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Seeing Citrulline: Development of a Phenylglyoxal-Based Probe To Visualize Protein Citrullination

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Supporting Information

ABSTRACT: Protein arginine deiminases (PADs) catalyze the hydrolysis of peptidyl arginine to form peptidyl citrulline. Abnormally high PAD activity is observed in a host of human diseases, but the exact role of protein citrullination in these diseases and the identities of specific citrullinated disease biomarkers remain unknown, largely because of the lack of readily available chemical probes to detect protein citrullination. For this reason, we developed a citrulline-specific chemical probe, rhodamine−phenylglyoxal (Rh−PG), which we show can be used to investigate protein citrullination. This methodology is superior to existing techniques because it possesses higher throughput and excellent sensitivity. Additionally, we demonstrate that this probe can be used to determine the kinetic parameters for a number of protein substrates, monitor drug efficacy, and identify disease biomarkers in an animal model of ulcerative colitis that displays aberrantly increased PAD activity.

The protein arginine deiminases (PADs) catalyze the post-translational conversion of peptidyl arginine to peptidyl citrulline, a process commonly called deimination or citrullination.[1,2] PAD activity is aberrantly increased in different diseases,[1,2] including rheumatoid arthritis (RA), ulcerative colitis (UC), Alzheimer's disease (AD), multiple sclerosis (MS), lupus, Parkinson's disease (PD), and cancer.[1,3−8] For example, increased PAD levels are observed in the RA synovium,[9] during the formation of neutrophil extracellular traps (NETs) in colitis and lupus,[10] within the brains of patients with MS,[11] in the joints of patients with osteoarthritis,[9] in UC lesions,[12] in the hippocampal extracts of AD patients,[5] and in several carcinomas.[6] Furthermore, the administration of Cl-amidine, a potent PAD inhibitor,[13] or a Cl-amidine analogue[14] has proven useful in decreasing disease severity in animal models of UC, RA, and cancer.[14−16]

Although abnormally high PAD activity and protein citrullination are observed in these and other diseases, the exact role(s) and target(s) of these enzymes are still poorly understood, in part because of a lack of chemical tools that can be used to identify the proteins that are citrullinated within a specific disease model. The ability to investigate the specific target and role of PADs in these diseases will inevitably allow for better, more tailored treatments and identify biomarkers that can be used to diagnose and monitor the efficacy of treatments for these diseases. Additionally, the ability to detect changes in protein citrullination rapidly in response to the addition of a PAD inhibitor will facilitate the development of such compounds.

Common methods for studying citrullination include the color development reagent (COLDER) assay[17−20] and a commercially available anti-citrulline (modified) detection kit (ACM kit; Millipore,; Billerica, MA).[21] Although widely used for studying small-molecule and peptide substrates of the PADs, the COLDER assay has a relatively high limit of detection (LOD) of ∼0.6 nmol, making it difficult to use with less concentrated protein substrates. The ACM kit has also proven useful but suffers from relatively high cost ($40/assay), a lengthy two-day procedure, and specialized analysis. Although other means of detecting citrullinated proteins have been described, these are also antibody-based (e.g., the F95 antibody[22] and citrullinated histones H3 and H4). In contrast, the probe described herein is readily synthesized, show excellent sensitivity, react with any citrulline bearing protein, and can be used by most researchers because detection uses reagents/instruments that are available in virtually all biochemically focused laboratories.

The design of the citrulline-specific probe described herein is based on the chemoselective reaction that occurs between glyoxals and either citrulline or arginine under acidic or basic conditions, respectively [Figure 1A and Figure S1 in the Supporting Information (SI)].[23] Although Fleckenstein and co-workers[23] reported the derivatization of phenylglyoxal onto the solid phase via the para position, this chemistry is not amenable to the solution phase (Figure S2). Therefore, we focused on synthesizing phenylglyoxal compounds with an azido group at the meta position that could be reacted with alkyne-modified rhodamine via the Cu-catalyzed azide−alkyne cycloaddition reaction (Figure S3).[24,25] The reaction between citrulline and phenylglyoxal likely proceeds as previously suggested (Figure S4).[20,23]

The probe selectivity against commonly reactive amino acids was first determined. Under acidic conditions, Rh−PG reacted selectively with citrulline, homocitrulline, and cysteine (Figure S5). Using a peptide substrate containing both citrulline and

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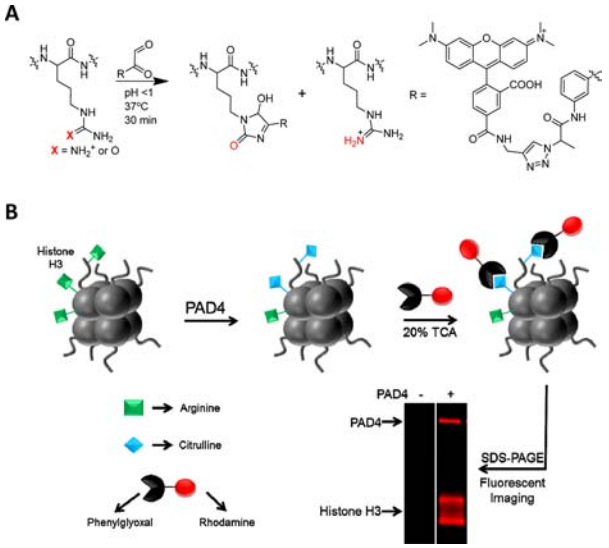

Figure 1. (A) The rhodamine−phenylglyoxal (Rh−PG) probe chemoselectively labels citrulline over arginine at acidic pH. (B) Schematic representation of the method used to monitor citrullination with the Rh−PG probe. Specific arginine residues (green squares) of histone H3 are converted to citrulline (blue diamonds) by PAD4. Treatment of the citrullinated protein with the Rh−PG probe under acidic conditions labels citrulline residues selectively. Subsequent separation by SDS-PAGE and fluorescent analysis reveals citrullinated proteins. An actual gel image is shown, demonstrating the selectivity of Rh−PG.

cysteine, we showed that the thiohemiacetal generated between Rh−PG and Cys is hydrolyzed at neutral pH, while the cyclic system formed between Rh−PG and citrulline is not (Figure S6). Since the gel-based screening method includes a neutralization step prior to gel loading, the problem of cysteine cross-reactivity is effectively negated; the selectivity versus Cys under these conditions is ≥650-fold. After demonstrating that our PG probe is capable of selectively reacting with either arginine or citrulline in a pH-dependent manner (Figure S1), we optimized the conditions for probe labeling, including the ideal probe concentration (i.e., 100 μM; Figure S7), the ideal labeling time (i.e., 30 min; Figure S8), and the probe sensitivity/LOD [i.e., ∼10 ng (∼0.67 pmol) of citrullinated histone H3 and ∼1 ng (∼12.7 fmol) of autodeiminated PAD4; Figure S9]. The different LODs are due to differences in the citrulline content of the two proteins. Since Rh−PG labeling is performed in 20% trichloroacetic acid (TCA) at 37 °C, it is important to note that increased labeling temperatures and times led to the formation of SDS-insoluble protein aggregates and decreased gel loading (Figure S10). Also, quenching of excess probe with citrulline after labeling reduced background labeling of arginine residues upon resuspension of the protein (data not shown).

To demonstrate that the Rh−PG probe detection method compares favorably to the commercially available ACM kit, we performed a comparative analysis of the two methods using PAD4 autodeimination as the end point. PAD4, autodeiminated for various amounts of time, was analyzed with Rh−PG (Figure 2A, top) and with the ACM kit according to the manufacturer's instructions (Figure 2A, bottom). Silver staining of the fluorescent gel was done to confirm equal protein loading (Figure 2A, middle). Analysis of these data (Figure 2B) indicated that the two techniques provide virtually identical results. The rate of recombinant histone H3 citrullination was also followed, and the two methods again showed essentially identical results (Figure S11). These data indicate that the Rh−PG method is comparable to commercially available techniques. However, relative to the ACM kit, the Rh−PG labeling method takes significantly less time (∼3 h vs ≥25 h), requires fewer steps (6 vs 12), and involves simpler analysis (fluorescent imaging vs Western blotting).

Given the high sensitivity of Rh−PG, we next determined whether this methodology could be used to quantify changes in citrullination accurately by determining the steady-state kinetic parameters for a protein substrate. Although we and others have used the traditional COLDER assay to do this type of analysis on protein substrates,[26] it is difficult to obtain highly accurate rates because of the limited sensitivity of the COLDER assay and the high concentrations of proteins required for accurate measurements of citrulline production. For the Rh−PG kinetic experiments, we first monitored H3 citrullination as a function of time to ensure that the kinetic parameters would be determined under initial velocity conditions (Figure S12). The Rh−PG fluorescence was converted to citrulline concentration using a citrulline standard curve (see the SI for details). Having identified a specific time that could be used to obtain the initial rates, we used this method to determine the steady-state kinetic parameters for the citrullination of histone H3 by PAD4 (Figure 2C). The results of this analysis provided a k_{cat}/K_m value of 4800 ± 1100 M^{-1} s^{-1}, which compares favorably with our previously reported value of 3700 M^{-1} s^{-1}.[26]

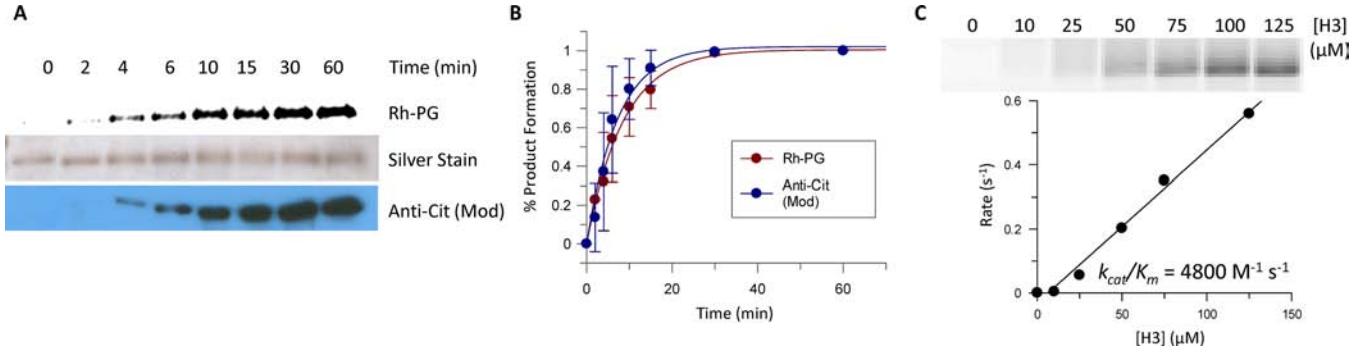

Figure 2. (A, B) The time course of PAD4 autodeimination is similar for the Rh−PG probe and commercially available antibody methods. (A) Rh−PG probed fluorescent image (top) and Anti-Cit (Mod) Kit Western blot analysis (bottom) of autodeiminated PAD4. Silver staining (middle) shows equal protein loading. (B) Plot of percent citrullination over time for each type of analysis, indicating that the two methods are comparable. (C) The Rh−PG labeling method can also be used to determine the k_{cat}/K_m value of histone H3 citrullination by PAD4.

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Similar analyses performed on the PAD4- catalyzed deimination of histone H4 gave a value of 2800 ± 200 M^{-1} s^{-1} (Figure S13), which is also similar to the previously reported value.[26] In view of the poor sensitivity and high signal-to-noise ratios of current methods for determining the rates of protein citrullination (i.e., the COLDER analysis and ammonia detection methods),[8,27] the accuracy and excellent sensitivity of our method (notably, the samples had to be diluted 60-fold before analysis) make it a unique tool to study protein deimination because it permits the determination of K_m values for high-affinity protein substrates and allows for the study of proteins that are difficult to produce in large quantities.

We envisioned that a key advantage of this probe would be its facile ability to visualize target engagement (i.e., decreased citrullination upon inhibitor treatment) as well as the detection/identification of disease-associated biomarkers. To demonstrate its utility for these types of analyses, we evaluated changes in protein citrullination using serum samples from our previous study demonstrating that Cl-amidine, a PAD inhibitor, reduces the disease severity in a mouse model of dextran sodium sulfate (DSS)-induced UC.[15] Citrullinated proteins present in these samples were labeled with the Rh−PG probe and separated by SDS-PAGE, and the fluorescent proteins were imaged (Figure 3A). We subsequently analyzed whole-lane fluorescence as a function of the addition of Cl-amidine (Figure 3B). Consistent with our previous studies, which used the COLDER assay to measure changes in total protein citrullination, analysis of the whole-lane fluorescence revealed a significant ($P < 0.01$) reduction in fluorescence intensity for the serum samples treated with Cl-amidine. Notably, selectivity analysis indicated the Cl-amidine itself does not react with the probe (data not shown). Unlike the previous analysis, which measures only total protein citrullination, the Rh−PG method allowed us to monitor the effects on specific proteins as opposed to global serum citrullination. On the basis of visual inspection of the fluorescent images, we identified five protein bands within each lane (at 10, 25, 50, 70, and 100 kDa) that appeared to show a marked change in protein citrullination as a function of Cl-amidine. From a comparison of the fluorescence intensities of these bands for the two groups, we observed that four of the five protein bands showed statistically significant decreases in citrullination in response to Cl-amidine ($P < 0.05$; Table S1 in the SI). The only protein band analyzed that did not show a significant decrease in response to Cl-amidine ($P = 0.44$) was the band at 10 kDa.

Given these findings, we hypothesized that direct correlations between the citrullination of these proteins and the disease severity may exist. To test this hypothesis, we compared the fluorescence intensity data for the five proteins described above to each of five different disease metrics obtained for the mice in this study (i.e., colon length, weight difference, white blood cell count, lymphocyte count, and inflammation score) (Table S2). Correlation plots (Figure 3C and Figure S14) and correlation coefficients (Figure 3D) were generated, and colon length (four out of six significant correlations) and inflammation scores (five out of six significant correlations) showed the highest number of correlations between disease severity and citrulline level. It is noteworthy that these two end points are the key indicators of disease severity.[15,28,29]

Interestingly, the 10 kDa protein, the only protein with no significant decrease in citrullination in response to Cl-amidine, also showed no significant correlation to any of the disease scores, so it essentially served as a negative control. The highest

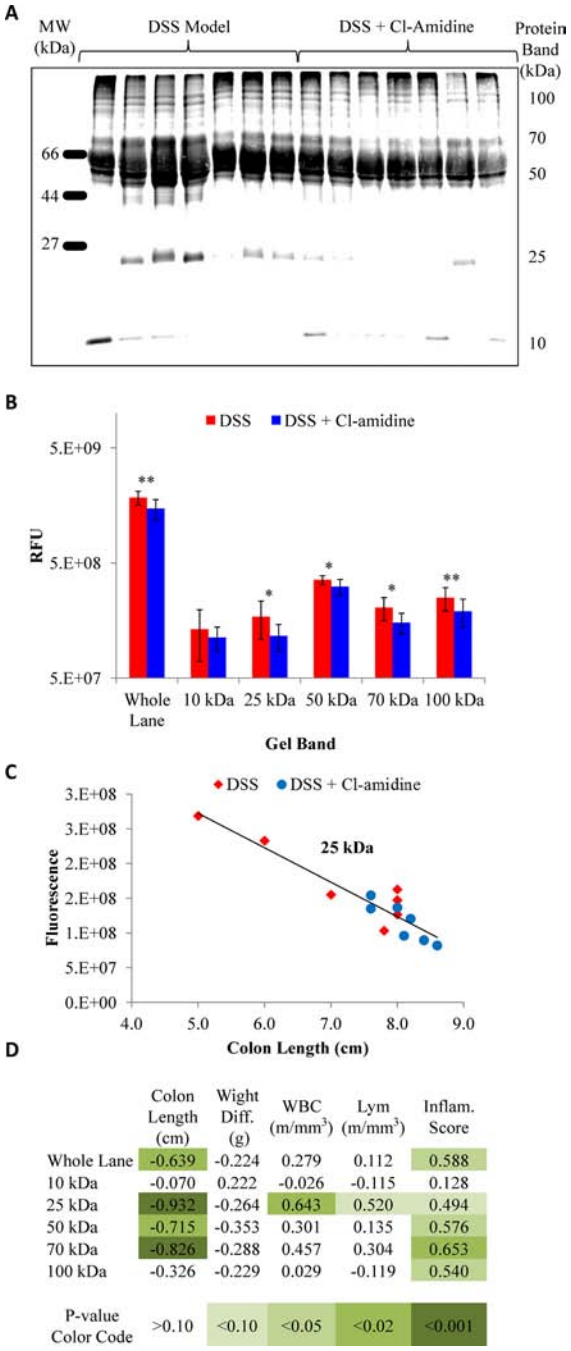

Figure 3. (A) Fluorescent image of Rh−PG-labeled serum samples from a mouse model of DSS-induced UC. (B) Analysis of the UC samples indicated a significant decrease in protein citrullination in response to Cl-amidine (*$P < 0.05$; **$P < 0.01$). (C) Correlation between colon length and fluorescence of the protein band at 25 kDa. (D) Table of correlation coefficients between various disease scores and fluorescence intensities of specific protein bands.

correlation coefficients, and therefore the most relevant correlations, exist between colon length and the levels of the citrullinated 25 kDa and 70 kDa proteins (correlation coefficients of −0.932 and −0.826, respectively; Figure 3C). It is important to note that the average protein levels, as analyzed by Coomassie staining, were the same for untreated and Cl-amidine-treated samples of each of these protein bands, indicating that the change in fluorescence was indeed due to a change in citrullination. The 25 kDa band also showed the

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greatest number of significant correlations with disease severity (i.e., four out of five disease activity scores). The only outlier was weight loss, which also did not correlate with any of the other protein bands analyzed. The strong correlation between citrullination of the 25 kDa protein and common disease activity scores, especially colon length, suggests that this protein is a biomarker of UC.

Although the Rh−PG probe is clearly useful for studying recombinant proteins, which is itself beneficial to the PAD field, this work demonstrates its true utility in studying more complex samples from disease states in which dysregulated PAD activity plays a role (e.g., UC, RA, AD, PD, and cancer, to name a few). For example, while anticitrulline protein antibodies (ACPAs) are well-established biomarkers for RA, less is understood about the targets of these antibodies. Additionally, we do not know the identities of citrullinated protein biomarkers for the other diseases in which the PADs are involved.

In total, these data demonstrate that Rh−PG is a powerful chemical probe of protein citrullination that will undoubtedly be useful in providing robust and telling insights into the role of particular PAD substrates in PAD-related diseases. Further work to determine the identity of the 25 kDa protein and to identify and characterize unique disease biomarkers in other diseases in which PADs play a role is currently underway.

■ ASSOCIATED CONTENT

Supporting Information

Synthetic procedures, experimental details, and supporting figures and tables. 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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