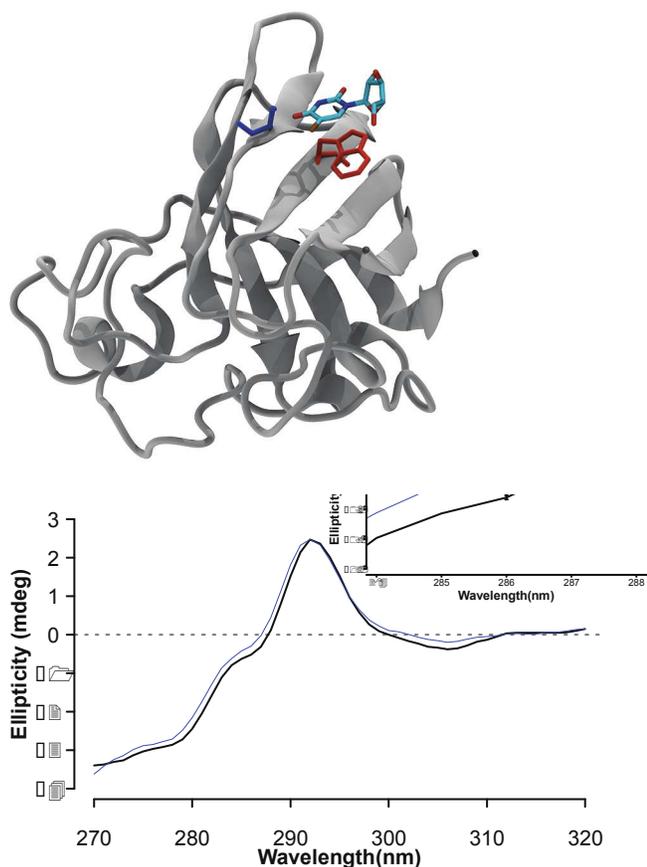


Figure 2. (Top) An X-ray crystallography-derived model of SOD-1 with the bound ligand, 5-fluorouridine, highlighted in (colour) [pdb accession code 4A7S⁶]. (Bottom) Selective detection of Trp in SOD-1, in the absence and presence of ligand. SRMCD spectra (270–320 nm) of SOD-1 (black line, 0.16 μ M) alone and in the presence of ligand, 5-fluorouridine (blue line, 1:1 molar ratio). All spectra are averaged over 4 scans (error bars show \pm one standard deviation). At 286 nm the spectra were significantly different ($p \leq 0.05$). These data had the featureless spectrum of the ligand alone deducted. See **Supplementary Figure 4** for raw data.

Magnetic circular dichroism (MCD) is the extension of magnetic optical rotation (MOR)⁷. As is the case with conventional circular dichroism (CD), MCD is observed for wavelengths at which an appropriate chromophore absorbs, and differs from MOR in that MOR is observable at all wavelengths. Unlike conventional CD, which is defined as the difference in absorbance by a chromophore of left and right circularly polarised light [$\Delta A = A_L - A_R$] arising from chirality, MCD relies on an applied magnetic field along the direction of light propagation inducing splitting of the ground and excited states. This results in the differential absorption of left and right handed circularly polarised light between CD measurements made with the magnetic field aligned first parallel and then anti-parallel to the direction of light propagation [$\Delta A = (A_- - A_+)/ (A_+ + A_-)$]. The signals that are present in MCD spectra have been described in terms of 3 components, types -A,

-B [Figure 1] and -C^{8,9} of which, types -A and -B are relevant to biological situations.

The information provided by MCD is largely complementary to that provided by conventional CD and in the past, MCD has been used to investigate proteins for two main reasons. The first was to study metal centres, particularly haem-groups, which exhibit strong MCD signals¹⁰, while the second was to determine Trp content, whose signals are much weaker. The MCD signal of Trp was shown to be sensitive to its local environment via a positional shift of the peak originally at 292 nm, although the amplitude of the major positive feature at 292–295 nm remained constant, and, being virtually free of contributions from other amino acids at pH 8 and below, allowed its use for quantification. Tyrosine residues also exhibit an MCD signal. The Tyr feature at 275 nm gradually red shifts as the pH increases and the chromophore becomes ionised (pK Tyr ~ 10.1), up to a maximum of 292 nm^{11,12}. The signal between 292 and 295 nm has been used to determine the concentration of protein^{12,13} and the shift in the feature arising from Tyr has been exploited to estimate Trp:Tyrosine ratios¹⁴.

The reported sensitivity of Trp to its local environment suggests that it may be possible to detect the binding of ligands to proteins in solution when the ligand is in the proximity of the chromophore. This has recently been exploited to study ligand binding with Trp by tracking hydrogen bond formation at 292 nm^{15,16}. Here an alternative method is presented, exploiting changes to a distinct feature at 286 nm, which is independent of any reported spectral changes relating to protein unfolding, such as a shift in the position of the feature at 292 nm¹² and provides unambiguous detection of Trp:ligand binding.

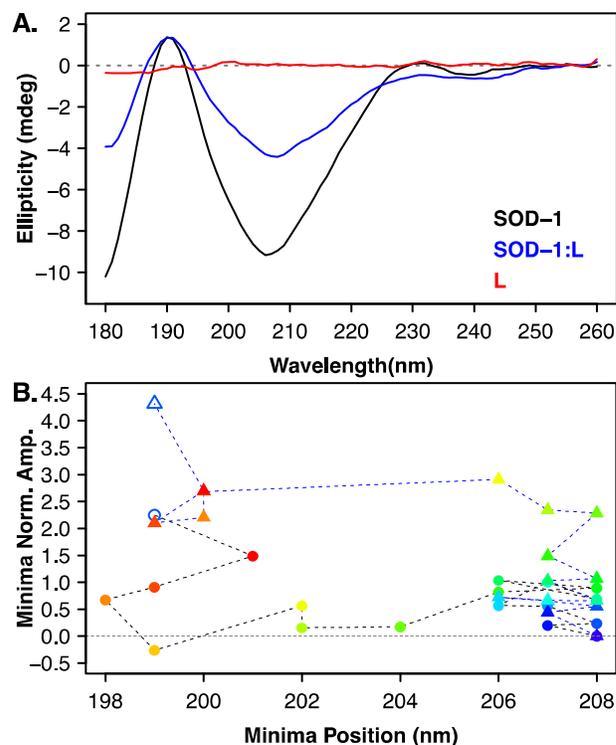


Figure 3. A. SRCD spectra of SOD-1 (black – SOD-1), SOD-1 bound to 5-fluorouridine (blue – SOD-1:L) and 5-fluorouridine (red – L) alone. On interaction with 5-fluorouridine the minima located at ~ 206 nm increase in amplitude, it should be noted that the ligand has no features in its' CD spectrum. B. Not

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only does the ligand change the conformation of SOD-1 on binding, it also affects the conformational trajectory the protein goes through when thermally denatured (SOD-1 circular symbols and black line, SOD-1:5-fluorouridine triangular symbols and blue line, the temperature was increased from 10 °C to 90 °C in 5 °C steps (blue to red) and then returned to 20 °C (blue, hollow symbols)). After heating neither of the samples returned to their original states.

Two contrasting examples of the use of MCD to study such protein-ligand interactions in solution based on established systems are presented here. The first is an MCD study of the well-defined interaction between a small molecule, 5-fluorouridine, a metabolite of the widely used chemotherapeutic, 5-fluorouracil, and the radical scavenging protein superoxide dismutase-1 (SOD-1). SOD-1 contains only one Trp residue, at amino acid 32, which is known from X-ray crystallographic studies, to bind the ligand^{6, 37}. The second example explores binding in solution of the mammalian extracellular glycosaminoglycan (GAG) polysaccharide, heparan sulfate (HS) to hen egg white lysozyme (HEWL). This example constitutes, as far as the authors are aware, the first reported detection of direct interactions between Trp residues on the surface of a protein with another biological macromolecule by synchrotron radiation MCD (SRMCD) and is detected by changes at 286 nm.

The ligand, 5-fluorouridine, exhibited a featureless SRMCD spectrum (**Supplementary Figure 1**) but induced changes in the SOD-1 SRMCD spectrum in solution. These changes included peak broadening of the main positive feature at ~292 nm and a positive influence continuing towards shorter wavelengths (**Figure 2** bottom). These are distinct from features arising from either component, or their superposition (**Supplementary Figure 1**), establishing that an interaction between them has occurred, and is in agreement with the literature^{6, 37}. That the interaction occurred in solution was also confirmed independently of both X-ray crystallography and SRMCD through observation of structural changes in the far UV SRCD spectrum (**Figure 3**), as well as by their distinct thermally-induced unfolding pathways, tracked using far UV SRCD spectroscopy (**Figure 3**). Since the changes observed in the SRMCD spectrum of SOD-1 with ligand (**Figure 2**) were relatively small, a Welch's t-test (to allow comparison of unequal variances) was performed on the centre of the shoulder of the main positive feature, confirming that the observed changes at 286 nm were significantly different from one another ($p \leq 0.05$).

In the second example, interactions between HS and HEWL, as well between HS in its modified Zn²⁺ form (HS:Zn²⁺), and HEWL, were studied by SRCD spectroscopy³⁸. In the near UV SRCD spectra, indications of interactions with surface aromatic amino acids were evident. HEWL contains 6 Trp residues (amino acids 28, 62, 63, 108, 111 and 123) of which, Trp 62 and Trp 63 are in the carbohydrate binding cleft^{19, 20} (**Figure 4** top). The SRMCD spectra of the protein alone and in complexes with HS and HS:Zn²⁺ are shown (**Figure 4** bottom). The SRMCD spectrum of HEWL alone (**Figure 4** bottom) agrees with previous literature^{31, 32} and contains the features expected at 292, 286 nm and the negative feature at 270 nm. It has been reported that, although they result in similar secondary structural changes, these complexes possess distinct surface interactions, particularly those involving Trp residues in the catalytic site³⁸, and the data presented here are consistent with this. Spectra were recorded with polysaccharide ligands present exhibiting positive features at 293

nm, blue shifted, from their original position at 294 nm and showed some differences around the positive shoulder at 286 nm (**Figure 3** bottom), indicating that, in the presence of a ligand, HS/HS:Zn²⁺, the Trp residues are in a distinct local environment compared to the native state ($p \leq 0.0002$, at 286 nm).

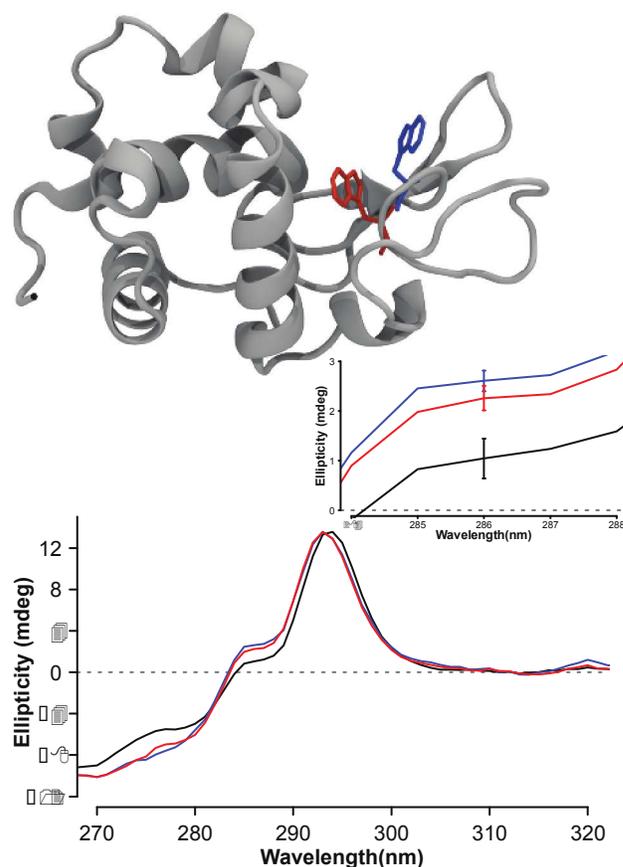


Figure 4 (Top) An X-ray crystallography-derived model (1.8 Å resolution) of HEWL (pdb accession code 1BWH²¹) with the two Trp residues of the carbohydrate binding region highlighted in red and blue. (Bottom) Selective detection of Trp in HEWL, in the absence and presence of polysaccharide ligand. SRMCD spectra of HEWL alone (black), HEWL:HS (blue) and HEWL:(HS:Zn²⁺) (red) in 12.5 mM PBS. All spectra are averaged over 4 scans (error bars show +/- one standard deviation) and at 286 nm both of the ligand spectra were significantly different from that of lysozyme alone ($p \leq 0.0002$).

SRMCD spectra were recorded on the purpose built beam line B23 at Diamond Light Source Ltd.²², (Harwell, UK) employing a 1.4 T magnetic field acquired from OLIS, UK. Spectra were collected first with a dummy cell holder to check for artefacts in the spectra, then recorded in both north to south and south to north orientations (in relation to the direction of the light). These were then deducted from each other, removing contributions from normal CD, and normalised to the average of 310-315 nm. The spectra containing ligands were then adjusted so that the maximal heights at the main positive feature between 292 and 295 nm were equal in all cases to that of the protein alone. All spectra of superoxide dismutase (SOD-1) and HEWL were normalised to the peak at 292 nm, allowing the direct comparison of line shape and relative amplitudes between spectra.

Spectra of HEWL were recorded with 1 cm path length in a 1.4 T magnetic field using 0.5 mm slits, 3 s integration time and were

averaged over 4 scans. HEWL was at 0.071 mM (0.4 mg/ml) and HS ligands were at 0.03 mg/ml. Hen lysozyme was obtained from Fluka, (Sigma–Aldrich, Gillingham, Dorset, UK). HS:Zn²⁺ was prepared from HS (10 mg/ml, in de-ionized water), shaken with Zn²⁺-cation exchange resin (Dowex W-50, Zn²⁺ form, 1 h), spun and the supernatant removed. Hen lysozyme samples were centrifuged once prior to data collection to remove aggregated protein HS and HS: Zn²⁺.

SOD-1 protein was used at 0.16 μM and, the ligand, 5-fluorouridine, was at a 1:1 molar ratio. Spectra of SOD-1 were recorded in 20 mM phosphate buffer with 1 mm path length using 1 mm slits and a 1.4 T magnetic field.

SRMCD can detect ligand binding to Trp residues in proteins for both small ligands and other biological macromolecules, in this case the polysaccharide, HS. These interactions alter the appearance of the SRMCD spectra around the Trp signals, principally the positive feature at ~286 nm which, hitherto, has not been studied extensively. The reduction of intensity of the MCD signal at 292 nm, as observed by Ali *et al.*²³ was not observed in the case of SOD-1 here.

Conclusions

SRMCD spectroscopy offers an additional tool for the study of ligand-protein interactions in solution, including those for which crystallography and solution NMR may not be appropriate, and has the capability for selective observation of ligand-Trp interactions. The information provided by SRMCD is complementary to that provided by conventional CD or SRCD and provides a method of quantifying binding to Trp residues on protein surfaces and could be used to determine binding constants. SRMCD signals are, in principle, much more widely available than those of conventional CD or SRCD, because they require only the presence of an absorbing chromophore. With the advent of commercial magnets of sufficient strength to enable MCD to be measured on bench top CD instruments, as well as synchrotron sources, the high sensitivity of the technique (0.16 μM of tryptophan has been recorded here: See Supplementary Figure 4 for comparison of near UVCD and SRMCD signal strength at 1.4 T) and the specific detection of Trp-ligand interactions will make it a useful adjunct to those techniques available for studying ligand-protein complexes in solution.

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Notes and references

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Electronic Supplementary Information (ESI) available: The files contains further methods and supporting CD and MCD spectra. See DOI: 10.1039/c000000x/

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TOC graphic and text

Magnetic circular dichroism can selectively detect interactions between macromolecules, here lysozyme and the cell-surface polysaccharide, heparan sulfate, in solution that involve tryptophan-ligand interactions through changes in a feature at 286 nm and also between proteins and small molecules, such as 5-fluorouridine and superoxide dismutase.

