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Review

Ligand- and drug-binding studies of membrane proteins revealed through circular dichroism spectroscopy ☆

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ABSTRACT

A great number of membrane proteins have proven difficult to crystallise for use in X-ray crystallographic structural determination or too complex for NMR structural studies. Circular dichroism (CD) is a fast and relatively easy spectroscopic technique to study protein conformational behaviour. In this review examples of the applications of CD and synchrotron radiation CD (SRCD) to membrane protein ligand binding interaction studies are discussed. The availability of SRCD has been an important advancement in recent progress, most particularly because it can be used to extend the spectral region in the far-UV region (important for increasing the accuracy of secondary structure estimations) and for working with membrane proteins available in only small quantities for which SRCD has facilitated molecular recognition studies. Such studies have been accomplished by probing in the near-UV region the local tertiary structure of aromatic amino acid residues upon addition of chiral or non-chiral ligands using long pathlength cells of small volume capacity. In particular, this review describes the most recent use of the technique in the following areas: to obtain quantitative data on ligand binding (exemplified by the FsrC membrane sensor kinase receptor); to distinguish between functionally similar drugs that exhibit different mechanisms of action towards membrane proteins (exemplified by secretory phospholipase A_2); and to identify suitable detergent conditions to observe membrane protein–ligand interactions using stabilised proteins (exemplified by the antiseptic transporter SugE). Finally, the importance of characterising in solution the conformational behaviour and ligand binding properties of proteins in both far- and near-UV regions is discussed. This article is part of a Special Issue entitled: Structural and biophysical characterisation of membrane protein–ligand binding.

Contents

Abbreviations: DDM, *n*-dodecyl-β-D-maltoside; SRCD, synchrotron radiation circular dichroism; CD, circular dichroism; GBAP, gelatinase biosynthesis-activating pheromone; UV, ultraviolet; TM, transmembrane domain; k_d, dissociation constant; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; DPPE, 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine

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1. Introduction

Circular dichroism (CD) is a well-known spectroscopic technique to study chiral molecules, in particular proteins in solution as well as in thin amorphous dry films [1–4]. Through pattern recognition of the CD spectral features, proteins can be classified in terms of their folding and secondary structure composition. A unique strength of CD spectroscopy is its high sensitivity to sample perturbations allowing proteins to be investigated qualitatively and quantitatively as a function of temperature, solvent composition, chemical agents, detergents, pH, and ligand binding interactions [5–9]. SRCD measured at Diamond B23 beamline shares the same advantage of other SRCD beamlines worldwide, by having higher photon flux and extended vacuum UV region down to 125 nm. This is the technique of choice for the characterisation of membrane protein property in solution. SRCD spectra measured at Diamond B23 beamline provide additional information content than those from bench-top CD instruments because of the beamline's higher photon flux and extended vacuum UV region down to 125 nm [8,11,12]. Also a unique feature of B23 is its highly collimated and small cross section beam light that allows measurements using very small volume capacity cells of a wide range of pathlengths, from a few microns to 10 cm [8,11,12] otherwise unattainable with bench-top instruments.

CD spectroscopy has proved highly useful for studies of ligand binding by soluble proteins [5–12], particularly as it is a relatively quick and easy spectroscopic measurement, requiring no extensive sample preparation, and it has the potential for use in rapid throughput technologies [13]. However, there have been fewer examples of its use in studies of ligand and/or drug binding to membrane proteins (see Section 2). CD and SRCD have most frequently been used to determine secondary structural content and integrity of membrane proteins such as histidine kinases [14–17], membrane transport proteins [18], protein fragments [19] and in membrane unfolding studies [16,18]. Sreerama and Woody [20] found that the CD analysis of secondary structure content of membrane proteins using soluble protein reference sets was slightly inferior to that obtained for soluble proteins. The inclusion of membrane proteins in the soluble protein reference sets – now common practise – has since improved the CD analysis for both membrane and soluble proteins [21–23]. It is not our intention here to provide a detailed review of all these aspects, but rather to describe recent developing advances in CD- and SRCD-based approaches that offer promising routes for progressing knowledge of membrane protein–ligand interactions in the future.

Perhaps the main reason why the application of CD and SRCD spectroscopy methods has proved more limited for ligand binding studies of membrane proteins in the past is largely due to the technical challenges associated with working with hydrophobic membrane proteins. Here we review some examples of the very recent advances in the use of CD and SRCD spectroscopy for qualitative and quantitative ligand- and drug-binding interactions by membrane proteins. A number of technical but addressable considerations have also been reported in these studies; they address some of the technical challenges, and have resulted in the successful use of the techniques for: (i) obtaining quantitative data on ligand and inhibitor binding to membrane proteins (FsrC) [24,25], (ii) identifying functionally similar but mechanistically distinct drugs that target membrane proteins (PLA_2) [26]; and (iii) characterising suitable detergent conditions for observing ligand interactions in membrane proteins [27–33]. A more detailed account is now described.

2. Quantitative data on membrane protein–ligand binding

CD methods have been used qualitatively to identify and characterise conformational changes in membrane proteins induced upon ligand binding. Examples of studies investigating ligand-induced changes on secondary structure and α-helical content, utilising the far-UV region, include: the studies of Fuertes et al. (2001) on the kinetoplastid membrane protein-11 of *Leishmania infantum*, in which pH- and temperature-dependent Ca^{2+} binding, was revealed [34]; successful application of CD techniques to immobilised protein membranes to investigate changes in α-helical content in response to hormone challenges [35]; and the studies of Dudzik et al. [36] on the neurological α-synuclein protein implicated in Parkinson's disease, in which far-UV CD spectroscopy was used to confirm that there was no change in the helicity of membrane-bound α-synuclein upon binding of its Cu^{2+} ligand [36]. Measurements in the visible regions involving time-resolved CD spectroscopy have also been used; for example, this approach was used to verify the assembly kinetics of the light harvesting chlorophyll *a*/*b* protein complex, in which it was shown that chlorophyll *a* binding possesses a fast kinetic phase whilst that of chlorophyll *b* was slower [37].

The first quantitative data of membrane protein–ligand interactions revealed through the use of SRCD spectroscopy was reported during our studies of the intact membrane sensor kinase FsrC [24] using Diamond beamline B23 [38,39]. FsrC belongs to the histidine protein kinase family of predominantly membrane proteins that constitute the sensory components of bacterial two-component signal transduction systems. These systems are the main mechanism by which bacteria sense and respond to environmental change [40,41]. FsrC is the sensory component of the Fsr signal transduction pathway involved in quorum-driven gene regulation of several virulence factor genes in the hospital-acquired infection agent *Enterococcus faecalis* and other enterococci (Fig. 1) [42–44]. FsrC senses changes in cell density or quorum by responding to changes in the external level of the gelatinase biosynthesis-activating pheromone (GBAP), a small cyclic peptide of 11 amino acid residues: H_3N^+-QNSPNIFGQWM-COO^- (lactone-linked between Ser-3 and Met-11). Following interactions between the GBAP ligand and the FsrC receptor, the pathway is activated through auto-phosphorylation of FsrC, followed by signal transduction to FsrA and culminating in activation of virulence genes such as gelatinase (GelE) and serine protease (SprE) and autoregulation of the *fsr* genes (Fig. 1; [44]). Production of purified intact FsrC protein that is active and responsive to the GBAP ligand was demonstrated in a wide-ranging study designed to evaluate the overexpression and purification of the genome complement of intact membrane sensor kinases of *E. faecalis* V583 [45]. In this study, the conditions required to successfully overexpress in *Escherichia coli* and to purify intact FsrC (including its transmembrane and sensing domains) were reported. Using in vitro activity assays, it was also shown that full-length purified FsrC was isolated as an active protein and that it responds to its GBAP signal in vitro, which is important information for confirming a link between conformational changes observed using CD and changes in membrane enzyme activity [45].

The initial ligand–receptor interactions of quorum sensing systems such as Fsr are suggested to be promising targets for the design of novel antibacterial drugs that might prove more effective against antibiotic-resistant strains of bacteria [46]. To assess the feasibility of novel drug design for disrupting GBAP–FsrC interactions, we employed SRCD spectroscopy as a means for observing, investigating and quantifying GBAP interactions with this detergent-solubilised membrane protein. Several technical considerations were established during these studies:

1) Reproducible SRCD spectra using beamline B23 [38,39] were obtained only when a sufficient incubation period was included for FsrC stabilisation following sample dilution and preparation from concentrated stocks [24]. For example, for FsrC in 0.02% DDM repeated scan measurements in the far-UV region under conditions that did not promote UV protein denaturation showed significant CD spectral changes that were used to determine the amount of incubation time needed to measure reproducible CD spectra. The incubation time required to equilibrate the structural

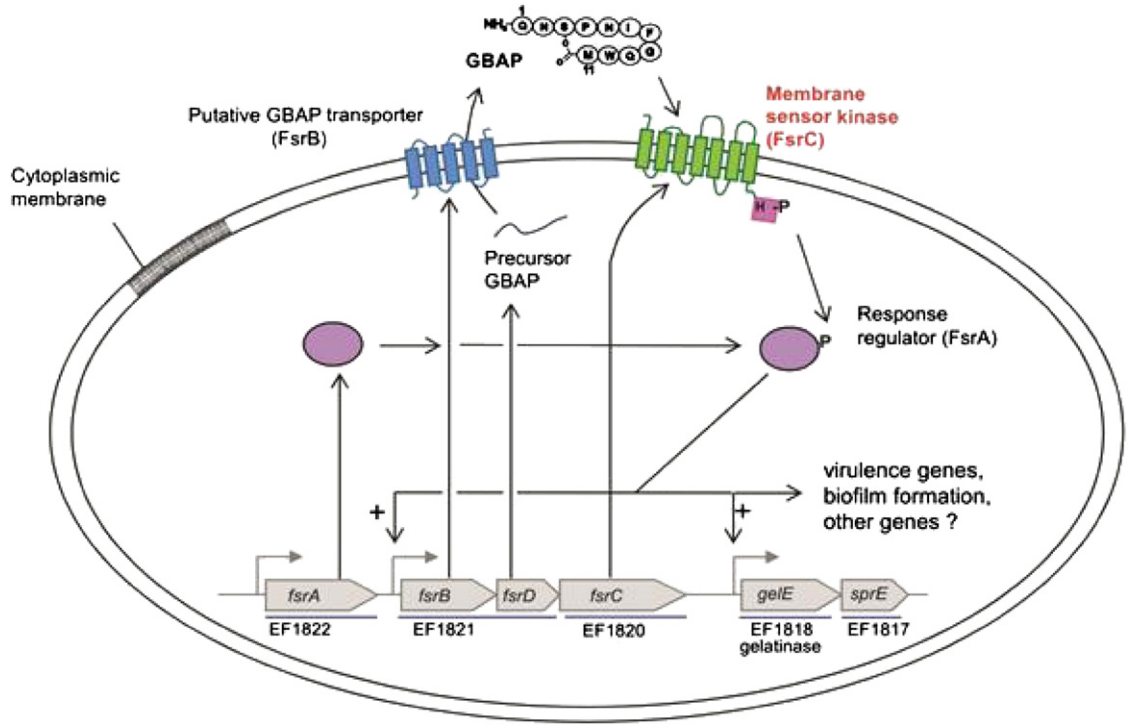

Fig. 1. The Fsr quorum (cell density) signal transduction pathway in *Enterococcus faecalis* (adapted from [44]). The first step in signal transduction involves interactions between the FsrC membrane sensor kinase (green) and the pheromone peptide GBAP (gelatinase biosynthesis-activating pheromone). GBAP is synthesised intracellularly and probably transported externally by the FsrB transporter (blue). Upon GBAP binding, FsrC is autophosphorylated. This phosphorylation signal is transferred to FsrA, a response regulator which is then able to significantly upregulate several genes including GelE, SprE and other virulence associated factors.

re-arrangements of this membrane protein in the DDM micelle environment was determined to be 1.5 h prior to data collection (Fig. 2A) [24]. A higher concentration of 0.05% DDM detergent reduced the incubation period required. It is conceivable that the effect of DDM concentration observed for FsrC is related to the protein:detergent ratio established following protein dilution and that this may occur as a general phenomenon amongst membrane proteins. The finding here of a requirement for a concentration- and time-dependent step for stabilisation of a purified membrane protein prior to SRCD measurements highlights the importance of detergent considerations for ensuring protein stability, including detergent and phospholipid types [30,47]. Indeed, the sensitivity of SRCD spectroscopy revealed here for detecting instability may identify the technique as a valuable tool for screening of membrane protein preparations prior to downstream applications such as protein crystallisation [48; see Chapter by de Moraes et al. in this Special Issue] and NMR [49] in which protein stability is an important consideration for success.

2) Titration experiments to obtain quantitative data included an incubation period of 20 min following each addition of ligand prior to obtaining spectral data.
3) Ten repeated scans and an integration time of 1 s were routinely performed per sample during the FsrC ligand titration work. This resulted in a good signal-to-noise ratio required for observing small changes in the spectral intensity following incremental additions of ligand. Thus, to acquire each spectrum, a total time of 75 min was required. For systems where the CD changes are larger, fewer repeated scans are required because better signal-to-noise ratio data can be acquired thereby reducing significantly the overall measurement times [7]. Other techniques such as surface plasmon resonance (SPR) spectroscopy [50–52] requiring sample immobilisation and often sample labelling, isothermal titration calorimetry (ITC) [53,54], and fluorescence resonance energy transfer (FRET) [55,56] requiring sample labelling, can also be successfully used to determine ligand binding but these techniques reveal less information regarding molecular interactions or direct conformational changes to the extent of CD and SRCD spectroscopy. See other chapters on such techniques in this Special Issue.
4) For small amounts of precious samples, appropriate small-volume capacity cells of various pathlengths can be used to interrogate different spectral regions that will convey information about secondary structure folding and aromatic side-chains [57] of Phe, Tyr, Trp and disulphide bonds [57] and prosthetic groups (heme, NAD, FAD, cofactors [5]). For the vacuum UV region (170–250 nm) very narrow cells (demountable or fixed) of 0.05 to 0.1 mm pathlength and 2–10 μl volume capacity can be used. In addition, it is advantageous to utilise a very small and collimated light beam that allows CD spectra to be measured through long pathlength cells but with very small volume capacity (20–60 μl for a 1 cm cell, 350 μl for 5 cm and 700 μl for 10 cm) [38,39]. This can be an important consideration when the amount of sample to be investigated is very small, as was the case for FsrC discussed above [24].

It is important to note that the vacuum and far-UV region from 170 to 200 nm is the region of instrumental limitation for top-bench CD instruments. This is often due to the combination of low photon flux as well as the high UV absorption of saline buffers. The ability to penetrate that region using SRCD beamlines allows the analysis without artefacts of more spectral features, in particular those associated with α-helix, β-turns and β-sheet conformations that otherwise would not be possible. For measurements in aqueous solutions, a reasonable cut-off limit should be 170 nm, which means that any CD spectral feature observed below this should be rejected as artefactual. Several such artefacts have been assigned to either super secondary structures of proteins or to charge-transfer transitions (reviewed in [38]). Longer pathlengths such as 1, 2, 5 and 10 cm of small diameter aperture with volume capacities of 30 to

80 μl, 155 μl, 350 μl and 750 μl respectively can be used to conduct ligand CD titrations at dilute concentrations without compromising the overall CD intensity. These long pathlength cells can only be used with a highly collimated SRCD-generated beam [38,39] but not with the bench-top CD instruments because of their highly divergent incident light beam. This is an important consideration when using precious and low abundance membrane protein samples, and can make all the difference as to whether useful data can be obtained or not.

Using stabilised purified FsrC and the technical considerations described above, far- and near-UV SRCD spectra revealed that whilst GBAP binding did not significantly affect FsrC secondary structural integrity, the local tertiary structure of the Tyr and Trp aromatic side-chain residues was significantly affected (Fig. 2B, C) [24]. CD titration experiments of GBAP into FsrC revealed a relatively loose binding interaction of 1:1 stoichiometry, with a dissociation constant k_d of 2 μM calculated from CD data using a non-linear regression analysis [5–7]. These studies indicate a promising future for the use of SRCD in quantifying ligand interactions and for identifying inhibitors of FsrC–GBAP and membrane protein–ligand interactions more generally. Indeed, SRCD spectroscopy was recently used to study an inhibitor of FsrC activity, siamycin I [25]. Previous studies had already shown that siamycin I inhibited gelatinase and GBAP production in *E. faecalis*, suggestive of possible inhibition of a component of the Fsr pathway [58]. Ma et al. [59] used purified intact FsrC in in vitro phosphorylation assays to establish that FsrC was a direct target for siamycin I inhibition. When FsrC–siamycin I interactions were interrogated using SRCD spectroscopy, a significant positive shift in the near-UV spectrum was identified, especially in the 270–290 nm region of the Tyr and Trp aromatic side-chain residues (Fig. 3), with only a very small change in secondary structure revealed in the far-UV spectrum [25]. Siamycin I itself possesses one Tyr and one Trp and therefore it is not possible to distinguish whether it is the local tertiary structure of Tyr and Trp residues in the protein or in the inhibitor that is affected upon binding. Nonetheless, the detection of a discernible and measurable CD spectral change upon inhibitor binding means that further investigation of FsrC–siamycin I interactions was possible using SRCD spectroscopy. Moreover, this SRCD-based study also revealed that no competition was observed between GBAP and siamycin I upon binding to FsrC, as the CD spectrum of the FsrC–GBAP–siamycin I mixture was the sum of the binary complex FsrC–GBAP and FsrC–siamycin I (Fig. 3). The siamycin I inhibitor binding site did not therefore overlap with the GBAP binding site.

In a study by Bettaney et al. [60], the SRCD spectra of three inositol membrane transport proteins (IolF, IolT, YfiG) measured with the Diamond B23 beamline showed a cut-off below 180 nm as indicated by the voltage of the photomultiplier tube (PMT) detector exceeding 600 V (Fig. 4). It is essential that for accurate secondary structure estimations the positive CD bands at about 190–195 nm of the α-helical conformation are measured with the lowest level of noise possible, which can be readily achieved with synchrotron CD beamlines. Repeated scans can also improve the signal-to-noise-ratio but at increased time (the noise is reduced by the root square of the number of scans). Following successful confirmation of secondary structural integrity and composition, the specificity of these three proteins for a large variety of inositols and sugars of D and L configuration was determined by measuring the cellular uptake of radiolabelled ^{3}H-*myo*-inositol in the presence of unlabelled competing compounds [60].

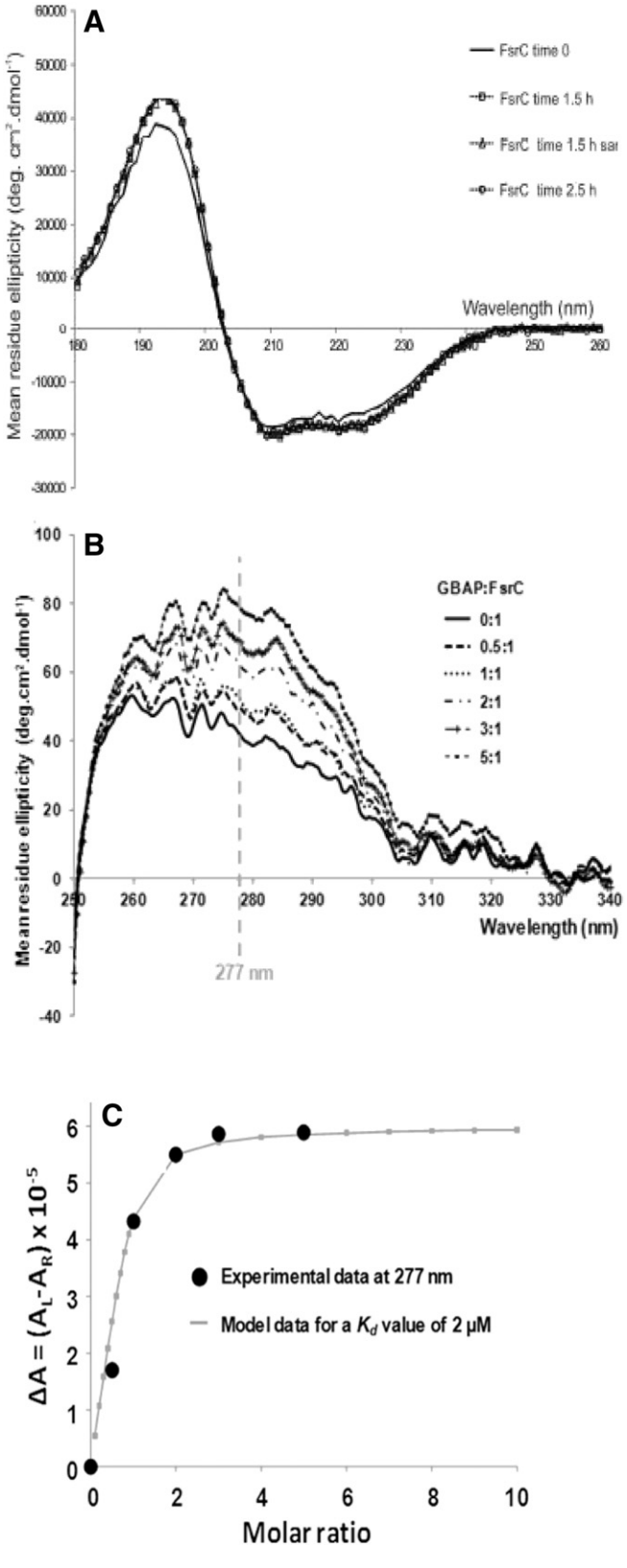

Fig. 2. SRCD spectroscopic study of FsrC. A) Average of four far-UV SRCD spectra of purified FsrC (6 μM) at 0 h (solid black line), 1.5 h (dashed line with unfilled square), and 2.5 h (dashed line with unfilled circle) following sample preparation in 10 mM sodium phosphate pH 7.5 containing 0.02% DDM at 20 °C. Repeat spectra after 1.5 h involving sample removal and reloading were also included (dashed line, with unfilled triangle) (adapted from [24]; B) SRCD titration of GBAP binding to FsrC using near-UV SRCD spectroscopy. The difference SRCD spectra of GBAP:FsrC at various molar ratios (0:1; 0.5:1; 1:1; 2:1; 3:1 and 5:1) were obtained by subtracting the spectra of GBAP at the corresponding equivalent molar ratios (0.5; 1; 2; 3; 5) (adapted from [24]). C) Dissociation constant k_d of GBAP:FsrC complex was calculated by fitting the CD data at 277 nm (solid circle) as a function of GBAP concentration using a non-linear regression analysis method [5–7]. As both FsrC and GBAP possess CD features in the near-UV region, the subtraction of the CD spectrum of GBAP from that of the GBAP:FsrC mixture reduces the system from a two to one CD component allowing the fitting to be of a Michael–Menten saturation type (solid line) (adapted from [24]).

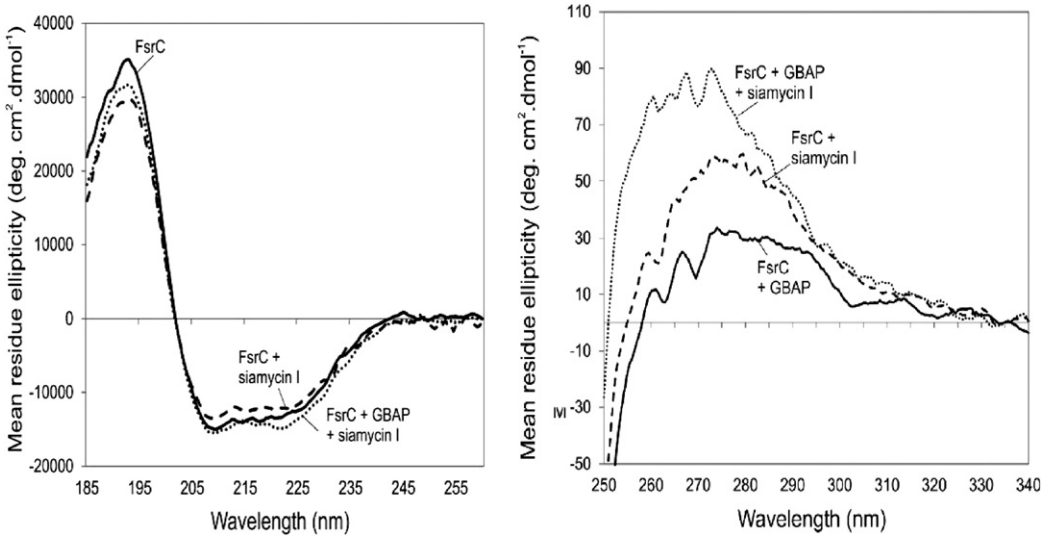

Fig. 3. Effect of GBAP on siamycin I binding to FsrC. *Left*) Far-UV region (185–260 nm) SRCD spectra of FsrC in 10 mM potassium phosphate, pH 7.5, containing 0.05% DDM (solid); difference CD spectrum of {[(FsrC + siamycin I) (1:5)] − [(siamycin I) (0:5)]} (dash); difference CD spectrum of {[(FsrC + GBAP + siamycin I) (1:3:5)] − [(GBAP + siamycin I) (0:3:5)]} (dot). In the case of FsrC alone, an equivalent volume of methanol used to dissolve siamycin I was also added to retain the same protein concentration in the mixtures with ligands. *Right*) Near-UV region (250–340 nm) difference SRCD spectra of {[(FsrC (20 mM) + GBAP) (1:3)] − [(GBAP) (0:3)]} (solid), {[(FsrC + siamycin I) (1:5)] − [(siamycin I) (0:5)]} (dash), and {[(FsrC + GBAP + siamycin I) (1:3:5)] − [(GBAP + siamycin I) (0:3:5)]} (dot) calculated by subtracting from the FsrC + ligand mixtures the SRCD spectra of the ligands alone. Upon each addition of ligand or inhibitor, the mixtures were incubated for 20 min at 20 °C before conducting the SRCD measurements.
Both figures were adapted from [25].

Aromatic residues (Tyr and Trp) have been found to have a high preference for binding carbohydrate ligands and sequence-based programmes are used to predict such binding sites [61]. Since chiral carbohydrates lack near-UV chromophores, CD spectroscopy and in particular SRCD (for low available quantities of membrane proteins) provides a powerful and sensitive means by which to study protein conformational changes upon carbohydrate ligand binding. Any interaction of the inositol/sugar ligands of L and/or D configuration with the Trp amino acid residues of the inositol transporters would show changes of the tryptophan CD like those illustrated in Fig. 5 for the CD titration of hen white egg lysozyme with N,N,N triacetylchitotriose (NAG_3).

CD measurements in the near-UV region of the spectrum are clearly valuable for obtaining both qualitative and quantitative information on membrane protein–ligand interactions. The wide range of wavelengths that can be investigated using a collimated synchrotron beamline that measures CD spectra from vacuum UV to far- and near-UV and the visible spectral regions is made possible because it operates a double grating monochromator with negligible stray light (e.g. [38]).

Taking these data on ligand- and inhibitor-binding of a membrane sensor kinase together, the suitability of beamline B23 for future quantification of drug- and ligand-binding to other membrane sensor kinases, and indeed other membrane proteins more generally, is established.

3. Identification of functionally similar but mechanistically distinct drugs

Although not an integral membrane protein, secretory phospholipase A_2 (PLA_2) of snake venom is a membrane interfacial enzyme that relies on tryptophan and other aromatic residues for interfacial binding to phosphatidylcholine and phosphatidylglycerol phospholipids, a

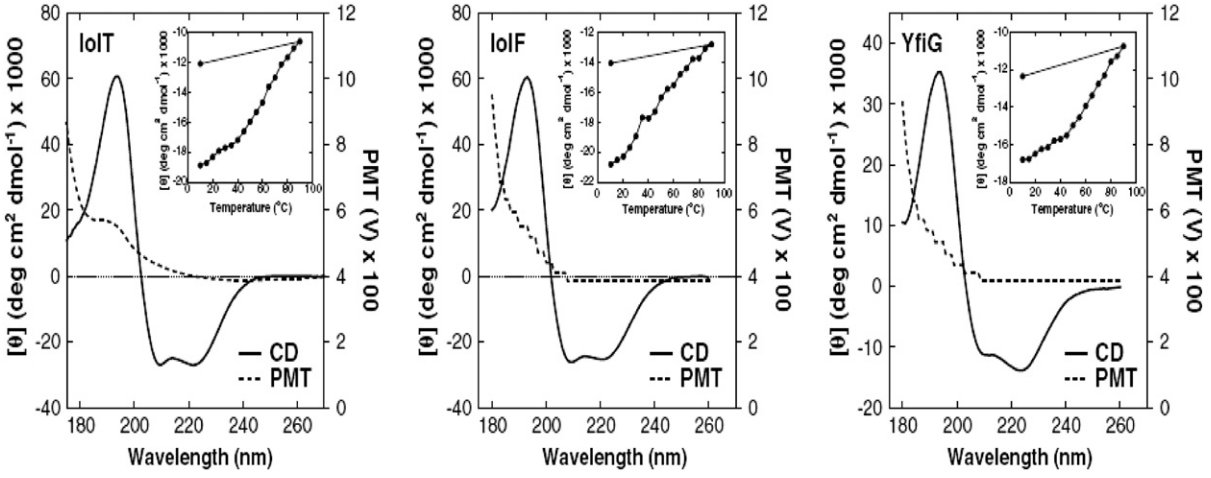

Fig. 4. Far-UV SRCD studies of bacterial inositol membrane transporter proteins. SRCD spectra (solid line) and PMT response that is proportional to the absorption (dashed line) of inositol transport membrane proteins. The insets are the plots of SRCD at 190 nm versus variable temperature in the range 10° to 90° every 5 °C and back to 10 °C.
Adapted from [60]).

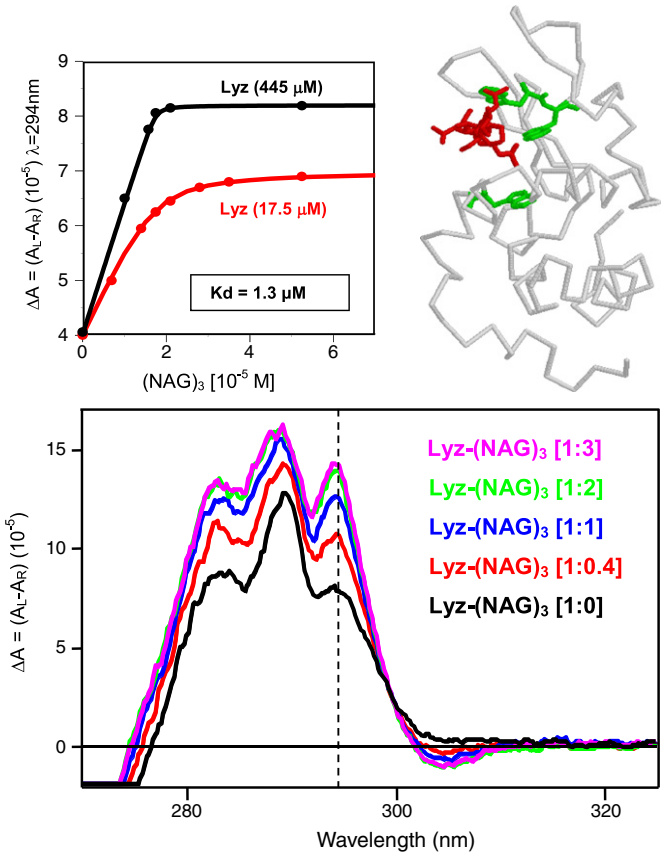

Fig. 5. CD spectra hen white lysozyme (Lyz) without and with N,N,N triacetylchitotriose [$(NAG)_3$] at various molar ratios (adapted from [5]). Top left inset) Plot of CD data at 294 nm versus $(NAG)_3$ concentration. The titration was repeated at two different Lyz concentrations: 17.5 μM (red solid circle) and 445 μM (black solid circle). For the titration with the higher Lyz concentration the $(NAG)_3$ concentration is about 25.4 times bigger than that reported for the lower Lyz in the plot axis. The data were fitted with a non-linear regression analysis [5–7] and for both titrations the fitting was obtained for a $k_d = 1.3$ μM. Top inset right) Rasmol cartoon of 1HEW PDB crystal structure of Lyz (grey) with $(NAG)_3$ (red) interacting with three Trp residues (green).

prerequisite step for PLA_2 enzymatic phospholipid hydrolysis [26]. PLA_2 lipolytic activity promotes the development of inflammatory processes and therefore represents a valuable therapeutic target for anti-inflammatory drugs such as the non-steroidal anti-inflammatory drugs (NSAIDs). CD spectroscopy was recently used alongside fluorescence measurements to study interactions between four NSAIDs (meloxicam, lornoxicam, nimesulide and tenoxicam) and snake PLA_2 [26]. The roles of the tryptophan and other aromatic residues were investigated using fluorescence spectroscopy whilst the effects of NSAIDs on enzyme secondary structural conformation was studied using far-UV CD measurements of PLA_2 in the presence and absence of DMPC:DPPE (7:3) liposomes. When PLA_2 was added to liposomes, the α-helical content increased from 34% in aqueous solution to 44% in the presence of lipid membrane. The authors suggest that this change in α-helical content is consistent with the role of the N-terminal amphipathic α-helix in surface binding and in facilitating phospholipid entrance into the hydrophobic substrate-binding pocket, both of which require a flexible enzyme with dynamic properties [26,62,63]. Far-UV CD measurements revealed that PLA_2 secondary structural conformation changed little upon addition of three of the NSAIDs (meloxicam, lornoxicam and nimesulide) in the absence of liposomes. When these drugs were added to the enzyme in the presence of liposomes, surprisingly there was no increased α-helical content for PLA_2; the α-helical content remained at approximately 34% [26]. This finding not only confirmed that PLA_2 is a target for these NSAID drugs, but also provided insight into a possible mechanism of inhibition, suggesting that these three NSAIDs may interact by affecting the flexibility of structural α-helical elements, reducing enzyme flexibility and ability to be activated at the interfacial surface of the membrane [26]. A fourth NSAID drug, tenoxicam, which performs the same function as the other three drugs through inhibition of PLA_2 activity, was shown by CD measurements to act through a different mechanism. Unlike the other three NSAIDS, tenoxicam was able to induce significant secondary structural changes in the enzyme directly [26].

It is important to note that the four NSAID drugs are non-chiral molecules that contain aromatic chromophores ($\lambda_{363\ nm}$ for meloxicam [64], $\lambda_{380\ nm}$ for lornoxicam [64], $\lambda_{394\ nm}$ for nimesulide [65] and $\lambda 3_{70\ nm}$ for tenoxicam [64]). In Ref. [26], CD measurements in the 260–300 nm near-UV region were not carried out otherwise they could have shown overlapping contributions arising from the Tyr and Trp residues of PLA_2 and the bound NSAIDs. However, only the bound species of NSAIDs would have shown induced CD spectra in the 360–400 nm region as the protein is devoid of any chromophore. This is, therefore, the ideal spectral region to assess unambiguously and determine quantitatively the binding stoichiometry and the dissociation constant k_d of the PLA_2–NSAID complexes by CD spectroscopy [5–8] rather than fluorescence spectroscopy [26] that required the approximation of data correction for the NSAID ligand re-absorption and inner filter effects. The CD approach could open up the possibility of further study to determine, for instance, drug competition as described above for the FsrC example.

4. Effects of environment conditions (detergents/surfactants, reducing agents, salt ionic strength and ligands) to observe membrane protein–ligand interactions

As described above, the importance of considerations of detergent environment for biophysical studies of membrane proteins has long been recognised. Particular considerations of the appropriate micellar environment to retain native protein folding, stability and activity have been reported, for example, Corin et al. [28] screened several detergents for their ability to produce and solubilise G-protein coupled receptors (GPCR) expressed in cell-free systems. CD spectroscopy was used to assess the folding of several purified GPCRs. In Fig. 8A, the CD spectra of mOR103-15 protein produced with and without Brij-35 showed the familiar high content of α-helical conformation (≈50%) only with the detergent. Without detergent only 10% of the protein was obtained showing mainly an irregular structure (Fig. 8A).

Kaiser et al. [29] reported the effects of increased NaCl salt concentration (50 mM) and the addition of reducing agent TCEP (1 mM) to the buffer (25 mM Tris, 10% glycerol, 3 times the critical micelle concentration (cmc) of Fos-Choline 14 (FC14) and 150 mM NaCl) enhanced substantially the amount of α-helical content of *E. coli* free-cell GPCR protein expressed in the presence of FC14 or Tri-mix detergents (Fig. 8B).

CD spectroscopy is the easier and faster technique to investigate whether ligand binding promotes protein conformational changes. O'Malley et al. [30] investigated the binding of several ligands, determined by fluorescence, revealing that no secondary structure conformational changes were indeed promoted to the GPCR protein $hA_2aR\text{-}His_{10}$ (Fig. 8C).

A CD- and NMR-based study of the $TatC_d$ membrane protein of the twin arginine translocation (Tat) system of *Bacillus subtilis* tested the effects of a range of different detergents on the secondary structure content following reconstitution in detergent micelles and lipid vesicles [31], whilst a SRCD spectroscopy study of cytolysin equinatoxin II tested the effects of micellar and vesicle environments of different lipid compositions on its pore forming function [32]. In addition, the stabilising effect of a bilayer environment over a micellar environment was noted by Miles et al. (2011) during their studies on thermal stability of two Na,K ATPases from different sources [33]. None of those works included CD measurements conducted in the near UV region characteristic of the local tertiary structure of the Phe, Tyr and Trp aromatic side-chain amino acid residues as natural molecular

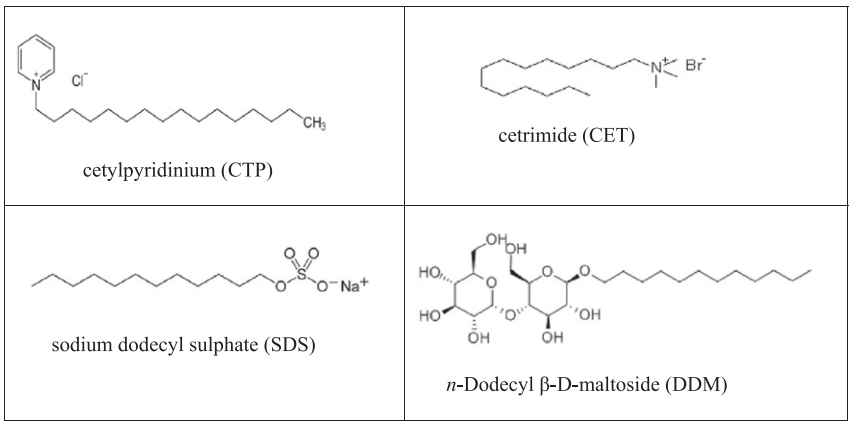

Fig. 6. Chemical formula of cetylpyridinium (CTP), cetrimide (CET), sodium dodecyl sulphate (SDS) and *n*-dodecyl β-D-maltoside (DDM).

probes to detect ligand binding interactions [5]. In Ref. [28] the function of the cell-free purified GPCR proteins was confirmed using microscale thermophoresis monitoring the changes of the Trp fluorescence of the protein as a function of carveol ligand. A CD titration in the near UV region would have determined the binding stoichiometry and the dissociation constant k_d as well as opened the possibility to investigate competition studies with other known ligands that might necessitate inner-filter fluorescence corrections hence introducing wider approximations to the data analysis. Similarly, for O'Malley et al. [30], a ligand binding CD titration monitored in the 250–350 nm near UV region not only could have complemented the fluorescence quenching experiment but also could have opened the study to ligand competition like that described in the FsrC work [25]. In general, the CD data acquisition should be conducted as far as possible in both far- and near-UV regions for a better and complete characterisation of the system. The examples described in this review exemplify the exploitation of the near UV region CD data for screening and diagnostic purposes.

Another important example is the effects of detergent environment on ligand binding by SugE recently investigated using CD spectroscopy [27]. SugE is a member of the SMR protein family of secondary active multidrug transporters found in bacteria and it binds antiseptic quaternary cation ligands (Fig. 6) such as cetylpyridinium (CTP) and cetrimide (CET) thereby conferring host resistance to these compounds. Using far-UV CD spectroscopy, SugE was shown to possess identical, high α-helical content in two different detergents, sodium dodecyl sulphate

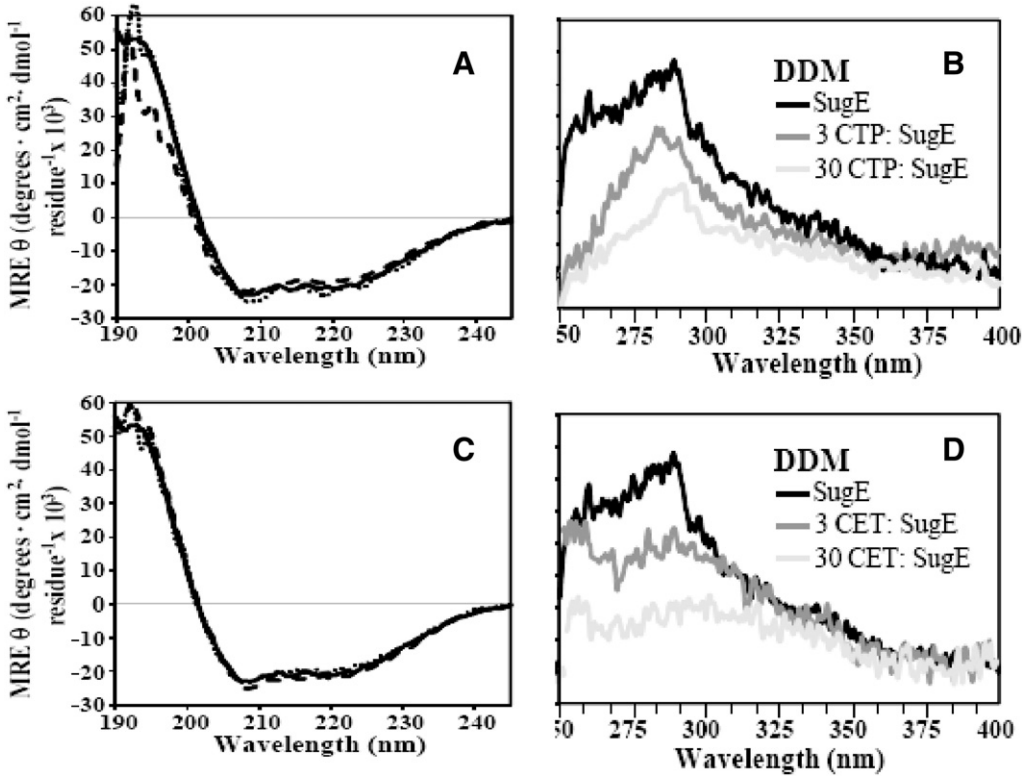

Fig. 7. CD spectra in the far- and near-UV regions of SugE membrane protein with and without CTP and CET respectively. A) Far-UV CD spectra of 5 μM SugE in DDM (3.9 mM):CTP at 1:0 (solid line), 1:20 (dashed line) and 1:200 (dotted line) molar ratios. B) Near-UV CD spectra of 31 μM SugE in DDM (3.9 mM):CTP at 1:0 (solid black line), 1:3 (solid dark grey line) and 1:30 (light grey line) molar ratios. C) Far-UV CD spectra of 5 μM SugE in DDM (3.9 mM):CET at 1:0 (solid line), 1:20 (dashed line), and 1: 200 (dotted line) molar ratios. D) Near-UV CD spectra of 31 μM SugE in DDM (3.9 mM):CET at 1:0 (solid black line), 1:3 (solid dark grey line) and 1:30 (light grey line) molar ratios.
Figure adapted from [27].

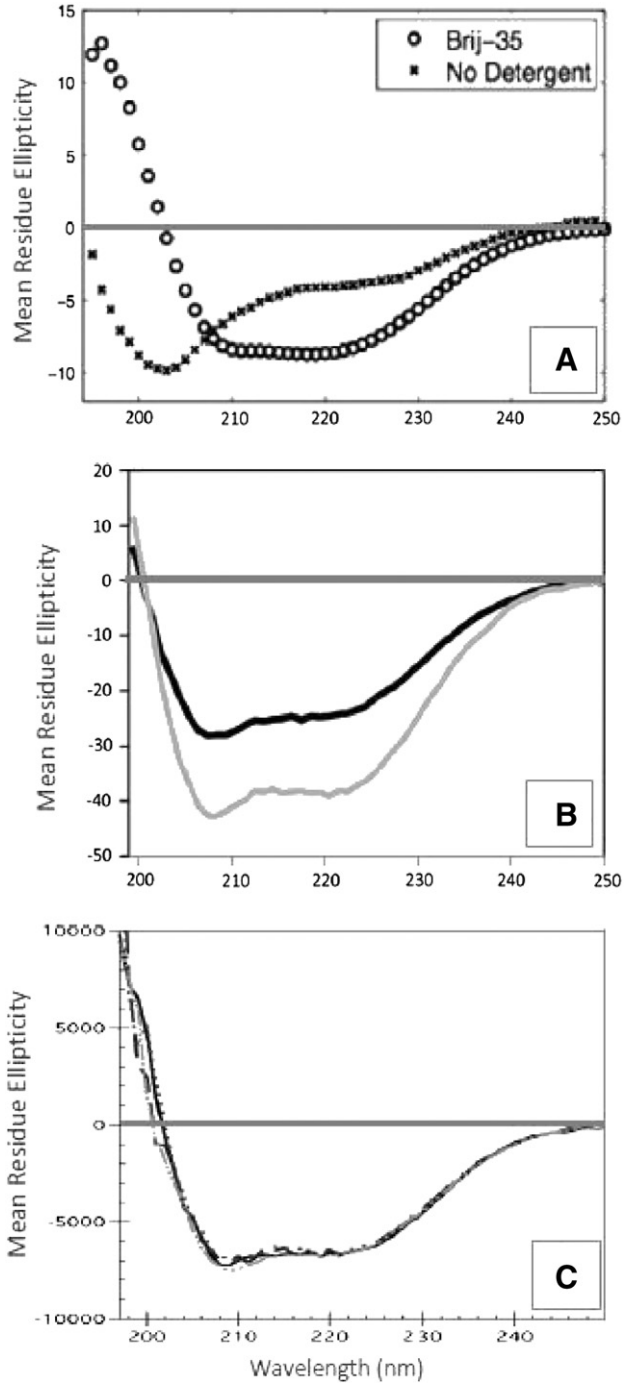

Fig. 8. CD spectra of GPCR proteins. A) mOR103-15 protein made with (open circle) or without (solid square) Brij-35 detergent (figure adapted from [28]); B) hOR17-4 protein in 25 mM Tris (pH 7), 10% glycerol, 3 × cmc Fos-Choline 14 (FC14) with 200 mM NaCl (black) or with 150 mM NaCl and 1 mM TCEP (grey) (figure adapted from [29]); C) $hA_2aR\text{-}His_{10}$ protein without (black) and with CHA agonist (dashed), theophylline (dotted) and 1 mM TCEP (dashed–dotted) ligands.
Figure adapted from [30].

(SDS) and *n*-dodecyl-β-D-maltoside (DDM) (Fig. 6). There were no significant changes in the secondary structure content of the protein upon addition of up to 200 fold CTP or CET in the presence of either SDS- or DDM-solubilised protein (Fig. 7) [27]. By contrast, measurements in the near-UV region revealed not only significant differences in tertiary structural arrangements depending on the detergent used, but also different effects of each ligand, though ligand effects were observable only in the DDM micellar environment (Fig. 7). The different effects of the two detergents are consistent with hydrophilic interactions between the quaternary cationic moieties of CTP and CET ligands and the anionic sulphate heads of SDS micelles, whereas the lack of such interactions with the maltoside moiety of DDM did not preclude CTP and CET binding to the SugE membrane protein. Thus this elegant study not only highlighted the importance of detergent choice but also provided further evidence of the versatility of CD spectroscopy in ligand binding studies using intact membrane proteins in micellar environments.

5. Summary

The work described in this chapter confirms and highlights the versatility of CD spectroscopy for studies of membrane proteins, particularly when a wide spectral range can be achieved for both protein and chromophore, including determinations of secondary structural integrity and composition, confirmation and quantitation of ligand binding, characterisation of (detergent) conditions suitable for observing ligand binding using stabilised protein, and identification of mechanistically distinct drugs that bring about the same function. It is the amide bond of the protein backbone structure that gives information about protein secondary structure, and the aromatic side-chains of Phe, Tyr and Trp residues about their local tertiary structure. The chromophore of the ligand can be used to determine unambiguously ligand binding in both far- and near-UV regions and in particular whether protein conformational changes are occurring upon ligand binding. This approach of achieving a complete CD spectroscopic study in solution for membrane proteins is clearly advantageous. In particular SRCD provides the extension of the vacuum UV region allowing measurements of far less transparent media and in small volumes that are unattainable with bench-top CD instruments.

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References

[1] S.F. Mason, Molecular Optical Activity and the Chiral Discrimination, Cambridge University Press, Cambridge, 1982.
[2] G.D. Fasman, Circular Dichroism and the Conformational Analysis of Biomolecules, Plenum Press, New York, 1996.
[3] N. Berova, K. Nakanichi, R.W. Woody, Circular Dichroism Principles and Applications, 2nd ed. Wiley-VCH, 2000.
[4] N. Berova, P.L. Polavarapu, K. Nakanishi, R.W. Woody, Comprehensive Chiroptical Spectroscopy: Applications in Stereochemical Analysis of Synthetic Compounds, Natural Products and Biomolecules, vol. 2, John Wiley & Sons, 2012.
[5] G. Siligardi, R. Hussain, Biomolecules interactions and competitions by non-immobilised ligand interaction assay by circular dichroism, Enantiomer 3 (1998) 77–87.
[6] G. Siligardi, B. Panaretou, P. Meyer, S. Singh, D.N. Woolfson, P.W. Piper, L.H. Pearl, C. Prodromou, Regulation of Hsp90 ATPase activity by the co-chaperone Cdc37p/p50cdc37, J. Biol. Chem. 277 (2002) 20151–20159.
[7] S.R. Martin, M.J. Schilstra, G. Siligardi, Biophysical Approaches Determining Ligand Binding to Biomolecular Targets, Detection, Measurement and Modelling, in: A. Podjarmy, A. Dejaegere, B. Kieffer (Eds.), Chapter 7: Circular Dichroism, RSC Publishing, 2011, pp. 226–246.
[8] G. Siligardi, R. Hussain, Applications of Circular Dichroism, in: J. Lindon, G. Tranter, D. Koppenaal (Eds.), Encyclopedia of Spectroscopy and Spectrometry, 2nd edition, vol. 1, Elsevier, Oxford, 2010, pp. 9–14.
[9] W.C. Johnson, Protein secondary structure and circular dichroism: a practical guide, Proteins Struct. Funct. Genet. 7 (1990) 205–214.
[10] S.M. Kelly, T.J. Jess, N.C. Price, How to study proteins by circular dichroism, Biochim. Biophys. Acta 1751 (2005) 119–139.
[11] R. Hussain, T. Jávorfi, G. Siligardi, Circular dichroism beamline B23 at Diamond Light Source, J. Synchrotron Radiat. 19 (2010) 132–135.
[12] T. Javorfi, R. Hussain, D. Myatt, G. Siligardi, Measuring circular dichroism in a capillary cell using the B23 synchrotron radiation CD beamline at Diamond Light Source, Chirality 22 (2010) E149–E153.
[13] S. Fiedler, L. Cole, S. Keller, Automated circular dichroism spectroscopy for medium-throughput analysis of protein conformation, Anal. Chem. 85 (2013) 1868–1872.

[14] C.A. Potter, A. Ward, C. Laguri, M.P. Williamson, P.J.F. Henderson, M.K. Phillips-Jones, Expression, purification and characterisation of full-length heterologously expressed histidine protein kinase RegB from *Rhodobacter sphaeroides*, J. Mol. Biol. 320 (2002) 201–213.
[15] Y.P. Kim, K.J. Yeo, M.H. Kim, Y.-C. Kim, Y.H. Jeon, Structural characterisation of the intra-membrane histidine kinase YbdK from *Bacillus subtilis* in DPC micelles, Biochem. Biophys. Res. Commun. 391 (2010) 1506–1511.
[16] N. Keegan, H. Ridley, J.H. Lakey, Discovery of biphasic thermal unfolding of OmpC with implications for surface loop stability, Biochemistry 49 (2010) 9715–9721.
[17] K.J. Yeo, S.-N. Kwak, H.J. Kim, C. Cheong, M.H. Kim, Y.H. Jeon, Expression and characterisation of the integral membrane domain of bacterial histidine kinase SCO3062 for structural studies, Biochem. Biophys. Res. Commun. 376 (2008) 409–413.
[18] G. Psakis, M. Saidijam, K. Shibayama, J. Polaczek, K.E. Bettaney, J.M. Baldwin, S.A. Baldwin, R. Hope, L.-O. Essen, R.C. Essenberg, P.J.F. Henderson, The sodium-dependent D-glucose transport protein of *Helicobacter pylori*, Mol. Microbiol. 71 (2009) 391–403.
[19] A.M. Powl, A.O. O'Reilly, A.J. Miles, B.A. Wallace, Synchrotron radiation circular dichroism spectroscopy-defined structure of the C-terminal domain of NaChBac and its role in channel assembly, Proc. Natl. Acad. Sci. U. S. A. 107 (2010) 14064–14069.
[20] N. Sreerama, R.W. Woody, On the analysis of membrane protein circular dichroism spectra, Protein Sci. 13 (2004) 100–112.
[21] CDPro software is available at the following website, http://lamar.colostate.edu/sreeram/CDPro/.
[22] N. Sreerama, R.W. Woody, Estimation of protein secondary structure from circular dichroism spectra: comparison of CONTIN, SELCON and CDSSTR methods with an expanded reference set, Anal. Biochem. 287 (2000) 252–260.
[23] S.W. Provencher, J. Glockner, Estimation of globular protein secondary structure from circular dichroism, Biochemistry 20 (1981) 33–37.
[24] S.G. Patching, S. Edara, P. Ma, J. Nakayama, R. Hussain, G. Siligardi, M.K. Phillips-Jones, Interactions of the intact FsrC membrane histidine kinase with its pheromone ligand GBAP revealed through synchrotron radiation circular dichroism, Biochim. Biophys. Acta Biomembr. 1818 (2012) 1595–1602.
[25] M.K. Phillips-Jones, S.G. Patching, S. Edara, J. Nakayama, R. Hussain, G. Siligardi, Interactions of the intact FsrC membrane histidine kinase with the tricyclic peptide siamycin I revealed through synchrotron radiation circular dichroism, Phys. Chem. Chem. Phys. 15 (2013) 444–447.
[26] D. Gaspar, M. Lúcio, S. Rocha, J.L.F. Costa Lima, S. Reis, Changes in PLA2 activity after interacting with anti-inflammatory drugs and model membranes: evidence for the involvement of tryptophan residues, Chem. Phys. Lipids 164 (2011) 292–299.
[27] D.C. Bay, R.J. Turner, Spectroscopic analysis of the intrinsic chromophores within small multidrug resistance protein SugE, Biochim. Biophys. Acta Biomembr. 1808 (2011) 2233–2244.
[28] K. Corin, P. Baaske, D.B. Ravel, J. Song, E. Brown, X. Wang, S. Geissler, C.J. Wienken, M. Jerabek-Willemsen, S. Duhr, D. Braun, S. Zhang, A robust and rapid method of producing soluble, stable, and functional G-protein coupled receptors, PLoS One 6 (2011) 1–6.
[29] L. Kaiser, J. Graveland-Bikker, D. Steuerwald, M. Vanberghem, K. Herlihy, S. Zhang, Efficient cell-free production of olfactory receptors: detergent optimization, structure, and ligand binding analyses, Proc. Natl. Acad. Sci. U. S. A. 105 (2008) 15726–15731.
[30] M.A. O'Malley, M.E. Helgeson, N.J. Wagner, A.S. Robinson, Toward rational design of protein detergent complexes: determinants of mixed micelles that are critical for the in vitro stabilisation of a G-protein coupled receptor, Biophys. J. 101 (2011) 1938–1948.
[31] O.V. Nolandt, T.H. Walther, S. Roth, J. Bürck, A.S. Ulrich, Structure analysis of the membrane protein TatCd from the Tat system of *B. subtilis* by circular dichroism, Biochim. Biophys. Acta Biomembr. 1788 (2009) 2238–2244.
[32] A.J. Miles, A. Drechsler, K. Kristan, G. Anderluh, R.S. Norton, B.A. Wallace, F. Separovic, The effect of lipids on the structure of eukaryotic cytolysin equinatoxin II: a synchrotron radiation circular dichroism spectroscopic study, Biochim. Biophys. Acta Biomembr. 1778 (2008) 2091–2096.
[33] A.J. Miles, B.A. Wallace, M. Esmann, Correlation of structural and functional thermal stability of the integral membrane protein Na, K-ATPase, Biochim. Biophys. Acta Biomembr. 1808 (2011) 2573–2580.
[34] M.A. Fuertes, J.M. Pérez, M. Soto, M.C. López, C. Alonso, Calcium-induced conformational changes in *Leishmania infantum* kinetoplastid membrane protein-11, J. Biol. Inorg. Chem. 6 (2001) 107–117.
[35] L. Boscá, F. Morán, Circular dichroism analysis of ligand-induced conformational changes in protein kinase C, Biochem. J. 290 (1993) 827–832.
[36] C.G. Dudzik, E.D. Walter, B.S. Abrams, M.S. Jurica, G.L. Millhauser, Coordination of copper to the membrane-bound form of α-synuclein, Biochemistry 52 (2013) 53–60.
[37] R. Horn, G. Grundmann, H. Paulsen, Consecutive binding of chlorophylls a and b during the assembly in vitro of light-harvesting chlorophyll *a*/*b* protein (LHCIIb), J. Mol. Biol. 366 (2007) 1045–1054.
[38] R. Hussain, T. Jávorfi, G. Siligardi, Spectroscopic analysis: synchrotron radiation circular dichroism, Comprehensive Chirality, Separations and Analysis, 8, 2012, pp. 438–448.
[39] R. Hussain, T. Jávorfi, G. Siligardi, Circular dichroism beamline B23 at the Diamond Light Source, J. Synchrotron Radiat. 19 (2012) 132–135.
[40] C. Fabret, V.A. Feher, J.A. Hoch, Two-component signal transduction in *Bacillus subtilis*: how one organism sees its world, J. Bacteriol. 181 (1999) 1975–1983.
[41] L.E. Hancock, M. Perego, Two-component signal transduction in *Enterococcus faecalis*, J. Bacteriol. 184 (2002) 5819–5825.
[42] J. Nakayama, Y. Cao, T. Horii, S. Sakuda, A.D.L. Akkermans, W. de Vos, H. Nagasawa, Gelatinase biosynthesis-activating pheromone: a peptide lactone that mediates quorum sensing in *Enterococcus faecalis*, Mol. Microbiol. 41 (2001) 145–154.
[43] X. Qin, K.V. Singh, G.M. Weinstock, B.E. Murray, Characterisation of *fsr*, a regulator controlling expression of gelatinase and serine protease in *Enterococcus faecalis* OG1RF, J. Bacteriol. 183 (2001) 3372–3382.
[44] P. Ma, Structure–activity Relationships of Membrane Proteins: The NCS1 Family of Transporters and Sensor Kinases of Two-component Systems, PhD Thesis University of Leeds, 2010.
[45] P. Ma, H.M. Yuille, V. Blessie, N. Gőhring, Z. Iglói, K. Nishiguchi, J. Nakayama, P.J.F. Henderson, M.K. Phillips-Jones, Expression, purification and activities of the entire family of intact membrane sensor kinases from *Enterococcus faecalis*, Mol. Membr. Biol. 25 (2008) 449–473.
[46] R.B. Raffa, J.R. Iannuzzo, D.R. Levine, K.K. Saeid, R.C. Schwartz, N.T. Sucic, O.D. Terleckyj, J.M. Young, Bacterial communication (quorum sensing) via ligands and receptors: a novel pharmacologic target for the design of antibiotic drugs, J. Pharmacol. Exp. Ther. 312 (2005) 417–423.
[47] G.G. Privé, Detergents for the stabilization and crystallization of membrane proteins, Methods 41 (2007) 388–397.
[48] C.G. Tate, Practical considerations of membrane protein instability during purification and crystallisation, Methods Mol. Biol. 601 (2010) 187–203.
[49] S.G. Patching, NMR structures of polytopic integral membrane proteins, Mol. Membr. Biol. 28 (2011) 370–397.
[50] P.J. Harding, T.C. Hadingham, J.M. McDonnell, A. Watts, Direct analysis of a GPCR-agonist interaction by surface plasmon resonance, Eur. Biophys. J. 35 (2006) 709–712.
[51] J.A. Maynard, N.C. Lindquist, J.N. Sutherland, A. Lesuffleur, A.E. Warrington, M. Rodriguez, S. Oh, Next generation SPR technology of membrane-bound proteins for ligand screening and biomarker discovery, Biotechnol. J. 4 (2009) 1542–1558.
[52] Y. Liu, Q. Cheng, Detection of membrane-binding proteins by surface plasmon resonance with an all-aqueous amplification scheme, Anal. Chem. 84 (2012) 3179–3186.
[53] C.W. Sikora, R.J. Turner, Investigation of ligand binding to the multidrug resistance protein EmrE by isothermal titration calorimetry, Biophys. J. 88 (2005) 475–482.
[54] N.G. Housden, J.A. Wojdylaa, J. Korczynskab, I. Grishkovskayaa, N. Kirkpatricka, A.M. Brzozowskib, C. Kleanthous, Directed epitope delivery across the *Escherichia coli* outer membrane through the porin OmpF, Proc. Natl. Acad. Sci. U. S. A. 107 (2010) 21412–21417.
[55] H. La, T. Zhou, B.D. Hamman, Q. Lin, A homogeneous G protein-coupled receptor ligand binding assay based on time-resolved fluorescence resonance energy transfer, Assay Drug Dev. Technol. 6 (2008) 543–550.
[56] A.D. Goddard, A. Watts, Contributions of fluorescence techniques to understanding G protein-coupled receptor dimerization, Biophys. Rev. 4 (2012) 291–298.
[57] G. Siligardi, M.M. Campbell, W.A. Gibbons, A.F. Drake, Conformational analysis of the melanin concentrating hormone (MCM) by CD spectroscopy: disulphide bridge and aromatic tyrosyl contribution, Eur. J. Biochem. 206 (1991) 23–29.
[58] J. Nakayama, E. Tanaka, R. Kariyama, K. Nagata, K. Nishiguchi, R. Mitsuhara, Y. Uemura, M. Tanokura, H. Kumon, K. Sonomoto, Siamycin attenuates fsr quorum sensing mediated by a gelatinase biosynthesis-activating pheromone in *Enterococcus faecalis*, J. Bacteriol. 189 (2007) 1358–1365.
[59] P. Ma, K. Nishiguchi, H.M. Yuille, L.M. Davis, J. Nakayama, M.K. Phillips-Jones, Anti-HIV siamycin I directly inhibits autophosphorylation activity of the bacterial FsrC quorum sensor and other ATP-dependent enzyme activities, FEBS Lett. 585 (2011) 2660–2664.
[60] K.E. Bettaney, P. Sukumar, R. Hussain, G. Siligardi, P.J.F. Henderson, S.G. Patching, A systematic approach to the amplified expression, functional characterization and purification of inositol transporters from *Bacillus subtilis*, Mol. Membr. Biol. 30 (2013) 3–14.
[61] A. Malik, S. Ahmad, Sequence and structural features of carbohydrate binding in proteins and assessment of predictability using a neural network, BMC Struct. Biol. 7 (2007) 1–14.
[62] S. Qin, A.H. Pande, K.N. Nemec, S.A. Tatulian, The N-terminal alpha-helix of pancreatic phospholipase A_2 determines productive-mode orientation of the enzyme at the membrane surface, J. Mol. Biol. 344 (2004) 71–89.
[63] S. Qin, A.H. Pande, K.N. Nemec, X.M. He, S.A. Tatulian, Evidence for the regulatory role of the N-terminal helix of secretory phospholipase A_2 from studies on native and chimeric proteins, J. Biol. Chem. 280 (2005) 36773–36783.
[64] E.A. Taha, N.N. Salama, L.S.A. Fattah, Stability-indicating chromatographic methods for the determination of some oxicams, J. AOAC Int. 87 (2004) 366–373.
[65] U.N. Kale, K.R. Naidu, M.S. Shingare, Simultaneous spectrophotometric estimation of chlorzoxazone and nimesulide from combined dosage form, Indian J. Pharm. Sci. 64 (2002) 168–169.