Circular Dichroism of Protein-Dye Complexes as a Characterization Tool for Protein Higher Order Structures

Christopher Sucato, Shreyas Laghate, Libo Wang, and Mario DiPaola

Blue Stream Laboratories, 8 Henshaw Street, Woburn, MA 01801 csucato@bluestreamlabs.com 617-234-0001

ABSTRACT

The fluorescent dye, 1-anilinonaphthalene-8-sulphonate (ANS), has a fluorescence quantum yield that increases significantly after binding to the hydrophobic regions of proteins. As a result of this effect, ANS has been widely used as a hydrophobic probe for the study of conformational changes in protein samples, especially when assessing the structural characteristics of two or more proteins in a comparability study. The change in fluorescent properties of the dye, upon association with the hydrophobic regions of protein structural domains, is a key feature of the ANS molecule. However, the information from extrinsic fluorescence spectra alone reveals little information about protein conformation at the level of secondary or tertiary structure. In this work we present advancements in methodology, using ANS dye, to gain further insight into the characterization of protein structure.

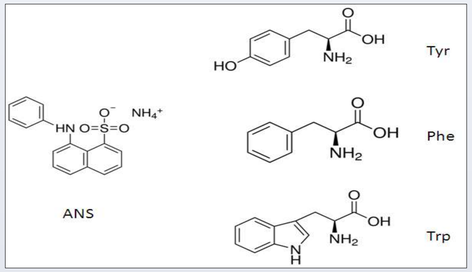


Figure 1: The chemical structure of ANS, and the three aromatic amino acids tryptophan, tyrosine, and phenylalanine. ANS shows fluorescence and also associates with hydrophobic sites in folded proteins. Because ANS has aromaticity and hydrophobic properties similar to the native residues above, we speculate ANS association with proteins may have an effect on the near-UV CD spectra.

BACKGROUND:

CD spectroscopy plays an important part in the development of protein pharmaceuticals, as a means to examine purified proteins for structural integrity and proper folding, and also to study relative conformational stabilities in response to changing pH, ionic strength, and other formulation variables. During production of biological products, CD spectroscopy can provide a non-invasive and non-destructive means of monitoring stability and checking batch-to-batch consistency of the drug product. CD spectroscopy in the near-UV wavelength region provides a tertiary structure fingerprint, including information on aromatic residues and disulphide bridges. Although the data from near-UV CD cannot be quantified in the same manner as the secondary structure deconvolution as done for far-UV CD spectra, the fingerprint obtained from near-UV CD is useful as a characteristic marker of protein tertiary structure, particularly in the region of aromatic residues.

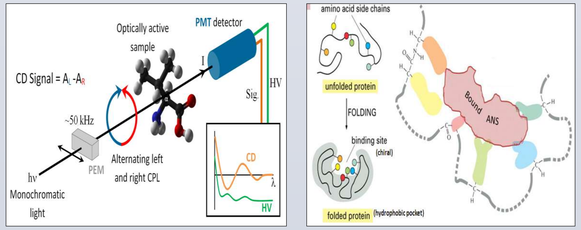


Figure 2: Measurement of a CD spectrum as illustrated in the upper panel. A beam of monochromatic light is polarized into left circular polarized light (CPL) and right circular polarized light. Light is passed through the sample and due to chirality the absorption is different between left and right CPL. The differential absorption results in the CD spectra. Lower panel: Unfolded or variable (random coil) regions of a protein will not give rise to CD signals because of a lack of chirality on the time scale of the measurement. Folded domains however, are likely to have asymmetry, and well-ordered side chains in such regions are the source of near-UV CD signal. If ANS associates in the same regions, we reason, the dye may have an effect on the near-UV spectrum. Image courtesy of www.isa.au.dk.

Here we present the results of circular dichroism studies of protein-ANS complexes that indicate the near-UV ellipticity spectral data of the complexes can be used to gain information that is complementary to both the ANS-protein fluorescence data, as well as the circular dichroism data on the native protein in the absence of ANS. Using Bovine Serum Albumin as a representative sample type, we prepare ANS-protein complexes for CD as follows:

Methods

For the initial study we prepared four sets of samples of different concentrations between 0.2 and 4.0 mg/ml. The ratio of BSA to ANS was kept as 1:1. A stock solution of BSA of 10 mg/mL was prepared and dilutions made from this stock. Buffer used for dilutions and BSA preparation was 1x PBS at pH 7.4. The samples prepared above were then were incubated at 25 ˚C for one hour before being tested. The CD spectra were collected on a JASCO 715 spectropolarimeter with parameters as follows: wavelength range: 250 nm- 450 nm; data pitch: 0.5 nm; scan speed: 20 nm/min; response: 8 secs; bandwidth: 1 nm.

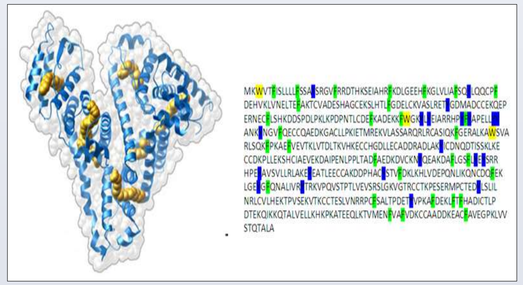
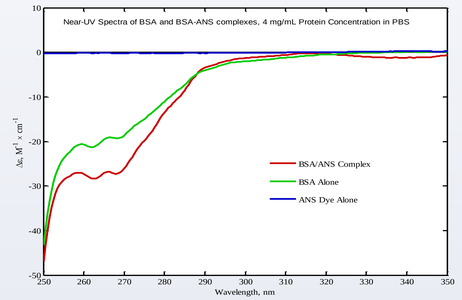


Figure 3: Ribbon structure of BSA, the yellow space-filling structures are sites where fatty acids are found, and are possible sites of attachment for ANS. The BSA primary sequence is shown to the right, wherein the aromatic CD-active residues are highlighted. Image courtesy of www.sbkb.org.

Results and Discussion

Our first experiment was to determine the effect of ANS dye binding on the near-UV spectrum of BSA; our hypothesis being that when ANS associates with the protein, it may do so in an environment that is chiral, and produce a CD signal, and may also effect other CD signals deriving from groups present in the native protein. Figure 4, below, shows the near-UV CD spectrum of BSA in the absence of ANS, and also after incubation with the dye. The CD spectrum of the BSA-ANS system has features that are not observed in the spectrum of the native BSA. Between 340 - 390 nm, a small but significant feature is seen in the BSA-ANS spectrum, comprised of a positive band near 390 nm and a negative one near 340 nm. Because this feature is observed in the region where ANS absorbs, and where absorption from the protein is low, we attribute this to an ANS-derived positive Cotton effect, which may arise because the ANS is associated with the protein in a chiral manner. Secondly, we also observe from the spectra in Figure 4 a notably strong signal enhancement of the negative CD band with dual minima at 262 and 268 nm, when comparing the BSA-ANS data to that of BSA alone. In this spectral region of the native protein, phenylalanine or tyrosine residues may contribute to the ellipticity. The effect of ANS on the near-UV spectrum of BSA is remarkable due to the magnitude of the signal enhancement in the region between approximately 250 - 280 nm. To a lesser extent, some differences are seen between the BSA and BSA-ANS spectra around 280 - 300 nm, where tryptophan contributes to the CD. The implication of such a significant shift, we notice, may have practical uses because if the enhancement is due to ANS interaction with aromatic residues in hydrophobic pockets of the folded structure, such an effect may be a sensitive probe for structural differences between protein samples.



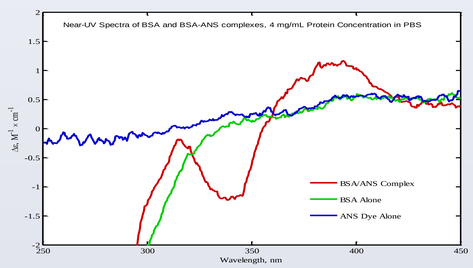


Figure 4: Upper Panel: near UV-CD data for BSA alone and BSA bound with ANS dye. CD signal in the region of tyrosine and phenylalanine with ANS dye is evident. It can be inferred that presence of dye intensifies the signal. Lower Panel: a Cotton effect where the signal drops into negative region and then increases into the positive region is present in the spectral range where ANS absorbs, and is likely due to a chiral orientation of the dye itself.

To further investigate the enhancement of CD signal upon ANS binding, we studied the near-UV response as a function of temperature. If the enhancement of the negative band is due to a close association of ANS with aromatic residues that contribute to CD in the region between 250 - 280 nm, and is a dynamic non-covalent association in thermodynamic equilibrium, then a temperature effect can be expected. Figure 5, below, shows the results of near-UV CD scans between ambient temperature and 4 deg. C.

The samples were prepared in similar fashion as the initial study. The sample was prepared at 4 mg/mL as it gave good results in the previous study. Two sets of samples were prepared. Both sets were first incubated at 25 ˚C for one hour before being tested. One set was then refrigerated at 4˚C and the other set was tested immediately.

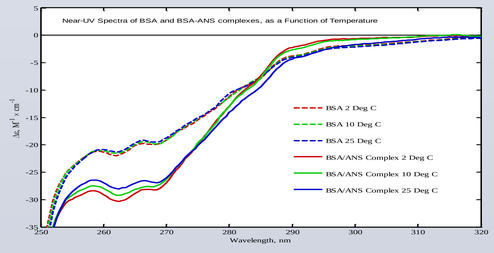


Figure 5: BSA and BSA-ANS near-UV CD spectra as a function of temperature. The amplitude of the CD signal in the region 260 – 270 nm correlates with the temperature in a manner consistent with a reversible association of ANS in proximity to Phe and Tyr residues.

As shown by the temperature study, a small but significant temperature effect is observed, consistent with an equilibrium binding effect of ANS in hydrophobic pockets shared by aromatic residues that have CD signal. A final study that we report on here is related to the point mentioned above, that the significant ANS-induced near-UV shift may have a practical use as a sensitive probe for structural differences between protein samples. In the section below is shown the results of a comparison of near-UV CD data on two different lots of BSA. The lots studied here are both commercially available BSA, both listed at greater than 95% purity, but were purified by different methods (c.f. Sigma website page reference). Prior to performing the CD assay, both samples were analyzed by size-exclusion chromatography (SEC) assay. The SEC results are shown in Figure 6 below, and reveal that only slight differences in the species profile are detectable by that method. In contrast, Figure 7 shows the results of near-UV CD, on the same two lots, on the native proteins and also after incubation with ANS dye.

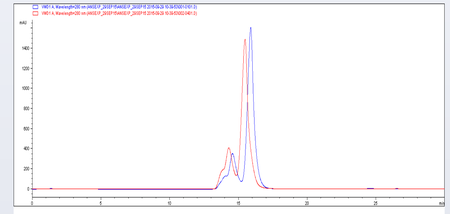


Figure 6: Size-exclusion chromatogram overlay for two lots of the BSA isolated by different purification methods. The Mobile phase was PBS, pH 7.4; run time 30 mins; pump rate 0.5 mL/min; 100 µL injection. BSA Sample Conc was 1mg/ml. Blue indicates the BSA non-heat shock fraction. Red indicates the BSA heat shock fraction.

The samples for Near UV-CD spectra was prepared similar to studies conducted earlier. Two different lots of BSA were used to prepare two different sets of samples having conc. 1 mg/ml. Both sets were incubated at 25 ˚C for one hour before being tested.

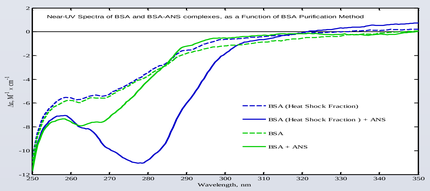


Figure 7: Near-UV CD spectra on the two lots. The BSA samples without ANS shows very slight difference as supported by the SEC data. BSA samples with ANS binding show large difference in signal.

The notable finding as shown in Figure 7 is that CD spectra measured with ANS binding could be helpful in differentiating between the folding patterns of similar protein as seen in the data above, where there is contrasting difference in the signal of Heat shock fraction and non-heat shock fraction.

CONCLUSIONS

From the studies conducted it was clear that ANS binding to the protein does enhance the Near UV-CD spectra. It can be concluded that binding of protein with ANS before CD analysis can help us in determining changes in the secondary or tertiary structure that may occur due to change in temperature or it can help us in comparing two different lots of sample. The results produced here are intriguing and needs further analysis as to why or what changes in the protein structure can be accurately determined using this procedure.

REFERENCES

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ACKNOWLEDGMENTS

We would like to acknowledge all the members of Blue Stream Laboratories for their support.

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