# Discriminating changes to protein structure using PTAD conjugation to tyrosine

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**ABSTRACT**

Many proteins of high interest to biology and biomedical research have few options that can provide even simple insights into structure, such as whether the protein conformation has changed between two states. Tyrosine is an abundant amino acid that balances hydrophobic and hydrophilic properties such that it can be found at protein surfaces, binding interfaces, and buried in a protein core. The reactive compound 4-phenyl-3H-1,2,4-triazole-3,5(4H)-dione (PTAD) can conjugate the phenolic side chain of tyrosine. We hypothesized that individual protein conformations could be distinguished by changes induced to tyrosine reactivity with PTAD. We quantified for a well-folded protein, bovine serum albumen (BSA), the complex distribution of PTAD labeling using LC-MS/MS. As a model conformational switch in BSA, we chose to label unfolded BSA using urea. We found that changes in PTAD reactivity for many tyrosine residues was easily quantified and several sites were highly sensitive to the changes produced by unfolding the protein. This study suggests that the reaction of PTAD to tyrosine may allow for the measurement of a covalent fingerprint that can discriminate conformational states in a protein.

## INTRODUCTION

Protein structure is driven by the interactions of the 20 amino acids with solvent and other amino acids[1, 2]. This basic property can be used to distinguish protein structures and conformational states[3-5]. Some proteins of high interest to biomedical research present unique challenges to structural biology techniques, such as intrinsically disordered and low sequence complexity proteins. Confirming the existence of a predicted protein structure in its native environment in the cell is challenging. Covalent modifications to a protein can be sensitive to protein structure. The strength of covalent bonds endures after proteins are denatured or digested for analysis, preserving a fingerprint that may identify a conformational state.

Tyrosine comprises 3.2% of amino acids contained in proteins, making it a relatively abundant target in native protein structures[6, 7]. The phenolic ring in tyrosine is partly hydrophobic and polar. Tyrosine residues can be found on a protein surface or buried in the hydrophobic core[7-9]. Protein surfaces that bind small molecules, nucleic acids, or protein partners are frequently enriched in tyrosine[8, 10, 11]. The most targeted amino acids for conjugation are lysine and cysteine[12, 13]. Lysine is abundant but usually found only on the protein surface because of its positive charge. Cysteine is rare in proteins and typically buried because of its ability to form disulfide bonds. Even after a conformation change, these residues are constrained to occupy similar positions in the protein.

Here we investigated changes to conjugation of tyrosine residues using a cyclic diazodicarboxamide with 4-phenyl-3H-1,2,4-triazole-3,5(4H)-dione (PTAD). We compared labeling of the folded and unfolded protein. The differences in tyrosine conjugation can demonstrate an approach useful to distinguish changes to protein conformation when traditional structural biology methods are limited.

## METHODS

**Materials.** PTAD conjugation reagents were purchased commercially from Sigma-Aldrich (Germany) and used without further purification: 4-Phenyl-3H-1,2,4-triazole-3,5(4H)-dione (PTAD, cat. # 42579), 4-(4-(2-Azidoethoxy)phenyl)-1,2,4-triazolidine-3,5-dione, N3-Ph-Ur for e-Y-CLICK (PTAD-$N_3$, cat. # T511552), 1,3-Dibromo-5,5-dimethylhydantoin (DBH, cat. # 157902), DBCO-Cy3 (cat. # 77366), DBCO-Cy5 (cat. # 777374), and tyrosine (cat. # T3754). Tris-HCl was purchased from Goldbio (cat. # T-400-5). Urea was purchased from Invitrogen (Carlsbad, CA). Angiotensin II was purchased commercially from Sigma-Aldrich (cat. # A9525). Bovine serum albumin (BSA) was purchased from VWR (cat. # 97062-508). Chemical structures can be seen in **Supplemental Diagram 1**.

**Nuclear Magnetic Resonance Spectroscopy.** For $^1$H NMR, 0.5 mM 3-(4-hydroxyphenyl)propionic acid, and 1.15 mM PTAD were incubated in 1 mL of water and 4% acetonitrile for 1 hour. For the 4x label sample, addition of PTAD was repeated at 15, 30, and 45 minutes, to a final concentration of 4.6 mM PTAD and 4% acetonitrile. Samples were lyophilized overnight and resuspended in $D_2O$. $^1$H NMR spectra were collected with a Bruker AVIII-400. Full spectra are found in **Supplemental Spectra 1**.

**Mass Spectrometry Analysis of Tyrosine and Peptides.** Tyrosine stocks were dissolved in 1 M HCl and diluted to 2.25 mM into 1:1 water and acetonitrile for conjugation reactions. The PTAD-$N_3$ pre-cursor (50 mM) was oxidized by briefly incubating with equimolar DBH until a color change to cranberry red was observed. These stock solutions were combined, vortexed, and incubated at room temperature for 1 hour. PTAD concentration of 2.25 mM was kept constant for

conjugations with tyrosine with 10:1 or 100:1 ratios PTAD to tyrosine. For analysis by UPLC-MS, 20 μL of samples were injected to a LCMS-2020 (Shimadzu) with C18 column (Onyx monolithic C18, 50 x 2.0 mm). Samples were eluted over 4 minutes over a linear gradient of acetonitrile from 5% to 20% over the first 3 minutes.

For peptide conjugations, angiotensin II (0.5 mM) was incubated with 5 mM PTAD and 100 mM Tris for 1 hour at room temperature. Peptides were analyzed by UPLC-MS using a Bruker AmaZon SL Ion Trap mass spectrometer (*Bruker* Daltonik GmbH, Germany) in-line with HPLC and ESI source with positive polarity. High resolution analysis of angiotensin II was performed using an LTQ Orbitrap Velos ETD mass-spectrometer (ThermoFisher Scientific, Bremen, Germany).

**CD spectroscopy.** To observe unfolding, 1 mg of BSA was incubated in 1 mL of 0 to 10 M urea overnight. Circular dichroism analysis was made with an OLIS DSM-20 CD spectrometer and a 1 mm pathlength cylindrical cell (Hellma, cat. # 121.000-QS). Three runs were averaged of spectra.

**Size-Exclusion Chromatography.** BSA (40 mg) was incubated in 1 mL of 0 to 8 M urea overnight. Protein at 114 μM was incubated with 2.25 mM PTAD or PTAD-$N_3$ for 1 hour at room temperature in buffer A (150 mM NaCl, 40 mM Tris-HCl). For PTAD-$N_3$ labeling, red·PTAD-$N_3$ (50 mM) was activated with equimolar DBH until color change before adding to protein. BSA unfolding was performed by incubating protein in phosphate buffered saline (PBS) with 0 to 8 M urea overnight. Size exclusion chromatography was performed by injecting 4 μg of BSA in 500 mL over a SuperdexTM 200 10/300GL column using an AKTA™ PureL system (GE Healthcare Life Sciences, Pittsburgh PA). For DBCO-dye conjugations, PTAD-$N_3$ and dyes were incubated together with proteins at a 10:1 molar ratio, respectively, for 1 hour at room temperature. Fluorescence was imaged after SDS-PAGE using a Chemidoc MP system (Biorad).

**LC-MS/MS.** BSA (5 mg) was incubated in 500 mL Buffer A (121 μM protein) and 2.25 mM PTAD for 1 hour. Protein was separated by SDS-PAGE using 5 μg of labeled or unlabeled protein and followed by staining with Instant Blue (Sigma-Aldrich, cat. # ISB1L). Gel slices were extracted and subject to digestion with Lys-C. Resulting peptides were purified by C18-based desalting, as previously described[14]. HPLC ESI-MS/MS was performed in positive ion mode on a Thermo Scientific Orbitrap Fusion Lumos tribrid mass spectrometer fitted with an EASY-Spray Source (Thermo Scientific, San Jose, CA). All solvents were liquid chromatography mass spectrometry grade. Spectra were acquired using XCalibur, version 2.3 (Thermo Scientific). Tandem mass spectra were extracted from Xcalibur 'RAW' files and charge states were assigned using the ProteoWizard 3.0.1 msConvert Script. The fragment mass spectra were assigned using Mascot (Matrix Science, London, UK; version 2.4) using the default probability cut-off score. Variable modifications used included PTAD (175 Da) conjugation to tyrosine, oxidation of methionine and phosphorylation of serine, threonine, and tyrosine. Cross-correlation of Mascot results with X! Tandem was accomplished with Scaffold (version Scaffold_4.8.7; Proteome Software, Portland, OR).

## RESULTS

**PTAD conjugation to tyrosine or peptides can yield distinctive products.** We assessed the products of PTAD conjugation with tyrosine to determine products our experiment designs may produce. Previous reports noted such additional reactions as conjugates with amines, second additions to tyrosine phenols, and short-lived conjugations to cysteine residues[15, 16]. We confirmed by $^{1}$H NMR the reaction with a tyrosine analogue, 3-(4-hydroxyphenyl)propionic acid (**Supplemental Spectra 1)**. We allowed PTAD to react with the tyrosine mimic for 15 minutes

and observed conversion to labeled tyrosine, consistent with previous reports (**Figure 1A**)[16, 17]. By repeated labeling up to four times, signals for the fully converted product exceeded those for the tyrosine analogue.

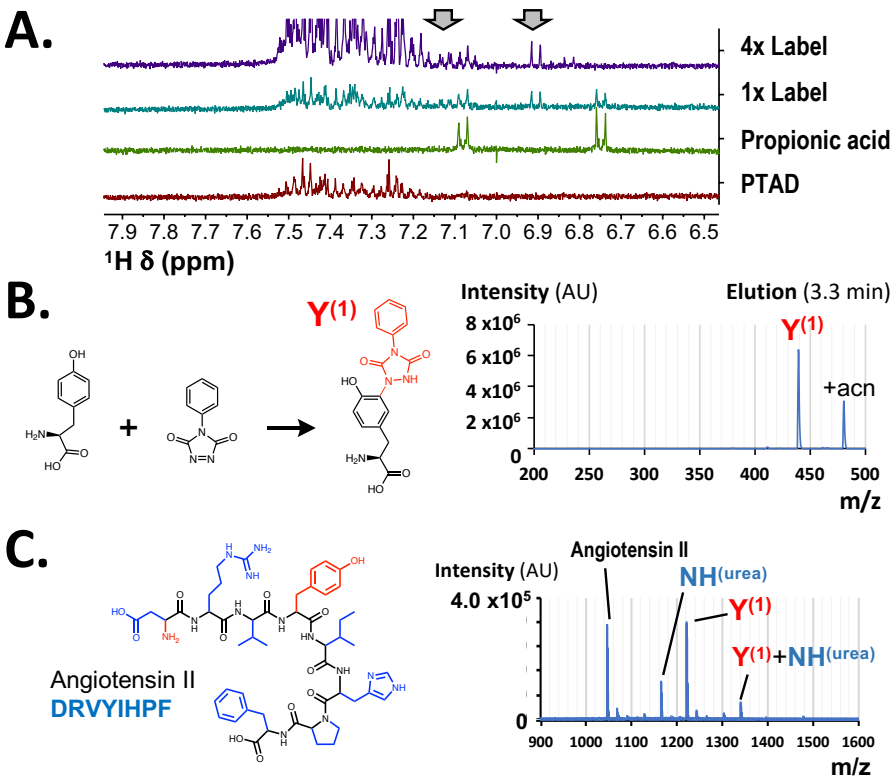


**Figure 1**. Products of PTAD reaction with tyrosine. **(A)** $^{1}$H NMR spectra for PTAD, a tyrosine mimic, a single incubation of the mimic with PTAD for 15 minutes (1x Label), and 4 repeated additions of PTAD to incubate with the mimic (4x Label). Peaks for the products formed are indicated by arrows. **(B)** The conjugation product of 1:1 PTAD to tyrosine at a single *ortho*-position, $Y^{(1)}$, on the phenolic ring was detected by UPLC-MS with the expected m/z of 440 Daltons. **(C)** Products of angiotensin II observed in MALDI-TOF. Products shown include PTAD conjugate with the tyrosine at position 4, $Y^{(1)}$, and a conjugate of a phenylisocyanate to the N-

terminal amine, $NH^{(urea)}$. AU indicates arbitrary units for MS intensities. All chemical structures can be seen in **Supplemental Diagram 1**.

We also visualized the products of PTAD and tyrosine using ultra-performance liquid-chromatography mass spectrometry (UPLC-MS). PTAD is available in two forms. The oxidized form, PTAD, can be used for reactions from fresh stocks. The reduced form, red·PTAD, can be stored in solution, but must be activated through oxidation before it can react with tyrosine. For our UPLC-MS analysis, we used a reduced PTAD-azide (red·PTAD-$N_3$, m/z = 262) and activated to PTAD-$N_3$ by including 1,3-dibromo-5,5-methylhydantoin (DBH) in the reaction. A 1:1 molar ratio of PTAD-$N_3$ to tyrosine yielded product of a single PTAD and tyrosine conjugate, $Y^{(1)}$ (m/z = 440, **Figure 1B**). We could also observe a double-reacted product of two PTADs on the phenolic ring, $Y^{(2)}$ (m/z = 700, **Supplemental Figure 1A**). PTAD can decompose to an isocyanate that is reactive toward amines[16, 18]. The third product we observed was the phenylisocyanate product conjugated with the primary amine of tyrosine, $NH^{(urea)}$ (m/z = 384, **Supplemental Figure 1A**). At 10 or 50-fold molar excess of PTAD over tyrosine, signals diminished for $Y^{(1)}$ and $Y^{(2)}$ products and were replaced by signals indicating those with the $NH^{(urea)}$ conjugation also added.

We assessed the products of PTAD (m/z = 175) and a peptide, angiotensin II. Angiotensin II is an 8 amino acid long peptide, DRVYIHPF (m/z = 1046, **Figure 1B**). In this reaction, we incubated PTAD in aqueous buffer containing 100 mM 2-amino-2-hydroxymethyl-propane-1,3-diol (Tris) to scavenge the isocyanate through its own primary amine[16, 19]. Matrix-assisted laser desorption/ionization and time of flight (MALDI-TOF) mass spectrometry was used to detect the PTAD conjugate (m/z = 1221). We also observed minor products with the urea conjugation (m/z

= 1165) or both urea and PTAD conjugations (m/z = 1340). We also noted that the amounts of urea conjugates observed diminished with higher Tris concentrations (**Supplemental Figure 1B**).

**Protein folding remains during PTAD conjugation.** We investigated whether the PTAD conjugation might unfold proteins. Changes to the protein might diminish the pattern in PTAD conjugation prompted by the protein conformation. We chose to test the well-folded bovine serum albumin (BSA, MW = 66 kDa) with 607 amino acids and 21 tyrosine residues.

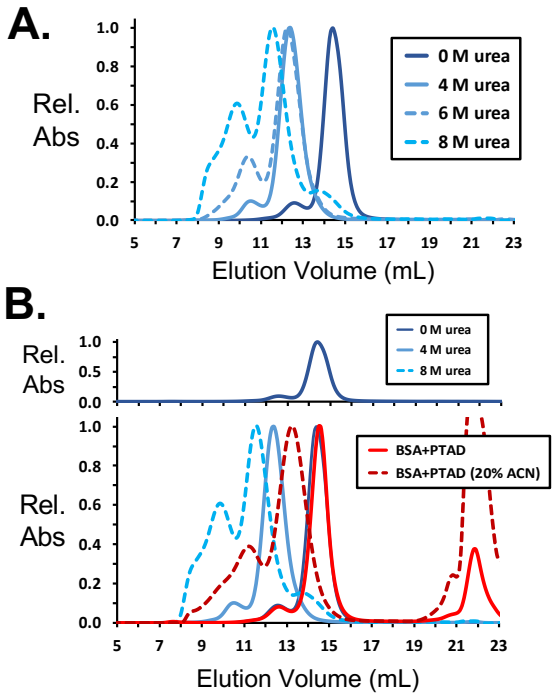


**Figure 2.** PTAD conjugation does not abolish protein structure. (**A**) Size exclusion chromatography of BSA, measured by UV absorbance and in titrating amounts of urea. BSA elutions were shifted by protein unfolding in urea. "Rel. Abs" in all SEC plots represents relative absorbance measured at 280 nm. (**B**) PTAD conjugated BSA elution (red) completely

superimposed over that of folded BSA. Labeling BSA in 20% ACN unfolded the protein and shifted its elution.

Size exclusion chromatography (SEC) can provide a straightforward assessment of protein structure. We first observed the change in elution during SEC for BSA as the protein unfolded in titrating amounts of urea[20]. Circular dichroism (CD) spectroscopy revealed the midpoint concentration to unfold BSA as 4.6 ± 0.1 M urea (**Supplemental Figure 2A**). For 4, 6, and 8 M urea, BSA eluted with an early and broader peak, indicating a more extended structure (**Figure 2A**). We labeled BSA with equal molar PTAD to tyrosine (121 μM BSA = 2.5 mM tyrosine, and 2.25 mM PTAD). The elution of BSA was not changed by PTAD conjugation (**Figure 2B**, solid red line). Labeling with PTAD in the presence of 20% acetonitrile/H2O produced a broad peak consistent with unfolding of the protein (**Figure 2B**, dashed red line). Using red·PTAD-$N_3$ and DBCO-Cy5 combined through click chemistry, we found the SEC profile of fluorescently labeled BSA to also indicate no evidence for protein unfolding (**Supplemental Figure 2B**).

**PTAD labeling is distinct for alternate folded states in a protein.** We next used liquid chromatography with tandem mass spectrometry (LC-MS/MS) to measure PTAD labeling for individual tyrosine residues in BSA. We were able to detect PTAD labels for all previously published sites in BSA except for Y393[21]. We also found previously unreported PTAD labeling at residues Y173, Y179, Y180, and Y520[19, 21-23]. We interpreted this to be a result of improved sensitivity by LC-MS/MS. We quantified the ratio of labeled to unlabeled tyrosine (L/U) for thirteen tyrosine residues (N = 4, **Supplemental Figure 3A–B**). Only single PTAD conjugations

to tyrosine were observed for residues positioned on or near the protein surface (**Figure 3A**). PTAD was quantified for eleven tyrosine residues. Three tyrosine residues were detected to be phosphorylated (**Supplemental Figure 3B**). Y286 had the highest phosphorylated signals and was never found labeled. Y520 occupied a disordered region of the protein with an adjacent threonine, T519, that was phosphorylated and also never found labeled (**Supplemental Figure 3B**).

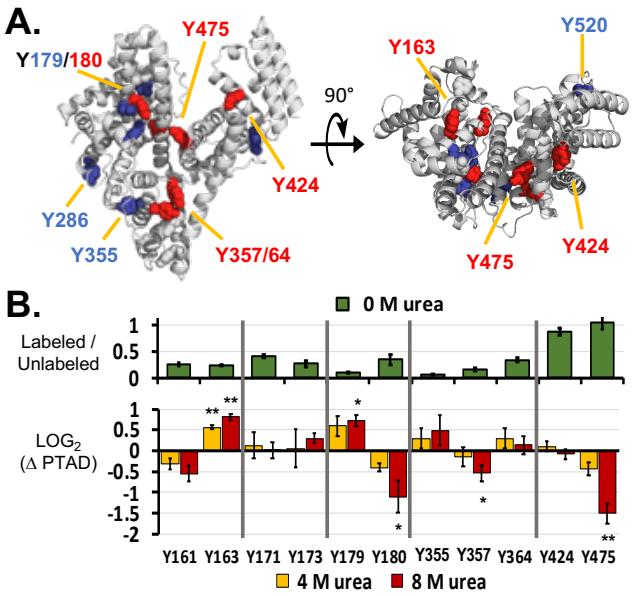


**Figure 3.** Quantitative analysis of PTAD labeling for BSA. (**A**) Two views of BSA (PDB: 3V03) are shown with tyrosine residues represented as spheres. Tyrosine residues are colored for those with more (red) or less (blue) than expected PTAD labeling. (**B**) The LOG2 of the fold change in PTAD L/U is shown for BSA incubated in 4 M and 8 M urea and normalized to the L/U of native BSA in 0 M urea. Below, the L/U values for each residue in the native BSA structure are shown (green). Error bars are standard error about the mean (N = 4 for all treatments). * $p < 0.05$, and ** is $p < 0.005$, student t-test assuming equal variances.

We labeled partially and fully unfolded BSA incubated in 4 M or 8 M urea, respectively (N = 4 for each treatment). PTAD conjugation was changed for several tyrosine residues. We compared the LOG2 of the fold change relative to the native state (**Figure 3C**). Levels of PTAD was unchanged for residues Y171, Y173, Y364, and Y424 in the unfolded proteins. Y163 increased enrichment the most ($p = 0.00014$ for 8 M urea; Student's t-test). Tyrosine residues with the lowest L/U ratios, Y179 and 355, were also increased in labeling ($p = 0.019$ and $p > 0.05$, respectively, for 8 M urea; Student's t-test). Residue Y475 fell from the most enriched for PTAD in the folded protein to comparable with other residues in the unfolded. Changes to PTAD enrichment from the native levels was greater for the 8 M than the 4 M urea samples. The most significant changes were found for Y180 and Y475 ($p = 0.009$ and 0.0034, respectively, for 8 M urea; Student's t-test). Phenylisocyanate (m/z = 121) conjugations were observed for sixteen lysine residues, twice as many as previously reported[16, 19]. However, this enrichment was little changed in the unfolded protein (**Supplemental Figure 3C**).

**PTAD conjugation is sensitive to protein structure.** We inspected the L/U ratios and position of tyrosine residues in the solved structure of BSA (PDB: 3V03). We also calculated the solvent exposure for each tyrosine based on the structure. Residues Y355, Y357, and Y364 were found with a diverse range in L/U ratios (3/113, 15/101, and 117/407, respectively, **Figure 4A**). These ratios were noted to closely correlate to their respective percent solvent exposures (2, 13, and 21 %). The highest L/U ratios were measured for Y424 and Y475. These were observed to have the most solvent exposure (**Figure 4B**).

Conjugation by PTAD was not strictly related to solvent exposure. Residues Y161 and Y163 were observed with similar levels of PTAD labeling (49/326 and 62/326, **Figure 4C**). However, the percentage of surface area exposed to solvent were quite different. The most exposed tyrosine, at 12%, was Y161. The residue Y163 had 2-fold less accessible surface area at 5%. Nevertheless, Y163 was seen to have its phenolic ring oriented with the hydroxyl and *ortho* positions faced outward toward solvent. The most accessible area for Y161 was the backbone and *meta* positions; whereas, the hydroxyl and *ortho* positions were mostly buried.

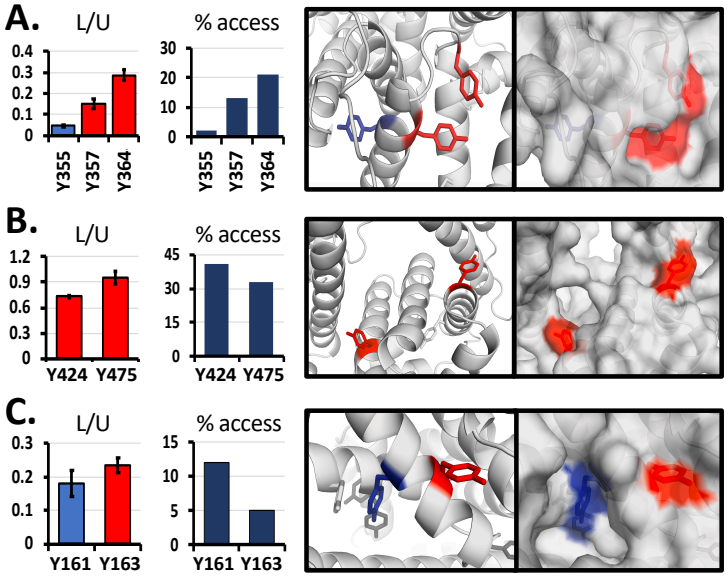


**Figure 4.** Effects of local structure to PTAD labeling. The ratio for labeled to unlabeled tyrosine residues (L/U) was measured. Residues enriched in PTAD are red in the left bar graph and structures. Those with relatively low ratios are blue. Also shown is the percent accessible surface area calculated for a solved structure for BSA [PDB: 3V03]. **(A)** For Y355, Y357, and Y364, the amount of PTAD labeled tyrosine is increased as the percent accessibility increases. **(B)** Y424 and Y475 were found to have the highest L/U ratios and percent accessibility. **(C)** Y163 was predicted to have considerably lower solvent accessibility but its enrichment for PTAD labeling is

approximately equal to Y161. In the structure, Y163 can be seen to have its *ortho* position oriented toward the surface.

Additional conjugated residues included Y171, which had slightly higher L/U ratio than Y173 (44/143 and 32/143, **Supplemental Figure 4A**). Their solvent exposures were similar. A reversal for L/U ratios compared to solvent exposure was observed for Y179 and Y180. These residues were packed exceptionally tight against each other in a narrow groove (**Supplemental Figure 4B**). Y179 appeared more solvent exposed (18%) and Y180 is among the least solvent exposed in the protein (5%). We noted the ring for Y179 lay nearly parallel to the protein surface. The ring for Y180 lay perpendicular to the surface with the *ortho* position most exposed. Additionally, the exposed *ortho* position of Y180 was positioned near a lysine, K187, whose basic nature may have been able to alter the local pH (**Supplemental Figure 4B**).

Our study found PTAD to effectively label tyrosine residues in folded or unfolded proteins. We found that the protein maintained its shape according to SEC after labeling with PTAD. Furthermore, partially and fully unfolded protein could be distinguished from the folded state based on their respective PTAD labeling. The results demonstrate that tyrosine conjugation can provide a chemical fingerprint to indicate contrasts in protein conformation.

## DISCUSSION

In agreement with earlier reports, we found PTAD to be highly efficient for conjugating tyrosine residues. We observed the three types of products: a single phenolic addition to tyrosine, a double addition, and an isocyanate reaction with an amine (**Figure 1**). Labeled tyrosine residues were

detectable to LC-MS/MS and this quantitative approach yielded a pattern in enrichment for PTAD distinguishable from another conformational state of the same protein(**Figure 3B**). This finding provides a proof of principle that tyrosine conjugation may be developed into a robust tool to probe protein structure-function relationships.

As expected, tyrosine residues in folded BSA varied considerably in their exposure to solvent, depending on their position in a folded protein. This fact can rationalize the strong differences observed at certain positions once the protein was unfolded. The orientation of the tyrosine phenol relative to the surface appeared to explain enrichment of PTAD conjugation at some tyrosine positions (**Figure 4**, **Supplemental Figure 4**). It can also be noted that the primary sequence for an unfolded protein or peptide can obstruct tyrosine accessibility to solvent or conjugation[24, 25]. Post-translational modifications, such as phosphorylation, correlated to reduced levels of tyrosine conjugation (**Supplemental Figure 3A**). To fairly demonstrate the advantage of tyrosine as a probe for structural changes, we monitored lysine modifications and found conjugation for this residue to be little changed upon unfolding to protein (**Supplemental Figure 3C**).

In conclusion, we consider that comparative or quantitative analysis of protein structure by tyrosine conjugation is a feasible approach with advantages that contrasts with other amino acids. The first reason is the relative abundance of tyrosine in proteins. Its amphipathic nature distributes those residues across the divide of the protein surface and hydrophobic core. Last, the functionality of the PTAD chemistry and strength of covalent conjugations can allow more adaptability to develop this approach for use in a variety of studies. In the future, application of this approach to whole cell proteomics and investigations of tyrosine-rich low complexity proteins should reveal how much this new window into protein biology can uncover.

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**Author Contributions**

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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**ABBREVIATIONS**

BSA, bovine serum albumin; CD, circular dichroism; DBCO, dibenzocyclooctyne; DBH, 1,3-Dibromo-5,5-dimethylhydantoin; LC-MS/MS, liquid chromatography and tandem mass spectrometry; $NH^{(urea)}$, amine conjugated isocyanate; PTAD, 4-Phenyl-3H-1,2,4-triazole-3,5(4H)-dione; PTAD-$N_3$, 4-(4-(2-Azidoethoxy)phenyl)-1,2,4-triazolidine-3,5-dione, N3-Ph-Ur for e-Y-CLICK; red·PTAD-$N_3$, reduced PTAD-$N_3$; SEC, size exclusion chromatography; UPLC-MS, ultra-high pressure liquid chromatography and mass spectrometry; $Y^{(1)}$, tyrosine with single PTAD conjugation; $Y^{(2)}$, tyrosine with two PTAD conjugations.

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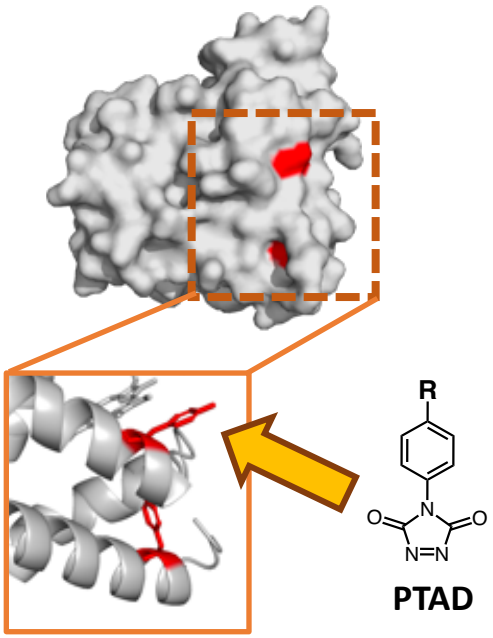
**Table of Contents Graphic**




For folded proteins, solvent exposure controls the efficiency by which tyrosine residues are available for conjugation. Changes to protein structure can change tyrosine accessibility considerably. Such changes are quantifiable by the extent of tyrosine labeling observed for distinctive folded or conformational states of a protein.