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Probing Tertiary Structure of Proteins Using Single Trp Mutations with Circular Dichroism at Low Temperature

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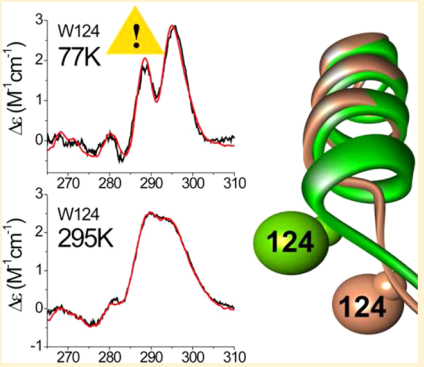
Supporting Information

ABSTRACT: Trp is the most spectroscopically informative aromatic amino acid of proteins. However, the near-UV circular dichroism (CD) spectrum of Trp is complicated because the intensity and sign of ^1L_a and ^1L_b bands vary independently. To resolve vibronic structure and gain site-specific information from complex spectra, deconvolution was combined with cooling and site-directed tryptophan substitution. Low temperature near-UV CD was used to probe the local tertiary structure of a loop and α-helix in tear lipocalin. Upon cooling, the enhancement of the intensities of the near-UV CD was not uniform, but depends on the position of Trp in the protein structure. The most enhanced ^1L_b band was observed for Trp at position 124 in the α-helix segment matching the known increased conformational mobility during ligand binding. Some aspects of the CD spectra of W28 and W130 were successfully linked to specific rotamers of Trp previously obtained from fluorescence lifetime measurements. The discussion was based on a framework that the magnitude of the energy differences in local conformations governs the changes in the CD intensities at low temperature. The Trp CD spectral classification of Strickland was modified to facilitate the recognition of pseudo peaks. Near-UV CD spectra harbor abundant information about the conformation of proteins that site directed Trp CD can report.



INTRODUCTION

UV absorption and circular dichroism (CD) spectroscopies are effective, inexpensive, and fast techniques for the study of aromatic amino acids and proteins to characterize environments and conformational mobility of chromophores.[1−4] Near-UV CD spectra of proteins reflect their tertiary structure via unique spatial arrangements of aromatic residues.[1,5] One challenge is the interrogation of the near-UV region for detailed local tertiary structural information conferred by Phe, Tyr, and Trp. At room temperature individual features often meld into a spectral ensemble. Among aromatic residues, the near-UV spectrum of Trp is most sensitive to its environment. Sequential single amino acid substitution conjoined with computer assisted deconvolution, a technique called site directed tryptophan circular dichroism, successfully permitted using Trp as a natural molecular probe. Specific structural information emerged about the environment of chromophores (for example, asymmetry) that is not accessible with other techniques.[6,7]

The near-UV absorption of Trp results from overlapping ^1L_a and ^1L_b transitions. ^1L_a and ^1L_b transition dipoles are roughly perpendicular.[8] The ^1L_a transition dipole is directed along a line joined by the center of the indole group and the sole N-atom. The involvement of polar group (NH−) and large increase of dipole moment with excitation to ^1L_a make this transition highly sensitive to the polarity of the environment.[8−10] The ^1L_a absorption band is broad and does not show resolved vibrational structure.[1,11,12] The ^1L_b band is much less sensitive to the environment and displays two prominent vibronic bands, 0−0 and 0 + 850 cm^{-1}. In aqueous solutions, the energy of the ^1L_a state is lower than that of ^1L_b. Therefore, the ^1L_a is a fluorescing state for most proteins.

Near-UV absorption and CD spectroscopies of Trp are advantageous because both ^1L_a and ^1L_b bands are revealed simultaneously. However, the sign and intensity of the ^1L_a and ^1L_b bands in the CD spectra vary independently complicating their assignments and interpretation. The prior observation that absorption spectra of biological compounds are sharpened at 77 K,[13] creates potential to separate the contributions of these bands.

Spectroscopic analysis at 77 K is predicated on avoidance of cold denaturation, (denaturation of protein upon cooling). Cold denaturation is a very common phenomenon for globular proteins.[14] Cold denaturation is not observed in most proteins above the freezing point of water. Therefore, denaturation studies use destabilizing agents such as urea or guanidinium chloride. The cold denaturation temperature (T_{cd}) is extrapolated from the zero concentration of the denaturant. For small molecular weight proteins, which show a two-state unfolding, cold T_{cd} is estimated to be about 20 K or more below freezing point of water.[15] Glycerol−water mixtures stabilize native protein structures to prevent cold denaturation.

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Protein in glycerol/water mixture is preferentially hydrated (or glycerol preferentially excluded).[16] Glycerol tends to favor the native state of the protein, i.e., increase free energy of unfolding. This will be evident in the CD spectra presented here for tear lipocalin (TL). Freezing TL in a glycerol/buffer mixture at 77 K yields transparent glass without crystallization (ice formation) of water molecules. To enhance resolution of vibronic structure and probe local tertiary structural elements, low temperature CD and absorption spectroscopies are applied to single Trp mutants of the protein (TL).

TL serves as an excellent paradigm for such experiments as it bears only one native Trp.[17,18] Trp is a very sensitive multispectroscopic reporter group that has been utilized extensively in protein research by fluorescence, CD, absorption, and Raman spectroscopies.[1,6,7,19−24] In TL, application of site directed Trp mutagenesis led to the development site directed tryptophan fluorescence (SDTF).[6,7] The solution structure of TL was resolved by SDTF, in which the nearest environment of each site has been accessed by fluorescence λ_{max} values.[19] The crystal structures of both apo- and holo-TL later confirmed the solution structure.[25,26] Furthermore, protonation/deprotonation triggered structural dynamics, determined by SDTF, have been confirmed by X-ray crystallography.[19,25−27] Rotamer distributions for Trp residues located in the main α-helix have been established by time-resolved fluorescence techniques.[28] The fluorescence data are particularly useful for Trp fluorescence featuring emission from the ^1L_a state.

In this study, Trp residues are sequentially introduced into two distinct segments of TL: the loop AB and the main α-helix. Fine spectral patterns observed at 77 K in the absorption and circular dichroism spectra are compared to those observed at room temperature. The CD spectra are deconvoluted into the ^1L_a and ^1L_b bands. The conformer/rotamer heterogeneity of Trp residues is assessed by comparing the intensities of the circular dichroism spectra recorded at 295 K and 77 K. This approach easily resolves pseudo peaks in published data and affords the opportunity to correctly analyze near-UV CD of Trp by deconvolution into the appropriate spectral components.

EXPERIMENTAL METHODS

Materials. All materials used in preparation of the solutions of the mutant proteins were purchased from Sigma-Aldrich (St. Louis, MO).

Site-Directed Mutagenesis and Plasmid Construction. cDNA from the lipocalin-1 (Lcn1) exon spanning bases 115−592 were cloned into pET 20b (Novagen, Madison, WI).[18,29] Flanking restriction sites for NdeI and BamHI were added to produce the native protein sequence with retention of the initiating methionine.[17] The previously well characterized TL mutant, W17Y,[30] served as the template to construct the mutants with a single Trp. Mutants plasmids were constructed with oligonucleotides (Invitrogen) using QuikChange II site-directed mutagenesis kit (Stratagene). cDNA with the introduced point mutations were sequenced. Amino acid 1 corresponds to His, bases 115−117.[18] For low temperature CD and absorption spectroscopies, single Trp mutants were produced in two specific segments of TL, the main α-helix and the loop AB. Single Trp mutants in the main α-helix include W17Y/L124W (for simplicity denoted as W124); W17Y/E125W (W125); W17Y/A126W (W126); W17Y/L127W (W127); W17Y/E128W (W128); W17Y/D129W (W129); W17Y/F130W (W130). The mutants with single Trp in the loop AB are W17Y/F28W (W28); W17Y/M31W (W31); W17Y/L33W (W33); W17Y/E34W (W34); W17Y/S35W (W35); W17Y/V36W (W36); and W17Y/T37W (W37). The mutants W17Y (Y17) and W17F (F17) were used in the CD spectral fitting to determine the aromatic contributions apart from the introduced single Trp. All mutant proteins have the native fold as previously shown by far-UV circular dichroism.[19,28]

Expression and Purification of Mutant Proteins. The plasmids of the mutants were transformed in *Escherichia coli*, BL 21 (DE3), cells were cultured and proteins were expressed, purified, and analyzed as described.[19,31] The expressed mutant proteins were used without additional enrichment with ligand. As shown previously, mutant proteins of TL expressed in *E. coli* as well as the native protein purified from tears contain a similar array of lipid ligands including palmitic acid.[30,32,33] Concentrations of the mutant proteins were determined using the molar extinction coefficient of TL (ε_{280} = 13760 M^{-1} cm^{-1}).[34]

Absorption Spectroscopy. UV absorption spectra of the single Trp mutants of TL were recorded at 295 K and 77 K temperatures using a Shimadzu UV-2400PC spectrophotometer. All experiments were performed in glycerol/buffer (1:1, v/v) solution. Buffer was 10 mM sodium phosphate, pH 7.3. The path length was 0.2 mm.

Sample Preparation for Absorption and Circular Dichroism Spectroscopies at 295 K and 77 K Temperatures. Sample preparation for low-temperature absorption and CD spectroscopies were identical. The protein solutions (1:1, v/v, glycerol/buffer) were frozen by gradually dipping the sample holder (0.2 mm path length) into the spectroscopic Dewar filled with liquid nitrogen, creating a slow-freeze condition. The frozen samples in 77 K appear transparent but with cracks. As shown previously, cracks in frozen water/glycerol samples do not cause depolarization using the path length up to 0.2 mm.[2] The sample holder inside the spectroscopic Dewar could be rotated and tilted in X, Y axes to achieve the smallest light-scattering condition possible (Supporting Information Figure S1). Utilization of the short path length at 77 K requires the use of high concentrations of TL. For near-UV CD measurements at 77 K the protein concentrations were about 30−40 mg/mL. TL, even at high concentrations, remains monomeric.[35] Care was taken to avoid any artifacts as described.[2] Each time the baseline was recorded and subtracted. The base lines from multiple sample preparations were similar indicating that no significant artifacts occur in frozen samples.

CD Spectral Measurements. Near-UV CD spectra were recorded for all mutants at 295K and 77 K on a Jasco J-810 spectropolarimeter. The path length was 0.2 mm. Each CD spectrum represents the average of at least 16 and 32 scans for 295 K and 77 K, respectively. Results were recorded in millidegrees and converted to molar $\Delta\varepsilon$ in M^{-1} cm^{-1}.

Fourth Derivative Absorption Spectra. A LabView program was designed to calculate fourth derivatives of the absorption spectra using a spectral shift method.[36] In summary, to calculate the first derivative, the spectrum was red-shifted ($\Delta\lambda$ = 1.6 nm), subtracted from original spectrum and, then, blue-shifted by $\Delta\lambda/2$. This procedure was repeated to obtain derivatives of higher order. The calculations were facilitated with a custom program written in LabView (National Instruments) so that the values of the spectral shift and the order of the derivative could be changed or monitored in real time. For noise reduction, the derivative spectra obtained from

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three $\Delta\lambda$ values were averaged, mean value $\Delta\lambda$ = 1.6 nm, as recommended[36] for Trp spectra at 295 K and 77 K.[37]

Fitting the Near-UV CD Spectra. Deconvolution of the CD spectra was carried out using a computer program written in LabVIEW (National Instruments, Austin, TX) as previously described.[7] The best-fit of the near-UV CD spectrum is composed of the best linear combination of the ^1L_b and ^1L_a bands of Trp, the remaining aromatic contribution (designated as Y17 or F17, see below for details), as well as a baseline with varying slope. The published set of ^1L_b and ^1L_a bands was varied in the fitting procedure.[7] The ^1L_b bands of Trp in proteins show variation in amplitudes of components.[6,7] Therefore, the $^1L_b(0-0)$ components of the ^1L_b bands were separated from the remaining components (designated as $^1L_b(r)$) to allow independent variation. The components of the ^1L_b band were shifted in tandem. However, a small independent shift (up to 1 nm to the blue or red side) was allowed for the $^1L_b(0-0)$ and $^1L_b(r)$ components. In the fitting at 77 K, the CD spectrum of Y17 or F17 was used to account for the nontryptophanyl aromatic contribution. The CD spectrum of F17 was used for the mutants that do not show ionized Tyr17 in their CD spectra at 77 K. The CD spectra of Y17 and F17 were allowed to vary within 10% in the fitting procedure. To facilitate the fitting, the initial parameters for the $^1L_b(0-0)$ was determined from fourth derivatives of the absorption spectra. A small spectral shift (up to about 1 nm) was allowed. In some cases, two ^1L_a components were required for acceptable fitting. All near-UV CD spectra were in the range of 260−320 nm with 0.1 nm increments. The goodness of the fit was judged by the normalized root-mean-square deviation (NRMSD) values between the experimental and the calculated spectra. The NRMSD was calculated as

$$\mathrm{NRMSD} = \sqrt{\frac{\sum_i (f_i - y_i)^2}{\sum_i y_i^2}}$$

f_i and y_i are the sequence of fitted and observed values, respectively. Examples of the CD spectra with pseudo peaks were digitized from published figures using OriginPro version 8 (OriginLab Corp., Northampton, MA).

RESULTS

Absorption Spectroscopy at 295 K and 77 K. Representative absorption spectra for the mutant proteins, in which single Trp was introduced sequentially spanning the loop AB or main α-helix, are shown Figure 1. Vibronic bands of all aromatic residues show enhanced resolution at 77 K compared to that of 295 K. In fact, vibronic enhancement is apparent in every single Trp mutant protein (Supporting Information Figure S2). Upon cooling, consistent with previous observations,[38] the spectral components of aromatic residues show a small (up to 0.8 nm) blue shift. Some mutants (Figure 1B) exhibit two additional bands about 299 nm and below 260 nm at 77 K (Supporting Information Figure S2). These bands are indicative of ionized Tyr.[39−42] However, not all mutants that possess Tyr17 show ionization at low temperature. The spectra of mutant W130, in multiple sample preparations at 77 K, showed both ionized and neutral Tyr17. Ionization did not correlate with the speed of immersion of the sample holder in liquid nitrogen, i.e., a fast plunge versus slow (gradually over several minutes). The basis of the ionized tyrosine at low temperature is the close interaction with arginine. An adjacent

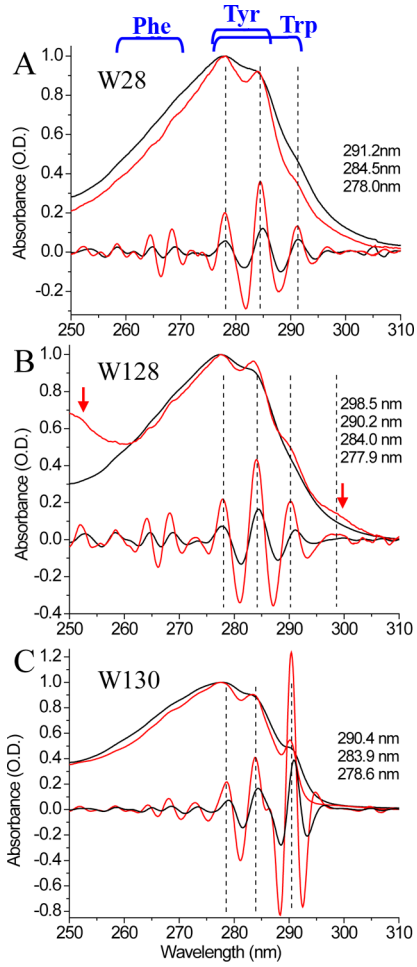

Figure 1. Representative absorption and their corresponding fourth derivative spectra of single Trp mutants of TL at 295K (black lines) and 77 K (red lines). The amplitudes of all spectra are normalized to unity. The fourth derivatives of absorption spectra have both positive and negatives values. The positive peaks correspond to the respective peaks of absorption. Trp residues in W28 (A) and W128 (B) are located in the loop AB and the main α-helix, respectively. W130 (C), located in the middle of the α-helix, shows very high resolution of vibronic bands. Red arrows in (B) indicate bands responsible for the ionized Tyr17. The wavelengths in the Figures show positions of dashed lines. Blue drawings indicate the spectral regions for the peak positions of particular aromatic amino acids (blue letters).

positively charged arginine may significantly decrease the pK_a value of tyrosine by 2.5 units.[43] The cation-π interaction energy is significant for native Trp17 and Arg118 in TL.[39] Perhaps, small differences in the side chain packing are responsible for the variation in ionization of Tyr17 at 77 K.

Beside the peaks of Phe (below 270 nm) and ionized Tyr (broad peaks around 299 nm and below 260 nm), all spectra show prominent peaks around 291 nm, 284 and 278 nm, which belong to Tyr and Trp residues. Two of these peaks (284 and 278 nm) can be attributed to overlapping bands of Tyr and Trp residues. However, the $^1L_b(0-0)$ band of Trp (around 291 nm) does not overlap with neutral Tyr residues and, therefore, is suitable to discern site-specific features.

The amplitudes of fourth derivatives of $^1L_b(0-0)$ bands of Trp are augmented more for the sites located in the main α-helix than in the loop region (Figure 2). At room temperature, the average amplitudes of the $^1L_b(0-0)$ bands of Trp that

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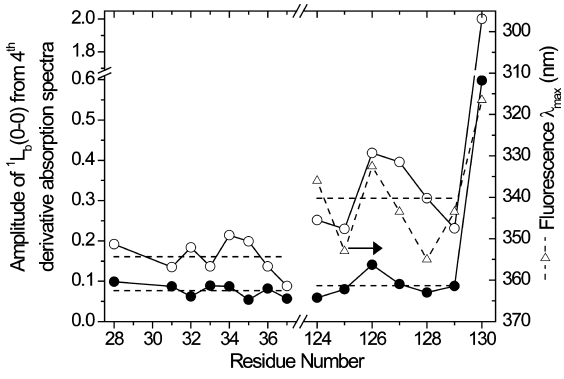

Figure 2. Site-specific features of the absorption spectra of single Trp mutants of TL. Open and filled circles represent the amplitudes of $^1L_b(0-0)$ bands of Trp at various sites (the loop AB: residue numbers 28−37; the α-helix: residue numbers 124−130) at 77 K and 295 K, respectively. Open triangles represent fluorescence (corrected) λ_{max} values of Trp at corresponding sites. The dashed lines show the average value of the amplitudes of $^1L_b(0-0)$ bands that were obtained from fourth derivatives of the normalized absorption spectra.

reside in the loop AB and the α-helix are very close: 0.08 ± 0.02 and 0.09 ± 0.03, respectively. However, at 77 K, the amplitudes for the loop AB and the α-helix are 0.16 ± 0.04 and 0.31 ± 0.08, respectively. The amplitudes of $^1L_b(0-0)$ bands and the fluorescence λ_{max} of Trp located within α-helix show similar trends. As evident from Figure 1, an exceptionally resolved $^1L_b(0-0)$ band was observed for Trp130, which corresponds to the most hydrophobic environment in TL.[19]

Far-UV CD Spectroscopy at 295 K and 77 K. Far-UV CD spectra of Y17, the TL mutant that was used as a template for single Trp mutations, at 77 K and 295 K were inspected (Supporting Information Figure S3) to make sure that no structural changes occur upon cooling. Indeed, at 77 K, the far-UV CD spectrum of Y17 retains both band position and shape. However, as can be expected, the CD spectrum of Y17 is narrower at 77 K than at 295 K. The data indicate that TL maintains its secondary structure at 77 K.

Near-UV CD Spectroscopy at 295 K and 77 K. Despite the better resolved vibronic bands at low temperature, the CD spectra of single Trp mutants at 77 K resemble those at 295 K. This fact indicates that low-temperature CD spectra are free of artifacts. New bands in the CD spectra at low temperature are observed only for the mutants in which Tyr17 was ionized upon cooling. The CD spectra of several mutants required two ^1L_a bands for satisfactory fitting.

Low-Temperature Near-UV CD Spectroscopy of Trp Residues Located in the α-Helix. The CD spectra of W124, W127, and W130 as well as their deconvolution into individual components are shown in Figures 3, 4, and 5, respectively. Other near-UV CD spectra of the Trp residues located in the main α-helix of TL are shown in Supporting Information, Figure S4. Upon cooling, the W124 shows the most enhanced ^1L_b band (Figures 3 and 6). The increase of the ^1L_b band of the CD spectra depends on position of Trp in the α-helix.

The trends of the ^1L_a band intensities of almost all of the Trp residues in the α-helix sites are remarkably similar at 295 K and 77 K. Unlike the ^1L_b, all Trp residues in the α-helix show enhancement ^1L_a bands upon cooling (Supporting Information Figure S4 and Figure 6). Furthermore, the intensities of the ^1L_a bands of the Trp residues at sites from 125 to 130 at 77 K correlate with their fluorescence λ_{max} values at room temper-

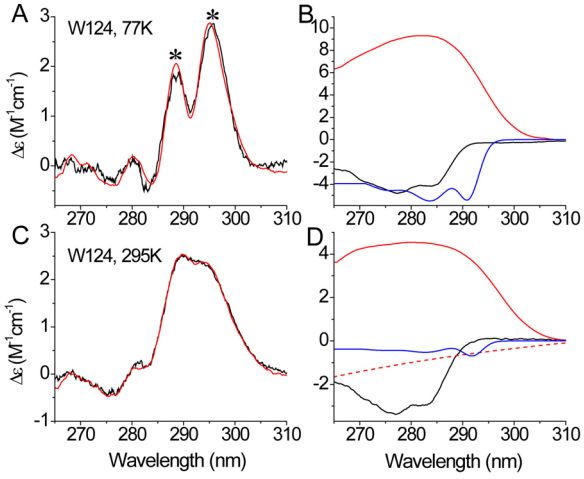

Figure 3. Deconvolution of the near-UV CD spectra of W124 at 295 K and 77 K. Black and red lines represent the experimental and best-fit spectrum at 77 K (A) and 295 K (C), respectively. Spectral components of W124 at 77 K (B) and 295 K (D) obtained by deconvolution: black, Y17 contribution; blue, ^1L_b band, solid and dashed red, ^1L_a bands. Asterisks connote pseudo peaks.

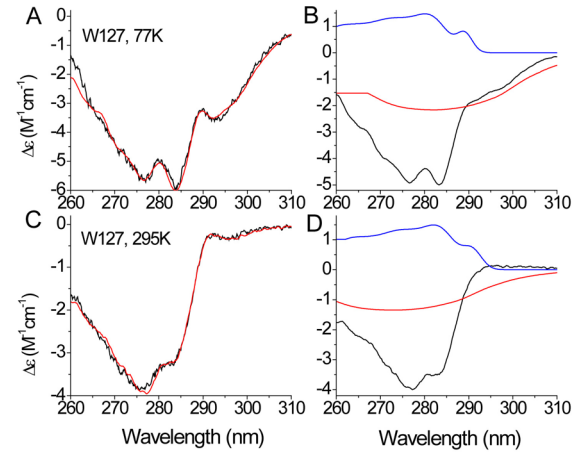

Figure 4. Deconvolution of the near-UV CD spectra of W127 at 295 K and 77 K. Assignments in panels A−D are the same as in Figure 3.

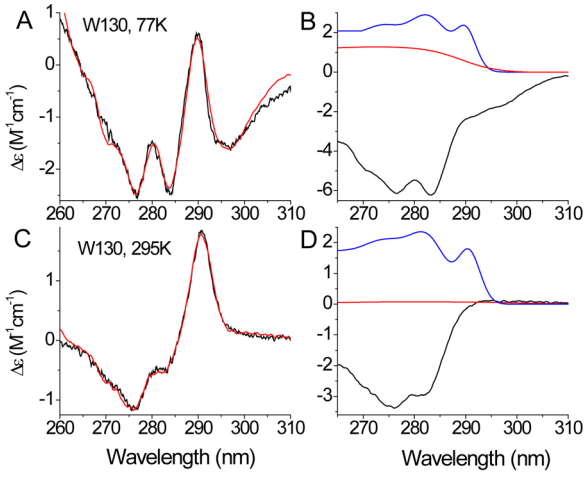

Figure 5. Deconvolution of the near-UV CD spectra of W130 at 295 K and 77 K. Assignments in panels A−D are the same as in Figure 3.

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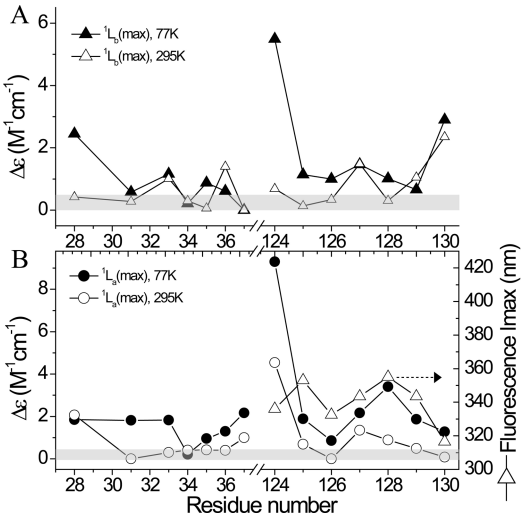

Figure 6. The amplitudes of ^1L_b (A) and ^1L_a (B) bands of Trp residues at 295 K and 77 K located in the loop AB and the α-helix. Triangle and circle symbols represent ^1L_b (A) and ^1L_a (B) bands, respectively. Only absolute values of the bands are plotted. When two ^1L_a bands were required for fitting, just the greater amplitude value was taken.

ature (Figure 6B). Explicitly, more hydrophobicity, which is reflected in decreased λ_{max} value, leads to less intensity of the ^1L_a band.

Low Temperature Near-UV CD Spectroscopy of Trp Residues Located in the Loop Regions. The near-UV CD spectra of W28 and W33 (Figures 7 and 8, respectively)

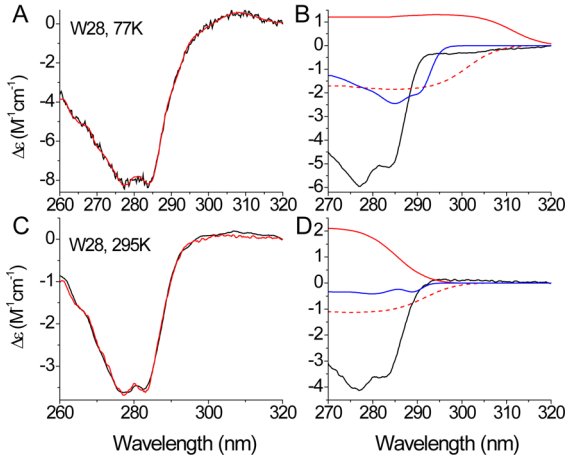

Figure 7. Deconvolution of the near-UV CD spectra of W28 at 295 K and 77 K. Assignments in panels A–D are the same as in Figure 3.

represent Trp in sites of the loop AB. CD spectra of other mutants along the loop AB are shown in the Supporting Information Figure S5. At 295 K, the Trp's that flank the loop AB, 28 and 37, show the highest intensity of ^1L_a bands therein (Figure 6 and Supporting Information Figure S5). Two ^1L_a bands are required for fitting the CD spectrum of W28 (Figure 7). Upon cooling to 77 K, most sites show enhanced ^1L_a bands in the CD spectra. Deconvolution of the CD spectra of W28 at 295 K and 77 K is revealing. There is no overall enhancement as absolute values of ^1L_a bands remain the same. However, significant red shifts are observed in the ^1L_a bands upon cooling (Figure 7). In contrast to W28, the CD spectrum of W33

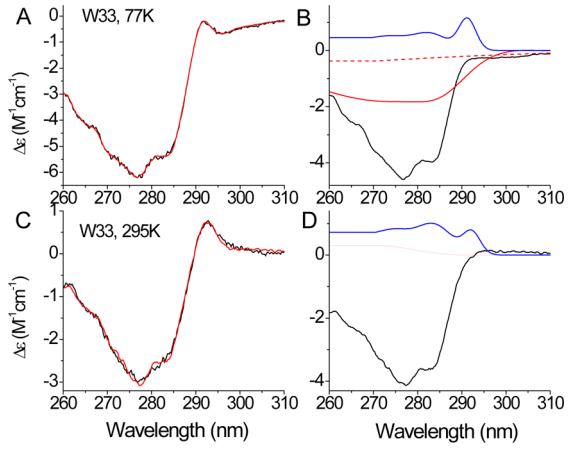

Figure 8. Deconvolution of the near-UV CD spectra of W33 at 295 K and 77 K. Assignments in panels A–D are the same as in Figure 3.

shows a minor ^1L_a band that is enhanced at 77 K (Figures 6 and 8).

For ^1L_b bands at room temperature, Trp residues in only two sites of the loop AB (33 and 36) show appreciable ^1L_b band intensities (Figures 6, 8, and Supporting Information Figure S5). In the CD spectra of W33 the ^1L_b band, unlike the ^1L_a, did not show enhancement upon cooling to 77 K. In general, differences in amplitudes of the ^1L_b bands at 77 K and 295 K vary for each position. At 77 K, only Trp28 shows significantly increased intensity of the ^1L_b band.

DISCUSSION

Several important findings emerge from this study. Trp is an effective reporter group to enhance resolution in low temperature absorption and CD spectroscopies. Alteration of the distribution of side chain rotamers/conformers at low temperature is evident in the deconvoluted CD components of Trp at 295 K and 77 K. Site-specific features emerge at low temperature in residues of the α-helix and the loop that have functional roles in ligand binding. Further, low temperature enhances many spectroscopic features seen at room temperature, validating a physiologic interpretation. Finally, careful fitting of individual components of near-UV CD spectra will prevent the erroneous interpretation of pseudo peaks caused by overlap of ^1L_a and ^1L_b spectra with opposing signs of rotary strength.

Low Temperature CD Enhances Vibronic Structural Resolution. The enhanced resolution at 77 K of the vibronic bands of Trp residues observed in both the near-UV absorption and CD spectroscopies of this study is governed by band narrowing of absorption bands as well as the relative positions of the ^1L_a and ^1L_b bands. In absorption spectra, the side chains of Trp residues in a hydrophobic environment show more enhanced ^1L_b bands compared to a hydrophilic environment. However, relative increase of the ^1L_b bands of CD spectra of Trp upon cooling does not correlate with the hydrophobicity of the environment.

Local Environmental Effects on the ^1L_a and ^1L_b Bands. The local environmental features evident in this study are critically reflected in the intensity and position of the ^1L_a and ^1L_b bands. The polar NH- group of the indole ring of Trp is involved in the ^1L_a transition.[12] Therefore, the ^1L_a band position is sensitive to polarity of the environment. This band is also sensitive to the formation of hydrogen bonds with the NH-

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group as well as the position and sign of a charged group relative to the indole group.[44,45] The maximum of ^1L_a band is observed around 279 nm for Trp in an aqueous environment.[11,12] Hydrophobic and polar environments shift ^1L_a band to the blue and red sides, respectively. In contrast, the polar group is not involved in the ^1L_b band. The transition moment of the ^1L_b band is approximately perpendicular to the ^1L_a band. Therefore, the ^1L_b band is much less sensitive to the environment. Because this band includes hydrophobic groups only, a small red shift is expected for the indole in a hydrophobic versus a hydrophilic environment. The 0−0 transition of ^1L_b band in proteins is situated between 287 and 293 nm.[1] Thus, in a hydrophobic environment ^1L_a and ^1L_b bands shift in opposite directions, blue and red sides, respectively. In this situation overlap of these bands is decreased. In addition, Trp in a hydrophobic environment is shielded from solvent molecules that results in decreased bandwidth for ^1L_b components. Therefore, the resolution of the ^1L_b is enhanced. Indeed, the two most enhanced ^1L_b components in absorption bands are the most hydrophobic sites (positions 126 and 130) for Trp (Figure 2). The side chains of Ala126 and Phe130 face toward the barrel of TL and, therefore, shielded from the solvent.[19] In contrast, the hydrophilic environment shifts the ^1L_a and ^1L_b toward each other. The components of the ^1L_b bands become broader and result in poor resolution of ^1L_b. The data obtained from absorption spectroscopy of the single Trp mutants, such as band positions and amplitude, are very useful in near-UV CD analysis.

Increased CD Intensity upon Cooling Reflects Heterogeneity of Local Conformations. Upon cooling reduced molecular motion results in decreased solute−solvent interaction energies. Inhomogeneous broadening of spectral bands is minimized, the band narrows and accurate band assignment is facilitated. However, these phenomena do not explain increased intensities of the near-UV CD bands at 77 K. The increased rotatory strength of the near-UV CD spectrum at 77 K compared to that of room temperature may indicate conformational mobility at 295 K.[2] Inherent of their dynamic nature, proteins in solution exist in multiple conformations simultaneously (backbone as well as side chain). Upon immersion of a sample in liquid nitrogen, freezing occurs in about 1 s or longer.[46] Under these conditions, the protein can repopulate conformational states. Even in crystal form, cryo-cooling changes the conformational distributions of more than 35% of the side chains of proteins.[47] Alteration in the CD intensity of the introduced Trp upon cooling uncovers conformational heterogeneity of a protein in a site-specific manner. Near-UV CD spectra of aromatic residues in proteins represent an ensemble of conformations. Cancellation may occur if various conformations show different signs of CD bands. Upon cooling the conformational population will be redistributed in accordance with the Boltzmann factor.[2] However, if the conformations are isoenergetic, then no significant redistribution in populations or changes in the CD intensity should be observed. If the conformational free energy difference between two states is about 0.34 kcal/mol then maximum redistribution (fractional population of lower energy state will increase by factor about 1.41) will occur upon cooling from 295 K to 77 K (Supporting Information Figure S6). However, the excited states (high energy) will be invisible if the free energy difference is above 2 kcal/mol (the fractional population at 295K will be <3%). Therefore, no significant gain in the populated low-energy conformations and, therefore, increase in CD spectra upon cooling is expected (Supporting Information Figure S6). Because the contributions of the low- and high-energy conformations to the CD spectra are expected to be different, a multifold increase in intensity may occur at 77 K. As an example, a very high increase of a CD signal upon cooling will occur if the low-populated (high-energy) state has a significantly enhanced CD spectrum that compensates for the contribution of the highly populated state with the opposite sign.

Low Temperature CD in the α-Helix. The ^1L_b bands in the CD spectra of Trp residues introduced to the α-helix sites are instructive. The enhancements of the ^1L_b bands at 77 K are not uniform and greatly depend on the position of Trp in the α-helix. The α-helix has much less backbone conformational flexibility than a loop. Usually both end sites of the α-helix show higher conformational mobility than in the middle. Unlike other lipocalins, TL shows significant conformational changes in the apo- to holo transition.[25,26,48,49] Accordingly, the position 124 shows marked displacement (4.90 Å) in the α-helix upon ligand binding (Figure 9). This suggests that the N-terminus residue, W124, is conformationally mobile to sample multiple conformations and is reflected in greater CD augmentation compared to other sites of the α-helix.

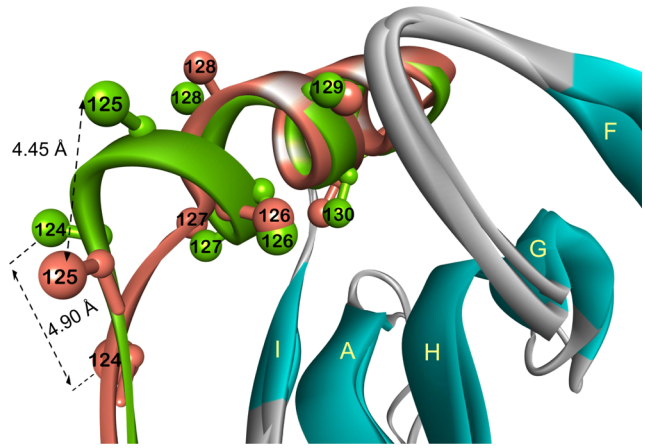

Figure 9. Ribbon diagrams to show the α-helix segment of TL in apo- and holo- forms. Green and orange backbones represent holo- and apo-forms, respectively. Upper case letters indicate the identity of the β-strands. Big and small balls are for C^α and C^β atoms, respectively. The ribbon diagrams of TL in apo and holo-forms were generated from the PDB files, 1XKI and 3EYC, respectively, with DS Visualizer 3.5 (Accelrys, Inc.).

The side chain of Trp residue at position 130 is positioned between hydrophobic residues of V113 and Leu115 of the strand H.[7,25,26] This interposition imposes constraints reflected by the most accentuated blue shift in the fluorescence λ_{max} value recorded for any site in TL.[7] In addition, fluorescence lifetime analysis of Trp130 indicates that its side chain assumes predominantly (84%) one rotamer (t). Trp residues in the α-helix conformation may assume only two (g^- (+60°) and t (180°)) out of three possible χ_1 rotamers.[50,51] The g^+ (+60°) rotamer is not feasible due to the steric restriction. Additional steric restriction originates from the long-range interaction sites (V113 and Leu115) so that the side chain of Trp130 is predominantly populated by one rotamer (t).

Upon cooling, the changes in the intensities of the ^1L_a bands of Trp in the α-helix sites differ significantly compared to that

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of ^1L_b. Despite these differences, both ^1L_a and ^1L_b bands show the largest increase for the Trp124 (Figure 6B). However, the enhancement of the ^1L_a bands at 77 K is more uniform than the ^1L_b. Because intensities of the ^1L_a bands at 295 K and 77 K as well as the fluorescence λ_{max} values along the α-helix sites all show correlation (Figure 6B), the intensity of this band is a reliable measure for the hydrophobicity. It is very interesting that this phenomenon was observed previously in the β-strand G of TL.[6] Trp at position 99 shows the second most blue-shifted fluorescence λ_{max} (~324.7 nm) for the entire TL protein[19] and a very small ^1L_a band.[19] Differential enhancement observed for the ^1L_b bands in the α-helical segment has a sound basis. It is difficult to generalize such a case for the ^1L_a observed in Figure 6B. More comprehensive data are needed for the ^1L_a band of Trp at various polarities.

Low Temperature UV CD of Loop Structures. Loop sites are particularly informative because rotamer/backbone restriction is insignificant. Conformational heterogeneity of the loop region is expected. The intensities of the CD signal of Trp in the loop AB are smaller than those of the α-helix. Reduced CD intensity was observed in some proteins with multiple Trp residues situated in loop regions.[52] Regardless of the origin of conformational heterogeneity (single Trp in multiple conformations or multiple Trp in various conformations), multiple conformations of Trp lead to the low-intensity CD signal. Because the ^1L_b band shows low intensity of most residues in the loop AB, 29−37, correlation between the positions of ^1L_b(0−0) band structure could not be made at 295 K versus 77 K.

However, the loop residue, Trp28, shows marked enhancement in the near-UV CD spectra upon cooling (Figure 6 and 7). The observed changes are corroborated by abundant structural information on TL.[19,25−27,39,49] Only the CD spectrum of Trp28 at 77 K required a DMSO-like ^1L_b band[7] for deconvolution. Prior work has shown that the DMSO-like ^1L_b band indicates a specific interaction with a nearest neighbor residue. For example in mutant W87 this band was indicative of the benzene ring of indole interacting with the methylene group of the nearest neighbor residue Lys76. In W28 (holo-TL) the benzene ring of indole interacts with its nearest neighbor residue His106. In the apo- to holo-transition Trp28 (originally Phe) shows significant displacement.[25,26,49] The g^- (−70.4°, +100.5°) conformation of Trp28 may be stabilized by both cation-π interaction with Lys108 [39] and interaction with His106 (Figure 10). Ligand binding in TL proceeds via conformational selection.[49] Therefore, several conformations of the loop AB are expected, including a specific conformation observed in holo-TL.[25] Upon cooling Trp28 may populate a holo-like conformation. This conformation also matches the observed red-shift in the ^1L_a band. Indeed, a positively charged group near the benzene ring of indole induces a red-shift in ^1L_a band.[44,45] His residues, as well as other aromatic residues, near (less than 10 Å) Trp enhanced the near-UV CD spectral signal due to μ- μ coupling.[1] Thus, the nearby His residue at position 106 may account for the increased ^1L_b band intensity of Trp28 at 77 K.

Types of CD Spectra Prone to Pseudo Peaks. CD spectra of Trp residues in proteins are classified according to the relative contributions of ^1L_a and ^1L_b bands.[1] Type I has a strong ^1L_b band (dominant 0−0 and 0 + 850 cm^{-1} vibronic bands) overlapping a weak ^1L_a band. Both bands have the same sign, negative or positive. Type II spectra display mainly ^1L_a bands that lack major vibronic structure. In type III spectra, both ^1L_a and ^1L_b transitions display noticeable CD bands. However, in some cases, it is difficult to differentiate actual from pseudo peaks in spectra of this type. Therefore, we propose that type III spectra should be subdivided into two groups. Type IIIa spectra feature ^1L_a and ^1L_b bands of the same signs (both negative or positive). In this case, the components of ^1L_b band are easily recognizable in the spectra over the ^1L_a band. In type IIIb spectra, ^1L_a and ^1L_b spectra have opposite signs. In this case, pseudo peaks arise that could be easily taken as ^1L_b bands.[1] Particularly, one should be very cautious if the 0−0 transition of ^1L_b is assigned to the relatively broad peak that resides in the region exceeding 293 nm. In most cases, for proper interpretation of type IIIb spectra, the position of the 0−0 transition of ^1L_b should be carefully examined from the absorption spectra.[1]

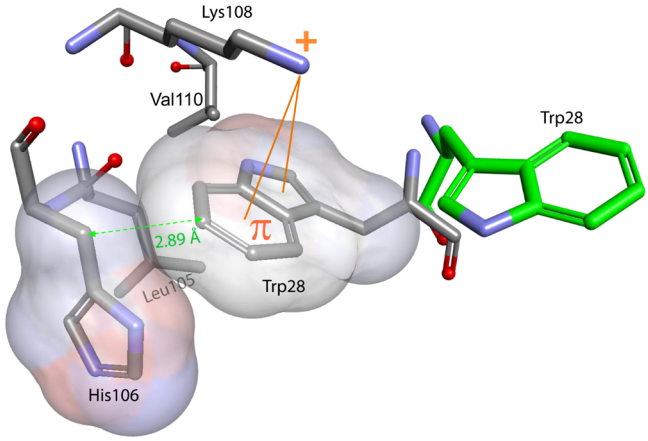

Figure 10. Positions of Trp28 (F28W mutant) in apo- and holo-TL, coordinates of which were taken from Protein Data Bank entries 1XKI and 3EYC, respectively. Molecules were superimposed using DS Visualizer 3.5 (Accelrys Inc.). The rotamers of Trp28 in apo- and holo-TL were generated by the software. Distance between centers (midpoints of atoms C8 and C9) of Trp residues in apo- and holo-forms is 8.01 Å. Color coding: green and gray for carbon atoms in apo- and holo-forms, respectively; red for oxygen, and blue for nitrogen.

W124 features a type IIIb spectra. The guidance provided about 40 years ago by Strickland works well to analyze the type IIIb spectra.[1] Deconvolution of the CD spectrum of *cyclo* (-His-Trp-) dissolved in water at pH 2 indicated that the negative peaks observed at 288.0 and 279.7 nm comprised the ^1L_b component, which overlap the positive ^1L_a bands (Figure 11A). Recognition of Type IIIb spectra is not trivial as apparent in prior publications. Consider the CD spectra of individual Trp residues in human hemoglobin (named as β37Trp) in oxy- and deoxy-forms (Figure 11B and 11C).[24,53] The ^1L_b 0−0 transition of the near-UV CD spectra of the β37Trp in both oxy- and deoxy-forms were assigned to the broad positive peaks at 297 nm. Consequently, other neighboring positive peaks 287 and 279 nm have been designated to the higher order vibronic bands.[24,53] In proteins, the 0−0 transitions of the ^1L_b bands lie between 287 and 293 nm.[1] The peaks previously assigned to the ^1L_b bands of β37Trp are suspect pseudo peaks. Deconvolution analysis unveils the origin of these spectra (Figure 11B and 11C). Both spectra are not type I[24,53] but type IIIb where the ^1L_b and ^1L_a bands have negative and positive signs, respectively. In the oxy-form, β37Trp of Hb A does not show positive[24,53] but rather negative vibronic peaks at 290.7 nm (0−0), 282.4 nm (0+ 1011 cm^{-1}), and 274.6 nm (0+ 2017

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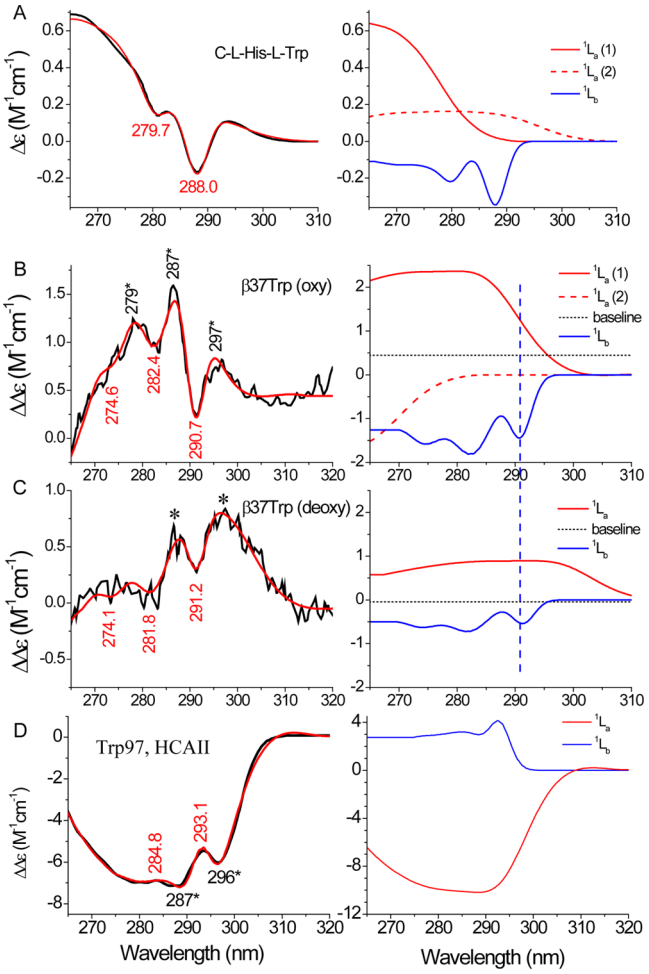

Figure 11. Deconvolution of the near-UV CD spectra of a type IIIb to separate pseudo and real peaks of the ^1L_b bands of Trp. The CD spectra are taken using a digitizer tool of the OriginPro version 8 (OriginLab Corp., Northampton, MA). (A), *cyclo* (-His-Trp-) dissolved in water at pH 2, (1). B and C, *β*37Trp of Hb A, from refs 16 and 46. * indicates pseudo peak positions previously assigned to the components of ^1L_b (refs 16 and 46–48).

cm^{-1}). Strikingly, the energy spacing of the ^1L_b (1011 cm^{-1}) exactly matches the authors' UV resonance Raman (UVRR) data for W16 band. In the deoxy-form, *β*37Trp demonstrates similar negative vibronic peaks at positions 291.2 nm (0–0), 281.8 nm (0+ 1146 cm^{-1}), and 274.1 nm (0 + 2142 cm^{-1}). However, the intensity of the ^1L_b components are significantly decreased (about 2.7 fold for 0–0 transition). The intensity of the positive ^1L_a band of the *β*37Trp in the deoxy-form is also diminished about 2.7 fold in the transition to the oxy-form. Furthermore, a significant red shift is evident for the ^1L_a band in this transition.

Spectral deconvolution reveals specific features of the CD spectra of *β*37Trp in the oxy- and deoxy-forms that are not apparent by conventional inspection.[24,53] Thus, deconvolution of the CD spectra is a valuable tool to correctly determine intensities, signs, as well as positions of the ^1L_a and ^1L_b bands of Trp. As recommended previously,[1] inspection of absorption spectra greatly facilitates identification of CD bands.

The near-UV CD spectrum of Trp97 in human carbonic anhydrase II is another good example for type IIIb spectrum, in which the spectrum is composed of relatively small positive ^1L_b and strong negative ^1L_a bands (Figure 11D).[54,55] Spectral deconvolution reveals that the peaks at about 296 and 287 nm are pseudo peaks. The real components of the ^1L_b bands are positioned at about 293.1 and 284.8 nm (Figure 11D).

The CD spectrum of W124 at 77 K exemplifies a type IIIb spectra with pseudo peaks at 295.5 and 288.3 nm. Deconvolution reveals a strong positive ^1L_a overlapped with a strong negative ^1L_b band (Figure 3B). The 0–0 transition of ^1L_b at 290.8 nm approximately corresponds to the first (from long wavelength side) downward peak position of the composite CD spectrum as well as the position of this band in the absorption spectrum, 291.2 nm (Supporting Information Figure S2). The spectra of W127 represent another example of the type IIIb CD spectra. However, in contrast to W124, the signs of the ^1L_b and ^1L_a bands are reversed (Figure 4). The 0–0 transition of the ^1L_b determined from deconvolution (288.7 nm) closely matches that determined from the absorption spectrum (290.0 nm) (Figure 4B and Supporting Information Figure S2). The significant difference in the shapes of the CD spectra of W127 at 295 K and 77 K is a result of the ionization of Tyr17 at 77 K. The absorption spectrum of W127 at 77 K shows peaks that are typical for the ionized Tyr, as seen for W128 (Supporting Information Figure S2 and Figure 1). The CD spectrum of W130 at 295 K is an example of a type I spectrum. However, at 77 K, the appearance of the positive ^1L_a band befits a type IIIa category (Figure 5).

Type IV spectra are characterized by the presence of the mismatch between the wavelengths of CD and absorption bands. For example, a type IV CD spectrum may arise if a Trp residue has species yielding two ^1L_a bands with shifted λ_{max} values and opposite signs.[1] This is a special case; not all these kinds of situations will yield a type IV CD spectra. Low temperature may enhance ^1L_a and ^1L_b bands differently, but in most cases, the type of the CD spectrum is unchanged.

Relevance of Low Temperature UV and CD Spectroscopy to Physiologic Conditions. Low-temperature absorption and CD spectroscopies may be underutilized due to the lack of systematic studies to show physiological relevance of low temperature CD measurements. Many features of absorption and CD spectra at 295 K and 77 K indicate that low-temperature measurements are relevant to extract site-specific features observed in solution at room temperature. In the *α*-helix region of TL, the trends of variations of the ^1L_b(0–0) band amplitudes are similar at 295 K and 77 K. Therefore, the measurements performed at 77 K identify environmental features of Trp residues relevant to room temperature. Such trends are less obvious for the loop AB (Figure 2), partly, because of small differences of the ^1L_b(0–0) band amplitudes at room temperature

Therefore, low temperature measurements are valid not only for the distance distribution studies but also for characterization of the conformation and the environment of the aromatic residues. Low temperature CD experiments may have advantages over other techniques at low temperature. For example double electron–electron resonance (DEER) performed at 77 K can determine intermolecular distance distributions.[46] [46] DEER experiments have to be conducted at 77 K and, therefore, there are no data at room temperature to compare. Specific data that relate the side chain properties (such as dynamics, polarity of environment, etc.) in frozen and solution are not directly available.

The combination of low-temperature and site-directed tryptophan absorption and CD spectroscopies is complementary.

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CONCLUSION

The CD spectra of Trp residues in proteins gain their rotary signal intensities from interactions from proximal aromatic residues.[1] At low temperature, aromatic amino acids within about 10 Å of a natural Trp probe were affected by conformational repopulations. The near-UV CD spectra at low temperature effectively revealed the low energy conformational states of the protein.

In this study, enhanced resolution of Trp residues at low temperature facilitated assignment and interpretation of the near-UV CD spectral components. The magnitude of the energy differences in the local conformations governs the changes in the CD intensities at low temperature. Upon cooling, repopulation of conformational states will not occur with very low- or very high-energy differences (in terms of k_BT). Under these circumstances, no changes are expected in the CD spectra. Low temperature CD signal enhancement is an effective tool to discern and repopulate the local conformational states with energy differences around k_BT.

In this work, some aspects of the CD spectra were successfully linked to specific rotamers of Trp obtained from fluorescence lifetime measurements. Trp rotamer distribution was considered a measure of conformational heterogeneity. Fluorescence lifetime measurements of Trp residues mainly reveal the χ_1 rotamers (−60°, 180°, +60°).[56] Because of fast (ns regime) interconversion, the lifetimes of the χ_2 rotamers (−90° and +90°) are averaged and, therefore, not time-resolved by fluorescence. However, the absorption phenomena are instantaneous, excluding averaging of χ_2 rotamers. Consequently, in CD spectral analysis of Trp residues, all possible rotamers (combination of χ_1 and χ_2) should be considered. Fluorescence lifetime measurements of Trp residues will be very useful to estimate or exclude the population of the χ_1 rotamers.

The implementation of site directed tryptophan near-UV absorption and CD spectroscopies at low temperature is nascent, but in this study unique features of local tertiary protein structure are revealed. The relatively simple sample preparation and commonly used instruments make these techniques accessible to most laboratories.

ASSOCIATED CONTENT

Supporting Information

Absorption spectra of some single Trp mutants of TL and their fourth derivatives. Near-UV CD spectra of some single Trp mutants of TL and their deconvolution into ^1L_a, ^1L_b, and remaining Tyr components. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

CD, circular dichroism; DEER, double electron−electron resonance; Hb A, human adult hemoglobin; HCAII, human carbonic anhydrase II; NATA, *N*-acetyl-L-tryptophanamide; SDCD, site-directed circular dichroism; SDTF, site-directed tryptophan fluorescence; TL, human tear lipocalin; UVRR, ultraviolet resonance Raman

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