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Exciton CD Couplet Arising From Nitrile-Derivatized Aromatic Residues as a Structural Probe of Proteins

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Abstract

Exciton coupling between two chromophores can produce a circular dichroism (CD) couplet that depends on their separation distance, among other factors. Therefore, exciton CD signals arising from aromatic sidechains, especially those of tryptophan (Trp), have been used in various protein conformational studies. However, the long-wavelength component of the commonly used CD couplet produced by a pair of Trp residues is typically located at around 230 nm, thus overlapping significantly with the protein backbone CD signal. This overlap often prevents a direct and quantitative assessment of the Trp CD couplet in question without further spectral analysis. Herein, we show that this inconvenience can be alleviated by using a derivative of Trp, 5-cyanotryptophan (Trp_{CN}), as the chromophore. Specifically, through studying a series of peptides that fold into either an α -helical or β -hairpin conformation, we demonstrate that in comparison to the Trp CD couplet, that arising from two Trp_{CN} residues is not only significantly red-shifted, but also becomes more intense due to the larger extinction coefficient of the underlying electronic transition. In addition, we show that a pair of *p*-cyano-phenylalanines (Phe_{CN}) or a Phe_{CN}-Trp_{CN} pair can also produce a distinct exciton CD couplet that is useful in monitoring protein conformational changes.

Introduction

Circular dichroism (CD) spectroscopy is one of the most commonly used techniques in assessing the secondary structural content of proteins [1–2]. This is because the far ultraviolet (UV) CD spectrum in the region of 190 – 250 nm [3], arising from the backbone of a polypeptide, depends on the exciton couplings among the individual π - π^* and n - π^* transitions of its amide units [4]. Similarly, when two amino acids with aromatic sidechains (chromophores) are in close proximity, exciton coupling between their π - π^* transition bands (1A_1 - 1B_b transition) could also lead to formation of unique CD signatures in this spectral region [5–6]. For example, the tryptophan (Trp) residues in the Trpzip β -hairpins give rise to a positive CD band at around 228 nm providing a convenient spectroscopic feature to monitor β -hairpin formation [7–9]. Similarly, a recent study by Gasymov *et al* [10] showed

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that a pair of Trp residues separated by less than 10 Å in a protein can produce an observable exciton CD band around 230 nm. In addition, Khan *et al.* [11] have observed the formation of a CD band resulting from the exciton coupling between two different aromatic sidechains in the enzyme phospholipid:lipid A palmitoyl transferase PagP.

The interaction potential (V_{AB}) between the electronic transition dipole moments (μ_A and μ_B) of two chromophores (A and B) can be expressed as:

$$V_{AB} \propto \frac{\mu_A \mu_B}{r_{AB}^3} (\cos \gamma - \cos \alpha \cos \beta), \quad (1)$$

where $\vec{r}_{AB}$ is the distance between A and B, $\alpha(\beta)$ represents the angle between $\vec{\mu}_A$ ($\vec{\mu}_B$) and $\vec{r}_{AB}$, whereas γ is the angle between $\vec{\mu}_A$ and $\vec{\mu}_B$. The extinction coefficient (ϵ) of the CD couplet arising from this exciton coupling is proportional to V_{AB} [12], as shown below:

$$\Delta \epsilon \propto V_{AB} R_{AB}, \quad (2)$$

where R_{AB} is the rotational strength [12], which is defined as:

$$R_{AB} \propto \pm \vec{r}_{AB} \cdot (\vec{\mu}_A \times \vec{\mu}_B). \quad (3)$$

Combining Eqs. (1–3) leads to:

$$\Delta \epsilon \propto \pm \frac{\mu_A^2 \mu_B^2}{r_{AB}^2} \Theta(\alpha, \beta, \gamma). \quad (4)$$

This equation indicates that $\Theta(\alpha, \beta, \gamma)$, which depends on the spatial orientations of $\vec{\mu}_A$ and $\vec{\mu}_B$, determines the sign of the long-wavelength component of the CD couplet. A convenient way to determine this sign is to first project $\vec{\mu}_A$ and $\vec{\mu}_B$ onto a common plane and then rotate the transition dipole moment of the front chromophore onto the one in the back. If a clockwise/counterclockwise rotation is needed in order to superimpose the two transition dipole moments, then the exciton CD couplet will have a positive/negative chirality; in other words, the long-wavelength component of the exciton coupling band will have a positive/negative value of molar ellipticity [12].

Eq. (1) indicates that the amplitude of an exciton CD couplet arising from two identical chromophores depends on the square of its absorption extinction coefficient (ϵ) [13]. Thus, we hypothesize that the far-UV CD signal produced by two 5-cyano-tryptophan (Trp_{CN}) chromophores would be larger than that induced by two Trp residues, when other factors (i.e., distance and orientation) are identical or similar. This is because, as indicated (Figure 1), the extinction coefficient of the π - π^* transition (i.e., the 1A_1 to 1B_b transition) of 5-

cyanoindole (the sidechain of Trp_{CN}) in methanol, is almost twice as large as that of indole (the sidechain of Trp). In addition, and perhaps more importantly, the far-UV absorption spectrum of 5-cyanoindole (and also Trp_{CN}) is red-shifted by ~20 nm from that of indole (and Trp), thereby resulting in a smaller overlap between the Trp_{CN} CD signal and that arising from the protein backbone. Indeed, in many cases, the far-UV CD signals arising from naturally occurring aromatic residues, including Trp, are difficult to discern due to such spectral overlaps [10]. Thus, we believe that these unique spectral features will make the CD couplet arising from a pair of Trp_{CN} residues a useful probe of protein structures.

To test this notion, we studied a series of Trp_{CN}-containing peptides that can form either an α -helical or a β -hairpin structure in appropriate solvents. Indeed, the CD spectra of those peptides show strong and distinct Trp_{CN} CD signals that depend on the peptide conformation, thus providing a direct validation of this idea. Since one of the π - π^* transitions of the unnatural amino acid *p*-cyano-phenylalanine (Phe_{CN}) is shifted to 240 nm, we also investigated the potential utility of the CD couplet arising from the electronic coupling between Phe_{CN} and Trp_{CN} and between a pair of Phe_{CN} residues. We found that when these two unnatural amino acids were brought into contact in a β -hairpin peptide, they produced an intense CD couplet with the long-wavelength component centered at around 247 nm. Similarly, we found that two Phe_{CN} residues, when placed next to each other in an α -helical peptide, also yielded an observable CD couplet. Thus, taken together, these results indicate that these nitrile-derivatized amino acids can be used to probe protein tertiary structural formation via CD measurements. Given the fact that the CN stretching vibration of Trp_{CN} and Phe_{CN} has been shown to be a useful site-specific infrared (IR) probe of proteins [14–16], we believe that the findings from the current study will further expand their spectroscopic utilities.

Experimental

All peptides were synthesized on a microwave assisted automated Liberty Blue peptide synthesizer (CEM Corporation, Matthews, NC) using Fmoc-protected amino acids purchased from Protein Technologies, Inc. (Tucson, Az). Peptide purification was achieved by reversephase high-performance liquid chromatography (HPLC) and the identity of each peptide was confirmed by matrix-assisted laser desorption ionization (MALDI) mass spectrometry. All peptide samples were prepared by dissolving lyophilized peptide solids in 10 mM sodium phosphate buffer (pH 7.0) or in a mixture of 10 mM sodium phosphate buffer (pH 7.0) and 2,2,2-trifluoroethanol (TFE). The peptide concentration was determined optically using the absorbance of the peptide at 280 nm and an $\epsilon_{280} = 5500 \text{ cm}^{-1} \text{ M}^{-1}$ for Trp_{CN} [17] and an $\epsilon_{280} = 850 \text{ cm}^{-1} \text{ M}^{-1}$ for Phe_{CN} [18]. UV-Vis spectra were collected on a Perkin-Elmer Lambda 25 UV-Vis spectrometer. All CD data were collected on an Aviv 62 DS spectrometer (Aviv Biomedical, NJ) using a 1 mm sample cuvette.

Results and discussion

To verify whether a pair of Trp_{CN} residues can produce a useful CD couplet, we first studied a Trp to Trp_{CN} mutant of an antimicrobial peptide, CP10A [19]. We then studied three alanine-based peptides, with each containing two but differently located Trp_{CN} residues.

Next, we studied a Trp to Trp_{CN} mutant of Trpzip5, a β -hairpin designed by Cochran *et al* [7]. We chose these peptide systems because they all contain at least two Trp residues that have been shown to engage in exciton coupling and hence exhibit an exciton CD band. Finally, we investigated the Trp to Trp_{CN} and Phe to Phe_{CN} double mutant of another stable β -hairpin that was designed through “loop optimization” by Andersen and coworkers [20].

CP10A Trp_{CN} Mutant

CP10A is an indolicidin-based antimicrobial peptide that contains five Trp residues (sequence: ILAWKWAWWARR-NH₂) and adopts an α -helical structure when bound to membranes [19, 21]. In addition, a previous study [19] reported the CD spectra of CP10A obtained under different solvent conditions. Therefore, this peptide can serve as a convenient model system to compare the CD signals arising from Trp and Trp_{CN} sidechains. Specifically, all of the five native Trp residues in CP10A were replaced with Trp_{CN} (hereafter referred to as Cp10A-Trp_{CN}). As shown (Figure 2), the CD spectrum of CP10A-Trp_{CN} collected in the presence of DPC micelles (at a peptide:lipid ratio of 1:100) shows the characteristics of an α -helical secondary structure (i.e., with two minima at 208 and 222 nm), indicating that the Trp to Trp_{CN} mutations have not changed the membrane-binding property of this peptide. More importantly, in both 20% TFE and DPC micelle solutions, the CD spectra of CP10A-Trp_{CN} exhibit an exciton CD couplet with a negative long-wavelength component (at about 247 nm for TFE and 243 nm for DPC micelles), confirming the notion that Trp_{CN}-Trp_{CN} electronic coupling can result in a strong CD couplet in the far-UV region.

The CD spectrum of CP10A-Trp_{CN} obtained in 100% TFE also supports this picture as its Trp_{CN} CD signal is significantly increased, as expected for an exciton coupling occurring in an environment with a much lower dielectric constant. For comparison, as indicated in the study of Friedrich *et al.*, [19] the CD spectrum of CP10A obtained in 100% TFE contains a much smaller contribution from the Trp sidechains. Thus, these results indicate that the CD couplet arising from a pair of Trp_{CN} residues could be a useful spectroscopic probe of protein structures.

Alanine-Based Peptides

To further substantiate the utility of the Trp_{CN} CD couplet as a probe of α -helix formation, we studied three Ala-based peptides with the following sequences: GKAAAKAAA-Trp_{CN}-Trp_{CN}-KAAAKG (referred to as H0-Trp_{CN}), GKAAAKAAA-Trp_{CN}-A-Trp_{CN}-KAAAKG (referred to as H1-Trp_{CN}), and GKAAAK-Trp_{CN}-AAA-Trp_{CN}-KAAAKG (referred to as H3-Trp_{CN}). As shown (Figure 3), the CD spectra of H0-Trp_{CN} and H1-Trp_{CN} show that both peptides are disordered in buffer solution and the Trp_{CN} CD couplet is not detectable. This latter finding indicates that in both cases the Trp_{CN} sidechains do not show any significant and preferential interactions, likely due to the excluded volume effect and/or rigidity of the backbone units separating them. In other words, the two Trp_{CN} sidechains can sample a wide range of spatial orientations with respect to each other and, as a result, leading to a (averaged) undetectable CD couplet. However, upon addition of 20% TFE, α -helix formation is induced for both the peptides and this fixes the Trp_{CN} sidechains to discrete rotameric states, with orientations that favor Trp_{CN}-Trp_{CN} coupling. The CD spectra of H0-Trp_{CN} and H1-Trp_{CN} clearly indicate the development of a CD couplet in the

wavelength region of ~230–255 nm. A closer comparison indicates that the Trp_{CN} CD couplets of these two peptides show measurable differences in both position and amplitude, reflecting the sensitivity of the underlying exciton coupling to the distance between the chromophores and their orientations.

Interestingly, as shown (Figure 4), the CD spectrum of H3-Trp_{CN} obtained in buffer solution contains a significant contribution from the Trp_{CN} residues, indicating that in this case they can preferentially interact with each other. This is consistent with the study of Finnegan and Bowler [22] and other studies [23–26] which showed that aromatic amino acids, particularly Trp and Phe promote residual structure formation under denaturing conditions. Furthermore, in 20% TFE, the Trp_{CN} CD couplet of H3-Trp_{CN}, unlike that of H1-Trp_{CN} (and H0-Trp_{CN}), has a positive long-wavelength component. This is in agreement with the expectation that when using the Trp_{CN} at position 12 as the front chromophore, the angle, γ , will first increase and then change sign when the back chromophore progressively moves away from the front one along an α -helix.

For comparison, we also studied a Trp analog of the H0-Trp_{CN} peptide i.e., H0-Trp (sequence: GKAAAKAAA-Trp-Trp-KAAAKG). As shown (Figure 3), the CD spectrum of H0-Trp obtained in 20% TFE indicates that it also adopts an α -helical conformation, as expected. However, there is no detectable CD couplet arising from the Trp-Trp exciton coupling, whose long-wavelength component is typically located at around 230 nm. Thus, this result and those obtained with the Trp_{CN}-containing helical peptides further corroborate the idea that Trp_{CN} is a more sensitive CD probe than Trp, due to its larger extinction coefficient.

Trpzip5-Trp_{CN}

Cochran *et al.* [7] have shown that a cross-strand Trp pair interacting in an edge-to-face manner can stabilize β -hairpin structures. Their CD measurements indicated that such Trp-Trp interaction produces a CD couplet with a positive long-wavelength component centered around 230 nm. Thus, to further test the utility of the Trp_{CN} CD couplet in monitoring β -hairpin formation, we studied a mutant of one of the β -hairpins designed by Cochran *et al.*, Trpzip5 (sequence: GEWTYDDATKTFTWTE), where the two Trp residues are replaced with Trp_{CN} (the resultant peptide is referred to as Trpzip5-Trp_{CN}). As expected (Figure 5), the CD spectrum of Trpzip5-Trp_{CN} shows the presence of a strong exciton CD couplet with a positive long-wavelength component centered at 248 nm. Further measurements of the CD signal at this wavelength at different temperatures (T) yields a CD T -melt curve (Figure 5, inset) that is similar to that of Trpzip5 [7]. Therefore, taken together, these results provide further evidence that the CD couplet produced by a pair of Trp_{CN} can be used to probe protein tertiary structures.

Phe_{CN}-Phe_{CN} and Phe_{CN}-Trp_{CN} exciton couplets

Since Phe_{CN} exhibits a strong absorption peak around 240 nm [27], which is well separated from the far-UV absorption spectrum of protein backbone, we also investigated whether a pair of Phe_{CN} residues or a Phe_{CN} and Trp_{CN} pair can produce an observable CD exciton couplet. To study the Phe_{CN}-Phe_{CN} coupling, we employed a peptide that has a similar

sequence as that of H0-Trp_{CN}, i.e., GKAAAAKAAA-Phe_{CN}-Phe_{CN}-KAAAAKG (referred to as H0-Phe_{CN}). As shown (Figure 6), the CD spectrum of this peptide collected in 20% TFE shows a detectable Phe_{CN}-Phe_{CN} exciton CD couplet with a positive long-wavelength component at about 242 nm, manifesting its potential utility as a structural probe.

In a recent study on the role of aryl-aryl interactions in β -hairpin folding, Andersen and coworkers [20] systematically examined the effect of various cross-strand interactions that can promote β -hairpin formation. In particular, they found that a peptide with the following sequence, KKYTFNPATGKWTVQE, can fold into a β -hairpin conformation at room temperature, due to a favorable Phe-Trp interaction with Trp occupying the face position and Phe at the edge position. Herein, we utilized this β -hairpin as a model to further test whether the exciton coupling between Trp_{CN} and Phe_{CN} can produce a useful CD couplet by mutating the Trp and Phe residues to Trp_{CN} and Phe_{CN}, respectively (the resultant peptide is referred to as β -Phe_{CN}-Trp_{CN}). As shown (Figure 7), this peptide indeed produces a CD spectrum that exhibits a strong CD couplet arising from Phe_{CN} and Trp_{CN} coupling, thus confirming the notion that a pair of Phe_{CN} and Trp_{CN} residues can be used as a CD probe to interrogate structure and conformational changes in proteins.

Conclusions

While the CD signals resulting from protein backbone units are routinely used to examine protein conformation and conformational changes, they do not offer any site-specific information. On the other hand, an exciton CD couplet arising from two aromatic sidechains is more useful in this regard as it can provide structural information between two specific sites. However, the most frequently utilized sidechain exciton CD couplet in the far-UV spectral region, i.e., that produced by a pair of Trp residues, is relatively weak and overlaps significantly with the protein backbone CD signals. In an effort to introduce alternative and perhaps better sidechain-based CD probes, we investigate the utility of the CD couplets arising from the exciton couplings between two Trp_{CN} chromophores, between two Phe_{CN} chromophores, and between a pair of Trp_{CN} and Phe_{CN} chromophores. Our results show that the exciton CD signals produced by those chromophore pairs are significantly red-shifted from those of protein secondary structures, thus making them more convenient to use in practice. Moreover, we find that under the same conditions, the Trp_{CN} exciton CD couplet is more intense than that arising from a pair of Trp residue, thus making it a more sensitive spectroscopic probe of proteins. Because nitrile-derivatized Phe and Trp unnatural amino acids can now be incorporated into proteins via *in vivo* methods [28, 29], we expect that the CD probes introduced herein will find various useful applications. Finally, a worthwhile future direction would be to develop unnatural amino acid-based CD probes that have not only a strong rotational strength but also absorb in the wavelength region above 300 nm, to completely avoid overlapping with the CD signals arising from protein sidechains and backbone units.

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Figure 1.

Absorption spectra of indole and 5-cyano-indole in methanol, as indicated. In both cases, the solute concentration was 28 mM, as determined by weight.

Figure 2.
CD spectra of CP10A-Trp_{CN} at 4.0 °C in different solvents, as indicated.

Figure 3.

CD spectra of H0-Trp_{CN} and H1-Trp_{CN} at 4.0 °C in 20% TFE and 10 mM sodium phosphate buffer (pH 7.0), as indicated. For comparison, the CD spectrum of H0-Trp at 4.0 °C in 20% TFE is also shown (black).

Figure 4.
CD spectra of H3-Trp_{CN} at 4.0 °C in 20% TFE and 10 mM sodium phosphate buffer (pH 7.0), as indicated.

Figure 5.
CD spectrum of Trpzip5-Trp_{CN} at 1.0 $^{\circ}\text{C}$ in 10 mM sodium phosphate buffer (pH 7.0).
Shown in the inset is the temperature dependence (T-melt) of the CD signal at 248 nm

Figure 6.
CD spectrum of H0-Phe_{CN} at 4.0 °C in 20% TFE.

Figure 7.
CD spectrum of β -Phe_{CN}-Trp_{CN} at 1.0 °C in 10 mM sodium phosphate buffer (pH 7.0).