Investigating the interaction of azobenzene moiety on the amino acid tryptophan

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Research Article

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Abstract

Molecular photoswitches are a series of compounds that can be isomerized upon irradiation with light. Photoswitchable molecules can modify the physical, chemical, and biological properties of a diverse range of materials. They can control protein structure and function with temporal and spatial precision. In this work, we investigated the possible interaction between azobenzene and aromatic amino acids. We hypothesized that aromatic amino acids, such as tryptophan, would show altered photochemical properties when conjugated with azobenzene. When irradiated at either 365 nm or 465 nm, the photoswitching behavior favors the cis isomer and is visibly fluorescent at 365 nm. To our knowledge, this is the first evidence to suggest that primary protein structure could affect photoswitch activity. The knowledge gained from this research will help to further the understanding of photoswitches as they are used in biomolecules.

Introduction

It is well known that photocontrol can effectively regulate processes in biological systems. Azobenzene moieties have proven to have a versatile structure as photoswitchable compounds, and they are widely used to control biomolecular structures (Ingerman & Waters, 2009; Zhu & Zhou, 2018). This is due to the connection between azobenzene structure and the electron density distribution of the bonds that contribute to the isomerization of the molecule. The cis and trans states of azobenzene show dramatic geometrical alterations when exposed to light (Fig. 1). Based on previous research studies, the conformational change is sufficient to modify the structure of a protein or a ligand and affect the protein's binding site (G. S. Kumar & Neckers, 1989; Strashkov et al., 2021). However, this phenomenon has not been fully studied when aromatic amino acids are near the azobenzene moiety. The work presented herein is a preliminary examination of the interaction between azobenzene and aromatic amino acids.

At room temperature, the azobenzene chromophore is naturally nonfluorescent in solution, with few notable exceptions in the literature (Watson LE., 2017). These included azobenzene in a bilayer structure, which showed fluorescence emission. This emission was attributed to excited states of aggregated azobenzene moieties (Huang et al., 2018). A decrease in absorption intensity around 365 nm from π◊π* transition and an increase in absorption intensity around 465 nm from n◊π* transition occurred (Blevins & Blanchard, 2004; Das et al., 2019). The trans-cis isomerization processes are reversible by photochemical stimuli. Extensive studies were done by substituting azobenzene amino acid derivative for proline residue in melittin (Ventura & Wiedman, 2021). Azobenzene moiety-coupled aromatic amino acids, however, have not been extensively studied. In this study, we synthesized the combined tryptophan (W), a naturally fluorescent amino acid, and 4(PhenylAzo)benzoic Acid (Z) using Fmoc solid-phase peptide synthesis (SPPS) to investigate the effects of the photoswitchable molecule and the interference with the isomerizing state of the azobenzene. We hypothesized that this combination would yield a molecule with unique optical characteristics. The absorbance measurement of the peptide was used to assess the molar extinction coefficient, an essential intrinsic characteristic (A. Kumar et al., 2006). In this study, we
aimed to comprehend the interaction of azobenzene moiety near aromatic amino acid, Tryptophan, and studied the aggregated states of the compounds.

**Materials And Methods**

**Chemicals**

The 4(PhenylAzo)benzoic Acid was purchased from TCI America (Portland, OR). The Fmoc amino acids used for synthesis were purchased from CreoSalus, Advanced Chem Tech (Louisville, KY). The Wang amide resin was purchased from EMD Millipore (Burlington, MA). O-(1H-6-Chlorobenzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HCTU) was purchased from Peptides International (Louisville, KY). Dimethylformamide (DMF), N,N-diisopropylethylamine (DIEA), acetonitrile, methanol, trifluoroacetic acid (TFA), acetic acid, diethyl ether, triethylsilane (TES), acetonitrile, and other chemical supplies were purchased from Sigma-Aldrich (St. Louis, MO).

**Peptide synthesis**

Three peptides were synthesized using tryptophan (W), 4(PhenylAzo)benzoic Acid (Z), and Alanine (A) to create WZ, AW, and AZ using solid support wang resin (100–200 mesh) along with standard solid phase peptide synthesis techniques (Hood et al. 2008). All amino acids used contained Fmoc as a protecting group along with various compatible side chain protecting groups. The first amino acid coupling was repeated four times to ensure quantitative labeling to wang resin 100–200 mesh before proceeding to the first deprotection. All other amino acids were coupled once. The peptides were coupled using 3:3:6:1 molar ratios of amino acids: HCTU: DIEA:wang resin. A solution of 10% piperidine in dimethylformamide (DMF) (v/v) was used to deprotect all amino acids. Amino acids were activated using HCTU in DIEA along with three times molar excess of the amino acid to be coupled. The coupling was monitored using a qualitative Kaiser Test. Peptides were cleaved using a mixture of 95% TFA, 2.5% TES, and 2.5% water (v/v/v) for two hours. The resulting solution was collected in a falcon tube. A steady stream of N2 gas was used to evaporate the liquid. The remaining dried material was diluted to 10 mL in 10% acetic acid in water. The samples were frozen in the −80°C freezer, lyophilized, and stored for further purification.

**Purification**

A Varian Prep Star chromatographic system (Palo Alto, CA) was used to purify the peptides. The Samples were diluted to 5 mL in 50% acetonitrile in water (v/v) with 0.1% formic acid (FA) buffer to make a 5 mg/mL concentration. The mixture was filtered through 0.45 mm filter and sonicated to remove air bubbles. The 5 mL filtrate was loaded into a 10 mL injection loop. The preparatory column used was a Phenomenex Luna 10 C18 column (250 × 21 mm, 10 µm, 100 Å). A 5 mL per minute flow rate was used at a gradient of 5–95% acetonitrile (ACN) with 0.1% formic acid (FA). The 220 nm and 280 nm channels were used to monitor and detect the sample via dual channel detectors. Fractions were collected in 50 mL falcon tubes and the volumes were reduced to 10-20mL on a roto-evaporator. Solutions were frozen in the −80°C freezer. The synthesized peptides were lyophilized to dry powder and characterized.
High-performance liquid chromatography (HPLC) Shimadzu Nexera-i LC2040c device equipped with a photodiode array detector was used to determine the purity of crude and purified peptides. A Phenomenex Hypersil 5 µm ODS (C18) 120Å, 125 x 4 mm LC Column was utilized as the stationary phase to determine the purity of the peptides. Concentrations of 1 mg/mL peptides were produced in 50% ACN containing 0.1% FA and 50% water containing 0.1% FA. Experiments were conducted using a flow rate of 1 mL/min and injection volumes of 10 µL. A gradient of mobile phase 5–95%, water containing 0.1% FA and ACN containing 0.1% FA. The data was gathered using the Photo Diode Array (PQD) detector with a spectral window ranging from 200 to 456 nm. The percent purity was determined as area under the curve of peaks in the chromatogram.

Mass characterization

Peptides were analyzed with a Hewlett Packard 1100 MSD Electrospray Mass Spectrometer to determine their properties. After producing peptides at a concentration of 1 mg/mL in 50% ACN containing 0.1% FA and 50% water containing 0.1% FA. Flow rates of 0.5 mL/min were used for direct injections of 10 µL. Chemstation software was utilized for peak deconvolution.

Spectrophotometer

A NanoDrop 1000 Spectrophotometer (Thermo Scientific) was used to measure absorbances of the synthesized dipeptides. The samples were diluted using various solvents depending on the experiment. UV light was used to irradiate the peptides depending on the experiments. This was accomplished with a Blak-Ray UV Lamp, 366 nm wavelength, 115 V, 60 Hz, 0.16 amps, manufactured by UVP Inc. of Upland, California, and the blue (visible) light at 450 nm wavelength. The samples were tested using the spectrum, 220nm-750nm. The NanoDrop 1000 Spectrophotometer is equipped with a 1mm path length and operating software 1000 version 3.8.1 for data analysis.

The Beer-Lamber law was utilized to calculate the extinction coefficients of the peptides:

Equation 1: \( \epsilon \lambda = A / (b * c) \)

Where \( A \) = absorbance value, \( \epsilon \lambda \) = wavelength-dependent molar absorptivity coefficient or extinction coefficient with units of \(/\text{M}/\text{Cm}\), \( b \) = the path length in centimeters, \( c \) = analyte concentration in moles/liter or molarity (M).

Plate Reader

Peptide measurements were evaluated using the Tecan Spark plate reader. Depending on the experiment, different solvents and parameters were used for the samples. The samples were analyzed using parameters of excitation wavelength of 280 nm, excitation bandwidth of 5 nm, emission wavelength start of 300 nm, emission wavelength end of 600 nm, and emission bandwidth of 5 nm. The purified peptides were analyzed using parameters of excitation wavelength 365 nm, excitation bandwidth 5 nm, emission wavelength start 380–600 nm, and emission bandwidth 5 nm. The purified peptides were analyzed using.
parameters used excitation wavelength start 280 nm, excitation wavelength start 380 nm, excitation bandwidth 5 nm, emission wavelength 400 nm, Emission bandwidth 5 nm. A 3D scan was performed using the following parameters: excitation wavelength starts 280-380nm, excitation bandwidth 5 nm, emission wavelength 400–600 nm Emission bandwidth 5 nm.

Results And Discussion

Azobenzene and several of its derivatives undergo reversible changes from the typically more stable \textit{trans-form} to the less stable \textit{cis-form} upon exposure to Ultraviolet light, resulting in a wavelength-dependent photostationary structure (Dokić et al., 2009; Fedele et al., 2022). Red-shifted azobenzenes tend to undergo rapid \textit{cis-to-trans} conversions. The conformational modification generated by isomerization of azobenzene derivatives has been exploited to regulate the biological characteristics of numerous systems (Beharry & Woolley, 2011; Song et al., 1997). As part of this research into the photoswitching behavior of 4(PhenylAzo)benzoic Acid, several peptides were synthesized and characterized, as shown in Fig. 2. Given the apparent significance of these molecules, these peptides, Z)-(4-(phenylidazenyl)(benzoyl)-L-tryptophan (WZ), L-alanyl-L-tryptophan (AW), and 4-(phenylidazenyl)(benzoyl)-L-alanine (AZ), were developed to determine if aromatic amino acids changed the activity of azobenzene and azobenzene derivatives.

Purification and characterization of each peptide using mass spectrometry and high-performance liquid chromatography were done and the data can be found in the supplemental results, refer to supplemental data in Figure S1. The purity for peptides was greater than 85%.

Determination of the concentration of the aqueous samples was performed by measuring their absorbance in the near-ultraviolet region using a spectrophotometer. The control Tryptophan was diluted at various concentrations and scanned from 220 to 750 nm. The maximum absorbance, 280 nm for Tryptophan, at each dilution was selected to calculate the extinction coefficient according to Eq. 1. The average extinction coefficient was 5714 /M/Cm when the control, Tryptophan, was dissolved in 50% acetonitrile. This determined average extinction coefficient for the Tryptophan is comparable to Mach et al. at 5540 /M/Cm (Mach et al., 1992). These data are found in Figure S2, Supplemental result, the extinction coefficient for Tryptophan. The overall extinction coefficient for the synthesized peptides was determined as shown in Fig. 3 and Table 1. For the synthesized peptides, the various concentrations were irradiated for 10 minutes using Ultra-Violet light, and the absorbance of the samples was measured. The measurements were repeated after incubating the samples using visible light. The maximum absorbance versus the concentration was plotted, and the R squared for each plot was greater than 0.99.

Based on the data, the initial Extinction coefficients are comparable to when the samples are irradiated with UV light and visible light, as shown in Table 1. The azobenzene within the peptide, WZ, remained the same when incubated with either visible or ultraviolet light. When irradiated with Ultraviolet light, the WZ is red-shifted and visibly fluorescent, as shown in Figure 4.
Table 1: The average Extinction Coefficient is shown for the peptides at their respective maximum absorbance wavelength.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Average $\epsilon_\lambda$ (M$^{-1}$cm$^{-1}$)</th>
<th>Wavelength (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>W</td>
<td>5714</td>
<td>280</td>
</tr>
<tr>
<td>WZ Initial</td>
<td>13331</td>
<td></td>
</tr>
<tr>
<td>WZ 10 min Vis</td>
<td>13186</td>
<td>280</td>
</tr>
<tr>
<td>WZ 10 min UV</td>
<td>13339</td>
<td></td>
</tr>
<tr>
<td>AZ Initial</td>
<td>6693</td>
<td></td>
</tr>
<tr>
<td>AZ 10 min Vis</td>
<td>6701</td>
<td>274</td>
</tr>
<tr>
<td>AZ 10 min UV</td>
<td>6735</td>
<td></td>
</tr>
<tr>
<td>AW Initial</td>
<td>11194</td>
<td></td>
</tr>
<tr>
<td>AW 10 min Vis</td>
<td>11066</td>
<td>265</td>
</tr>
<tr>
<td>AW 10 min UV</td>
<td>11608</td>
<td></td>
</tr>
</tbody>
</table>

The fluorescence intensity of light-induced isomerization was measured for the synthesized peptides. All purified samples were serially diluted at various absorbances. Using the following parameters on the spectrofluorometer: excitation at 365 nm and emission wavelength 380–600 nm, the samples were initially measured using a 96-well black plate. The samples were irradiated with 365 nm light. They produced fluorescence color for the aromatic amino acid, tryptophan when combined with azobenzene, while the other samples were not, as shown in Fig. 4, for the other two peptides. When exposed to either visible light or ultraviolet light, the azobenzene in the W-Z peptide was locked in the cis isomer, as seen in Fig. 5.

The data in Fig. 6 shows that when the WZ sample is excited at 365 nm, the sample emits maximum Relative Fluorescence Unit (RFU) at around 475 nm. This value did shift significantly upon exposure to UV light or to visible light in a similar manner to the data presented in Fig. 5. This suggests that the molecule created generates visibly fluorescent compounds that favor the cis isomerization of the azobenzene rings.

The aggregation characteristics of photoresponsive azobenzene-containing compounds have been investigated in the past by other researchers (Lu et al., 2016; Zhu & Zhou, 2018). In high concentrations or aggregation, fluorophores exhibit strong intermolecular π–π interactions, resulting in either weak emissions or non-emission in aggregates. To study this effect, the visibly fluorescent materials, WZ, were
serially diluted, and the visibly fluorescent, red-shifted were observed when irradiated using the UV light at various concentrations, as shown in Fig. 7. The persistence of fluorescence at low concentrations suggests that this is not merely an aggregation effect. To further prove this point, the WZ peptide was denatured by adding 2 µL of concentrated Hydrochloric acid (HCl). The red-shifted peptide was no longer visibly fluorescent and the RFU intensity was significantly reduced when excited at 365 nm with an emission 380 nm to 600 nm as shown in Fig. 8.

The Stokes shift is the difference in wavelength between the spectral position of the maximum of the first absorption band and the maximum of the fluorescence emission. Small Stokes shift limits the range of applications and is especially problematic for uses (Más-Montoya et al., 2023). In this research study, the peptides were subjected to a 3D fluorescence scan, excitation wavelength 280 nm to 380 nm, emission wavelength 400 nm to 600 nm as shown in Fig. 8a, 8b, and 8c, and the stokes shifts were calculated as seen in Table 2 by identifying the point of maximal RFU and subtracting the excitation wavelength from the emission wavelength.

<table>
<thead>
<tr>
<th>Proportion</th>
<th>Excitation λ (nm)</th>
<th>Max intensity</th>
<th>Stokes Shift</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>288</td>
<td>6783</td>
<td>120</td>
</tr>
<tr>
<td></td>
<td>268</td>
<td>12862</td>
<td>36</td>
</tr>
<tr>
<td></td>
<td>268</td>
<td>6509</td>
<td>42</td>
</tr>
</tbody>
</table>

The peptides clearly exhibited an apparent Stokes shift that was much higher than the tryptophan-alannine or the azobenzene-alanine complex. This again highlighted the fact that proximity to an aromatic amino acid can lead to unusual photochemical properties.

**Conclusion**

The structure and function of a protein can be successfully regulated by a photoswitchable molecule like azobenzene if an appropriate motif is designed. It is therefore crucial to comprehend how amino acids like tryptophan interact and influence the isomerization of a photoswitchable molecule. Our research study revealed that the WZ peptide was visibly fluorescent, red-shifted favored cis isomer when irradiated with Ultraviolet light. This phenomenon was further evaluated as the serially diluted materials' Spectrofluorometer results indicate that the fluorescence is not caused by intermolecular π – π stacking, but rather by the aromatic ring’s influence on the 4(PhenylAzo)benzoic Acid molecule to remain in the cis isomer.

**Declarations**
There are no financial or non-financial interests to report for the authors. There are no declared conflicts of interest between the authors and the actual content of this research. Each author attests that they are not connected to or a part of any group or organization with a financial or non-financial stake in the subjects or materials included in this article. There are no proprietary or financial interests held by the authors in any of the topics covered in this article. Starting funding for this research study was provided by Seton Hall University.

Author Contribution

All writers contributed to the content of this research study. Every author has approved the final version of this research article. All writers made equal contributions.

References


**Figures**
**Figure 1**

The geometric change of cis and trans isomer when 4(PhenylAzo)benzoic Acid compound irradiates with light.

**Figure 2**

Structure of the peptides (a) (Z)-(4-(phenylidazenyl)(benzoyl)-L-tryptophan, (b) L-alanyl-L-tryptophan, and (c) 4-(phenylidazenyl)(benzoyl)-L-alanine. Peptides were all constructed using SPPS and mass was verified using Mass Spec.
Figure 3

The graph of the Extinction coefficients versus the concentration for the synthesized peptides at initial state, after incubating for 10 minutes with visible light, 465 nm, and after irradiating for 10 minutes with UV light, 365 nm.
Figure 4

The synthesized peptides irradiated with UV light, (a) WZ is visible fluorescence red-shifted, when compared to (b) AZ and (c) AW.
Figure 5

The graph of absorbance measurements for WZ at various concentrations. The samples were irradiated for 10 minutes using UV light and incubated using visible light. No changes were observed among UV and visible light samples indicating a lack of conformational change in structure. Values are shown as
Figure 6

Representative traces of peptide fluorescence with and without previous light exposure. The peptide maximum fluorescence was determined initially, after exposure to 365nm light (UV) and after relaxation in visible light (Vis). All purified samples were diluted using 50% of ethanol using various absorbance measurements. Though the RFU values varied, the wavelength of maximum RFU showed no statistically significant difference (ANOVA, p >0.05).
Figure 7

Serially diluted peptides under UV light. The serially diluted WZ peptides exhibited red-shifted characteristics irrespective of concentration and clearly shown by photograph above at concentrations of (a) 1.8mM, (b) 1.3mM, (c) 1.0mM, (d) 0.08 mM, and (e) 0.06 mM.
Figure 8

The peptide bond was disrupted using HCl. Upon cleavage, the peptides were no longer visibly fluorescent and the fluorescence intensity decreased between 450nm and 550nm.

Figure 9

The synthesized peptides were 3D fluorescence scanned, WZ (a), AZ (b), AW (c), using the excitation and emission wavelengths.
Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementalResults.docx