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Double Tryptophan Exciton Probe to Gauge Proximal Side Chains in Proteins- Augmentation at Low Temperature

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Abstract

The circular dichroic (CD) exciton couplet between tryptophans and/or tyrosines offers the potential to probe distances within 10 Å in proteins. The exciton effect has been used with native chromophores in critical positions in a few proteins. Here, site-directed mutagenesis created double tryptophan probes for key sites of a protein (tear lipocalin). For tear lipocalin the crystal and solution structures are concordant in both apo- and holo-forms. Double tryptophan substitutions were performed at sites that could probe conformation and were likely within 10 Å. Far-UV CD spectra of double Trp mutants were performed with controls that had non-interacting substituted tryptophans. Low temperature (77K) was tested for augmentation of the exciton signal. Exciton coupling appeared with tryptophan substitutions at positions within loop A-B (28 and 31, 33), between loop A-B (28) and strand G (103 and 105), as well as between the strands B (35) and C (56). The CD exciton couplet signals were amplified 3–5 fold at 77K. The results were concordant with close distances in crystal and solution structures. The exciton couplets had functional significance and correctly assigned the holo-conformation. The methodology creates an effective probe to identify proximal amino acids in a variety of motifs.

Keywords

circular dichroism; lipocalin-1; tear lipocalin; conformational state; excited state interaction; short distance assessment

INTRODUCTION

Distance measurements between amino acid residues in proteins can be accomplished by several methods. X-ray crystallography of proteins is one of the most accurate methods and is considered the standard by which other methods are compared. But some proteins, particularly membrane proteins, are difficult to crystallize and certain regions of many proteins may reveal poor electron density. Proteins in solution are highly dynamic so a

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SUPPORTING INFORMATION

Far UV CD spectra of double Trp mutants of tear lipocalin in 60% TFE at 295K. This material is available free of charge via the Internet at <http://pubs.acs.org>.

single crystal structure may underestimate inherent variations in conformation.¹ Nuclear magnetic resonance is an excellent method for proteins less than 20–25 kDa when millimolar concentrations of proteins and sophisticated equipment are available. Electron paramagnetic resonance methods capture a range at close distance starting from about 8–20 Å.^{2–3} Disulfide bonds and cysteine-rich proteins may complicate mutagenesis and labeling. Fluorescence methods, e.g. fluorescence resonance energy transfer, are quite good for distance measurements of 10–100 Å between a donor –acceptor pair.⁴ Site-directed tryptophan fluorescence has developed in our laboratory as a method to specifically assign specific amino acids to secondary structural motifs. This site-resolved protein structure permits homology modeling, observation of loop motion, and identification of specific rotameric configurations.^{5–11}

Woody proposed an alternative method for probing distance measurements by Trp-Trp excited state interactions manifested as circular dichroic exciton coupling.^{12–13} Exciton coupling may occur between a combination of native tryptophans and/or tyrosines that are proximal (<15 Å apart) and within proper geometric constraints in proteins or peptides.^{12, 14} However, in some cases, predicted and observed exciton coupling signals did not correlate despite elaborate calculations. The potential obfuscation by other aromatic chromophores in concert with variable side chain orientation of the target chromophores may have contributed to the disparity. The use of CD exciton coupling has generally been limited to proteins that have proximal native tryptophans/tyrosines.^{12–13, 15} In some of these cases substitution of tryptophan by weaker chromophoric amino acids mitigates exciton coupling to reveal the functional nature of specific tryptophan residues.^{13, 15} An opportune extension of this work and the focus here is to substitute 2 potentially proximal native non-tryptophanyl amino acids with tryptophans to induce exciton coupling in the far UV region. Recent work with the near UV CD showed that low temperature altered the distribution of side chain rotamers to populate the lower energy conformation states.^{5, 16} It follows that reducing flexible conformations at low temperature should enhance the bisignate exciton coupling signal by increasing the populations of energetically favored conformations in Trp-Trp interactions.

EXPERIMENTAL METHODS

Materials

Reagents for solutions were purchased from Sigma-Aldrich (St. Louis, MO).

Site-directed mutagenesis and plasmid construction

The lipocalin-1 (tear lipocalin or LCN1) gene, spanning bases 115–592 of the sequence,¹⁷ was cloned into pET 20b (Novagen, Madison, WI). Synthesized tear lipocalin cDNA¹⁸ was used as template. Flanking restriction sites, NdeI and BamHI, were inserted. The native protein sequence retained the initiating methionine.¹⁹ The previously characterized tear lipocalin mutant, W17Y,²⁰ was the template to construct mutants with a single Trp. Mutants were constructed with oligonucleotides (Invitrogen) using QuikChange II site-directed mutagenesis kit (Stratagene) to introduce point mutation in the cDNA. All mutations were confirmed by sequencing. Amino acid 1 corresponds to His, bases 115–117, as previously

published.¹⁷ For exciton CD signal detection, single and double Trp mutants were produced. The sites of single tryptophan mutations were chosen to avoid interaction with Tyr (Figure 1). Single Trp mutants of tear lipocalin include: W17Y/F28W (for simplicity W28); W17Y/M31W (W31); W17Y/N32W (W32); W17Y/L33W (W33); W17Y/E34W (W34); W17Y/S35W (W35); W17Y/V36W (W36); W17Y/L56W (W56); W17Y/G103W (W103); W17Y/L105W (W105) and W17Y/V110W (W110). Double Trp mutants of tear lipocalin include: W28W31; W28W32; W28W33; W28W103; W28W105; W28W110; W35W56.

Expression and purification of mutant proteins

The plasmids of the mutants were transformed in *E. coli*, BL 21 (DE3), cells were cultured and proteins were expressed, purified, and analyzed as described.^{10–11} The expressed mutant proteins were used without additional enrichment with ligand with the exception of W28W33. As shown previously, mutant proteins of tear lipocalin expressed in *E. coli* as well as the native protein contain various fatty acid ligands including palmitic acid.^{20–22} Concentrations of the single-Trp mutant proteins were determined using the molar extinction coefficient of tear lipocalin ($\epsilon_{280} = 13760 \text{ M}^{-1}\text{cm}^{-1}$).²³ For tear lipocalin with two Trp residues, the extinction coefficient of $\epsilon_{280} = 19260 \text{ M}^{-1}\text{cm}^{-1}$ was used.

Absorption Spectroscopy

UV absorption spectra of the single-Trp mutants of tear lipocalin were recorded at 295K and 77K using a Shimadzu UV-2400PC spectrophotometer. All experiments at 77K were performed in glycerol/buffer (1:1, v/v) solution. Buffer was 10 mM sodium phosphate, pH 7.3. Path length was 0.2 mm. Protein concentrations were about 2 mg/ml.

CD spectral measurements

Far-UV CD spectra were recorded for all mutants at 295K and 77K temperatures on a Jasco J-810 spectropolarimeter. The path length was 0.1 mm. The protein solutions for CD measurements were the same as in absorption spectroscopy shown above. Each CD spectrum represents an average of at least sixteen and twenty-five scans for 295K and 77K, respectively. Results were recorded in millidegrees and converted to mean residue ellipticity $[\theta]$ in $\text{deg}\cdot\text{cm}^2\cdot\text{dmol}^{-1}$.

Sample Preparation for Absorption and Circular Dichroism Spectroscopies at 295K and 77K

Sample preparation for low-temperature absorption and CD spectroscopies were identical. The protein solutions (1:1, v/v, glycerol/buffer) in the sample holder (0.1 mm path length) were frozen by dipping into the spectroscopic Dewar filled with liquid nitrogen under slow-freeze conditions.²⁴ The frozen samples at 77K appear transparent but with cracks, which did not cause much scattering in both absorption and CD measurements. As shown previously, cracks in frozen water/glycerol samples do not cause depolarization using a path length up to 0.2 mm.²⁴ The sample holder inside the spectroscopic quartz dewar could be rotated and tilted in X and Y axes to achieve the smallest light-scattering condition possible. Each spectrum at 77K represents the average of at least 25 runs. For far-UV CD measurements at 77K the protein concentrations were about 2.4 mg/ml. Care was taken to

avoid any artifacts as described in reference 24. Each time, the base line was recorded and subtracted. Base lines from multiple sample preparations were similar indicating that no significant artifacts occur in frozen samples. Exciton coupling was determined from difference spectra by subtracting the averaged single-tryptophan mutant spectra of the tryptophan pair from that of the double-tryptophan mutant or from subtraction of that another second double-tryptophan mutant from a tryptophan pair that showed no exciton coupling. In this way the influence of potential Tyr-Trp coupling was vitiated. To account for inherent error in protein concentration (up to 5%) the difference spectra were calculated allowing absorption spectra to vary up to 5% in cases where the lobes of the couplet were asymmetric. This invariably resolved the couplet without a significant effect to the overall spectrum (Figure S1).

RESULTS AND DISCUSSION

The key findings in this work include: 1) tryptophan substitution of native amino acids can be used in combination with CD exciton coupling to verify close proximity in proteins (less than 10 Å), 2) Gauging distances with exciton coupling is effective for residues in loops, between strands and loops, and between strands. 3) Low temperature enhances the exciton coupling CD signal by populating low energy conformational states. 4) Exciton coupling of carefully chosen tryptophans can reflect a functional conformational state (apo- versus holo-forms). Overall, exciton coupling between introduced pairs of Trp residues can be used as an effective tool for monitoring structural and conformational changes in proteins especially at low temperature.

Single Tryptophan Mutations

The introduction of single tryptophan substitutions (Figure 1) did not induce excited state interactions with Tyr residues because the distance between the center of any introduced Trp residue and any Tyr residue in tear lipocalin is greater than 10Å. The rationale for the other mutations was to gauge distances between residues in sites with various conformations 1) within a loop (loop AB), 2) between a loop and B strand (loop AB and strand G), and 3) between 2 strands (strands B and C).

Exciton Coupling from a Trp Pair in a Loop Conformation

Interactions in loops may be missed by crystallographic assessment as poor resolution due to the motion of flexible loops. The mouth of the cavity of tear lipocalin is formed by four flexible loops, A-B, C-D, E-F, and G-H.^{6-7, 11, 25-26} Particularly, for residues Asp-25 to Met-31 of the loop AB of apo-tear lipocalin, the electron density was unclear in crystallographic analysis.²⁷ The loop A-B is the longest within the lipocalin family of proteins and has proven functional relevance. Loop A-B was successfully assigned by site-directed tryptophan fluorescence and positions 28 and 31 were predicted to be in proximity.¹⁰

The results from CD in Figure 2A demonstrate a characteristic bisignate CD curve in subtraction spectra for W28W31 indicative of exciton coupling between these residues. The positive long-wavelength component has a maximum at 229.6 nm. Since the spectrum

averaged from individual spectra (W28 and W32) is virtually identical to W28W32, this double tryptophan mutant can be used for subtraction as well (Fig 2B). Such a strategy may yield less uncertainty since errors in measurements need to be considered only for one mutant protein instead of two. A double Trp protein W28W33 also shows exciton coupling with the positive long-wavelength component positioned at 229.4 nm (Figure 2C). Figure 3 shows that the exciton contribution can also be calculated as a difference of double Trp mutants. Figure 4 shows the distance between rotamers using the predominant rotamer modeled with substituted tryptophans in holo- as well as apo-forms. As predicted those 10 Å yield a coupling signal, while W28–32 is predicted to be about 14 Å apart in both apo- and holo forms and shows no appreciable coupling signal. Interestingly, W28–W33 is modeled to be 10.1 Å (holo-form) and 15.1 Å (apo-form). Yet the amplitude of the signal appears greater than for W38W31, whose separation is only 6.1 Å (holo-form) and 9.7 Å (apo-form). This demonstrates that exciton coupling is not simply a function of distance separation. Therefore the amplitude of the signal alone does not provide a proportional ruler for distance. Rather correct geometric orientation must be produced by various populations of conformers.¹⁴ The theoretical maximal couplet strength has been predicted to occur if two indole rings are in parallel planes and one ring is rotated by 45° relative to the other.²⁸ The exciton effects in the A-B loop confirm that a population with the holo-conformation is likely to exist in expressed tear lipocalin as the distances between W28 and both W31 and W33 are predicted to increase beyond 10 Å in apo-tear lipocalin (Figure 4B). Expressed tear lipocalin is known to be bound to some lipids.⁵ Indeed the exciton effect was further enhanced by saturating W28–W33 with palmitic acid (Figure 5). The conformational basis of the exciton effect in the loop residues is evident as the signal is completely abolished when solvated in trifluoroethanol along with a CD far UV transition of secondary structure from beta sheet to helical (Figure S2). Trifluoroethanol is known to alter the protein secondary structure of the homologous lipocalin, beta-lactoglobulin.²⁹

The exciton effect is enhanced at low temperature (77K) in loop residues (Figure 6 and 7). W28 is known to have energetically favored rotamers that promote a holo-conformation at low temperature.¹⁶ The selection of energetically favorable conformations of Trp at low temperature enhances the exciton signal and permit establishing the proximity of these residues within the loop. The enhancement of the amplitude of the couplet for W28W33 is as much as five fold (Figure 6). Therefore CD exciton coupling of substituted tryptophans at low temperature effectively reveals loop interactions as well as plasticity of the tested regions.

Exciton Coupling of a Trp Pair Between a Loop and β -strand

Strand to loop interactions may also be missed by crystallographic assessment because flexible loops have multiple conformations that cannot be resolved, e.g. apo-tear lipocalin.^{26–27} The positions of the loops were better resolved by site-directed tryptophan fluorescence.¹⁰ The Trp-Trp exciton signals between the loop A-B (Trp28) and the strands G (Trp 103 and Trp105) or H (Trp110) reveal the relative spatial rearrangement of potential long-range interactions (Figures 8 and 9). Despite the small amplitudes the positive long-wavelength components positioned at 229.0 nm and 227.6 nm are consistent with exciton coupling for W28W103 and W28W105, respectively (Figure 8).. The distance

measurements of these residues as well as W28W110 are within about 10 Å. Positions 103 and particularly 105 have been shown to have functionally relevant interactions with a host of ligands.^{8–10, 30} Residue 105 is positioned at the calyx rim, poised to interact with both ligands as well as other residues that could impact conformational selection. The timescale of detecting these interactions by exciton coupling is essentially instantaneous so that the amplitude of the coupling signal is by nature an average of several conformations. Nonetheless, the exciton CD couplet is strong enough for Trp to permit detection even in a heterogeneous environment.

Exciton Coupling of a Trp Pair Between β -strands

There is a precedent for using exciton coupling to monitor cross strand interactions in β strands. Very intense exciton coupling has been shown with the tryptophan zipper peptides consistent with an edge to face orientation of indoles.^{31–34} This arrangement provides an extremely chiral Trp environment and has provided an important model for calculations of electronic CD.³⁵ Exciton coupling was observed from an interaction of Tyr26-Trp66 buried in a β barrel.¹³ Exciton coupling linked to a pyramid of closely interacting native tryptophans from different strands was used to monitor conformational changes and may have an important functional role in plant photoreception responses.¹⁵ In tear lipocalin strands B/C are flexible and make large excursions in the transition from the apo- to holo-forms.

The Trp-Trp exciton effects between the strands B and C (positions 35 and 56) are shown in Figure 10. A positive exciton couplet is evident with a peak at 231.5 nm in the difference CD spectrum but it is of relatively low amplitude. A comparison at 295K and 77K shows an increase in amplitude and a more defined bisignate curve at low temperature (Figures 7 and 10). The augmented CD signal suggests that low temperature selects for energetically favored rotameric conformations and indicates the protein's plasticity (Figures 7, 10 and 11). Figure 11 illustrates that the tryptophans modeled in tear lipocalin would be much closer in the holo-form commensurate with an exciton effect. Inserting tryptophans as a probe for exciton coupling at low temperature may result in discovery of favored conformations in long-range interactions and sample multiple conformations such as those that accommodate ligand binding.

The technique is quite applicable to study native tryptophan residues that are located in functionally important sites. Introducing a second Trp in close proximity to that of native Trp may be used to follow functionally important conformational states of proteins. Caveats for the use of this technique include the complexities of obfuscating signals from adjacent tyrosines and tryptophans as well as the relative orientation of the indole rings in the aromatic residue probes. Similar to others¹² we did not find a clear proportional effect with couplet signal that correlated with gradations below 10 Å. At room temperature, signals were quite meager compared to that observed in tryptophan zipper peptides.^{31–32, 34} The lack of an exciton effect between 2 tryptophan residues in a protein of unknown structure simply means that either the distance and/or geometric constraints to obtain a couplet signal are not met.

CONCLUSION

An exciton couplet with double tryptophan probes seems to work very well as an intra-molecular feeler gauge to confirm a distance of 10Å or less. Low temperature augments the exciton signal. Functional conformational states that are dependent on close interaction of amino acid side chains can be identified using this method.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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ABBREVIATIONS

CD circular dichroism

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Figure 1.

Ribbon diagram of apo-tear lipocalin to positions of Tyr and introduced Trp residues. Gray balls represent native Tyr (except 17, which is Trp in the native protein) residues. Brown balls represent positions of introduced Trp residues. Trp residues were introduced in pairs to observe exciton effect in far-UV CD measurements. Except positions 35 and 36, all introduced Trp (C^α atom) residues are $\sim 12\text{\AA}$ from Tyr residues, therefore unlikely to show exciton effects from these residues. The closest distance is $9.1\text{\AA}$ for residues 18 and 36 (C^α - C^α).

Figure 2. Far UV CD of double Trp mutants compared to the average CD spectra of the individual single Trp mutants. Insets are the difference spectra to show exciton coupling.

Figure 3. Method of estimating exciton coupling using double Trp mutants. Upper, far UV CD of double Trp mutant and CD difference spectra from the Trp pair that does not show coupling (lower).

Figure 4. Ribbon diagram of holo-tear lipocalin (A) and apo-tear lipocalin (B). The ribbon diagrams (blue, holo) and yellow (apo) were generated from Protein Data Bank entries 3EYC (holo) and 1XKI (apo) with DS Visualizer 3.5 (Accelrys Inc.). For apo-tear lipocalin missing loop fragments (part of the loops AB and GH) were modeled using DeepView/Swiss-PdbViewer version 3.7 (GlaxoSmithKline R&D) in accord with solution structure data (Figure 2 and reference 10). Numbers represent positions of the tryptophans with associated distances between geometric centers of the indole rings (green balls).

Figure 5. Far UV CD spectra of Trp mutants with and without ligand saturation. Inset shows difference CD spectrum to show enhanced exciton coupling. (see also Fig. S2)

Figure 6. Far UV CD at 77K for double tryptophan mutants (upper). Difference CD spectra to show exciton coupling using double Trp mutants (lower).

Figure 7.

Amplitude of exciton coupling versus distances calculated from indole centers using distances as shown in Figures 4, 9 and 11. Black line is the calculated theoretic upper limit for Trp-Trp exciton coupling for corresponding distances adapted from Grishina and Woody.¹² The ordinate of the graph is the ellipticity per Trp pair, not per residue. Therefore, for proper comparison the amplitudes of the exciton signals from this work are multiplied by the number of amino acid residues. Amplitudes are shown at 77K, (open blue circles) and at 295K (closed red circles).

Figure 8.

Far UV CD spectra of double-Trp mutants (upper) and difference spectra (lower) with subtraction of double-Trp mutant spectrum (W28W110) that shows no exciton coupling.

Figure 9.
Ribbon diagram to show positions of Trp residues for exciton CD effects.

Figure 10.

Upper, far UV CD spectra of double-Trp mutant between strands B and C with subtraction spectra (inset). Lower, comparison of far UV CD spectra at 295K and 77K with the difference spectra (inset) to show marked enhancement of exciton coupling signal.

Figure 11. Ribbon diagram for holo-tear lipocalin (cyan) and apo-tear lipocalin (yellow) with Trp side chains colored green (holo) and gold (apo). Distances between the geometric centers (green) of the indole rings are shown.