Detection of Protein−Protein Interactions by Proximity-Driven S$_N$Ar Reactions of Lysine-Linked Fluorophores

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S *Supporting Information*

ABSTRACT: Critical protein−protein interactions are ubiquitous in biology. To provide a new method to detect these interactions, we designed and synthesized fluorinated bromopyronins as molecular probes. These electrophilic compounds rapidly react with amines via a S$_N$Ar mechanism to form modestly electrophilic aminopyronin fluorophores. To investigate whether proteins modified with aminopyronins might selectively transfer these fluorophores between proximal lysine residues at protein−protein interfaces, immunoglobulin-G (IgG) was conjugated to fluorinated pyronins and added to unlabeled Protein A (SpA) from *S. aureus*. Analysis by gel electrophoresis and mass spectrometry revealed transfer of this fluorophore from IgG to specific lysines of its binding partner SpA but not to bovine serum albumin (BSA) as a nonbinding control. Examination of an X-ray structure of IgG bound to SpA revealed that the fluorophore was selectively transferred between amino groups of lysines that reside within ∼10 Å at the protein−protein interface. To evaluate whether this approach could be used to identify interactions with endogenous cellular proteins, pyronin-modified Rnase A was added to crude extracts of human HeLa cells. Analysis of interacting proteins by gel electrophoresis revealed the endogenous ribonuclease inhibitor as the primary cellular target. Given that proximal lysine residues frequently reside at protein−protein interfaces, this method may facilitate identification of diverse protein−protein interactions present in complex biological matrices.

Protein−protein interactions control diverse biological processes. To detect these interactions, numerous methods have been used including a variety of fluorescence-based techniques.[1] If a protein is covalently labeled with a fluorophore, interaction with a second protein can potentially alter the intensity, wavelength, or polarization of the fluorophore, enabling detection of the interaction. However, this approach generally requires that the primary protein be labeled with a single fluorophore on a unique residue that is sensitive to complexation. If proteins are more extensively labeled by conjugation to multiple lysines or other residues, spectroscopic changes at the fluorophore most affected by the protein−protein interaction can be difficult or impossible to detect above background fluorescence.

We report here a new method for detection of protein−protein interactions. This method allows lysine residues on the surface of a protein of interest to be randomly or extensively conjugated to novel fluorinated fluorophores. These fluorophores are designed to form stable protein conjugates. However, the high effective concentration of proximal lysines at interfaces of protein−protein complexes provides a driving force for reversible exchange between lysines of interacting proteins. This transfer of a fluorophore from a donor to an acceptor protein can potentially be detected by gel electrophoresis and/or proteomics methods. Several methods for proximity-driven alkylation of cysteine residues,[2−5] acylation of lysine residues,[6−8] and other reactions of proteins[9−11] have been described and reviewed,[12,13] but proximity-driven exchange of fluorophores between Lys residues at protein−protein interfaces has not been previously reported. This new approach is illustrated in Figure 1.

We recently published[14] the synthesis of fluorinated xanthones such as **1**−**3** (Scheme 1) via iterative nucleophilic aromatic substitution. To create novel reagents for protein modification, we treated these xanthones with triflic anhydride and tetrabutylammonium bromide to generate the fluorinated bromopyronins **4**−**6**. These compounds are stable solids, but they readily react with amines to yield fluorinated amino-pyronins such as the ethylamine derivatives **7**−**9**. Similar to other known pyronins,[15,16] these compounds are highly fluorescent (Figure 2). Furthermore, **7**−**9** exhibit large Stokes shifts of ∼100 nm, which can beneficially limit self-quenching when multiple fluorophores are conjugated to biomolecules,[17] and high quantum yields of ∼0.8 were measured for both **7** and **8**. The absorbance spectra of **7**−**9** were insensitive to changes in pH in the range of 4−9, but deprotonation was observed under more basic conditions, and pK_a values of 9.9 to 11.2 were measured (Figure 2, pH-dependent spectra are provided in Figure S4 of the Supporting Information). Analysis of reactivity by mass spectrometry revealed that aminopyronins **7**−**9** are highly stable in buffered aqueous solutions under physiological conditions (pH 7.4). Moreover, treatment of **7**−**9** (50 μM) with a high concentration (10 mM) of methylamine in buffered water (pH 7.4) resulted in <1% conversion to **10**−**12** (Scheme 1) after 96 h at 37 °C. However, under the same conditions in nonbuffered methanol/water (9:1), the addition of excess methylamine (10 mM) resulted in clean conversion to **10**−**12**, with half-times of 1.9 to 3.8 h under pseudo-first-order conditions (Figure 2C), resulting from nucleophilic aromatic substitution. Under these conditions, the more hindered pyronin **9** was ∼2-fold more reactive than **7** or **8**, and this

Figure 1. Proximity-driven transfer of fluorinated pyronin fluorophores between lysine residues for detection of protein–protein interactions.

Scheme 1. Synthesis of Bromopyronins (4–6) and Aminopyronins (7–12)

Figure 2. (A) Absorbance and fluorescence emission spectra of pyronins 5 and 8 in PBS (pH 7.0). (B) Physicochemical properties of 4–9 in PBS. (C) Kinetics of conversion of 7–9 to 10–12 upon reaction with excess methylamine.

Figure 3. Analysis of transfer of pyronins between proteins. Human IgG was conjugated to pyronins 4 (A), 5 (B), and 6 (C), added at 5 µM to PBS (pH 7.4) containing unlabeled SpA (5 µM) and BSA (5 µM), allowed to react at 37 °C for 0–16 h, and analyzed by SDS-PAGE with fluorescence detection. Quantification of the fluorescence of the SpA bands is shown on the bottom.

Figure 4. Analysis of fluorophore transfer from IgG conjugate 14 to SpA by tryptic digestion and MS/MS. The amino acid sequence of mature SpA (Uniprot ID P02976) is shown with fragments A–E aligned by homology. Lysine residues are colored red. Four dominant pyronin-modified tryptic peptides, identified with >99% probability, are shown boxed, with arrows illustrating sites of lysine modification. The underlined residues in fragment B represent the residues visible in the X-ray structure of the IgG Fc-SpA complex (PDB ID 1FC2).

increased reactivity was correlated with a lower quantum yield and decreased pK_a, consistent with less conjugation of the t-Bu-substituted amine.

We reasoned that the high stability of aminopyronins 7–9 under physiological conditions, in conjunction with their high reactivity toward amines under more basic conditions, might be useful for detection of protein–protein interactions. Exchange of these fluorophores between proximal lysine residues at protein–protein interfaces, driven by the high effective concentration of donor and acceptor lysines, might reveal interacting proteins by the labeling of protein partners.

To evaluate this hypothesis in a model system, we conjugated bromopyronins 4–6 to lysines of human IgG protein (150 kDa) to afford conjugates 13–15 (Figure 3) at fluorophore:antibody ratios of 3–4. These conjugates were purified by size-exclusion chromatography and added to an aqueous buffer (pH 7.4) containing both Protein A (SpA, 42 kDa) from *S. aureus* and bovine serum albumin (BSA, 66 kDa). SpA binds the Fc region of human IgG with high affinity,[18] whereas BSA
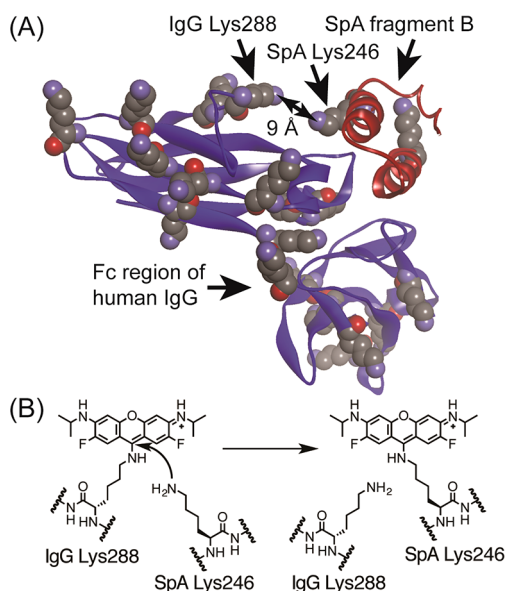
Figure 5. (A) X-ray structure of the Fc region of human IgG (blue) bound to Fragment B of SpA (red, PDB ID 1FC2). Carbohydrates of the IgG were omitted for clarity. Lysine residues are shown as CPK models. (B) Mechanism of molecular transfer of pyronin between lysine residues of SpA and IgG.

was included as a nonbinding but Lys-containing internal control. As shown in Figure 3, transfer of the fluorescent tag from IgG to SpA, but not BSA, was observed both qualitatively and quantitatively by SDS-PAGE. Although all three of these conjugates showed some transfer to SpA after 16 h, **14** labeled SpA to the greatest extent (Figure 3), and this conjugate was the focus of subsequent studies because it was more reactive than **13** and exhibited a higher quantum yield compared with **15**. Moreover, in further support of the mechanism shown in Figure 1, when lysines of IgG were conjugated to 5-carboxyfluorescein or fluorescein isothiocyanate (FITC) through more stable amide or thiourea bonds, no transfer of fluorophore from IgG to SpA was observed after 16 h (Figure S6, Supporting Information). These controls further confirm that the unique reactivity of these pyronin fluorophores toward amines is critical for detection of protein–protein interactions.

SpA contains five homologous domains with an affinity for the constant Fc region of most IgG subclasses.[18] This bacterial protein also binds the variable Fab fragments of some IgGs.[19] However, conjugation of **5** to purified Fc and Fab regions of human IgG revealed that the labeled Fc fragment exclusively transferred the pyronin to SpA (Figure S5, Supporting Information). To further examine the mechanism of transfer of pyronins from IgG to SpA, after the fluorophore was transferred, unreacted conjugate **14** was removed from labeled SpA using resin-bound SpA, and modification of specific SpA residues was analyzed by tryptic digestion and MS-based protein sequencing. Remarkably, as shown in Figure 4, among the 118 total lysine residues of mature SpA, only four homologous residues, Lys-69, Lys-130, Lys-188, Lys-246, predominantly reacted with the pyronins of IgG. To investigate the molecular basis of this pattern of reactivity, we examined an X-ray crystal structure of the Fc region of human IgG bound to Fragment B of SpA.[20] As shown in Figure 5, this structure revealed that the amino groups of Lys-246 of SpA and Lys-288 of IgG uniquely reside within 5–9 Å, depending on side-chain rotamer conformations, and represent the most proximal
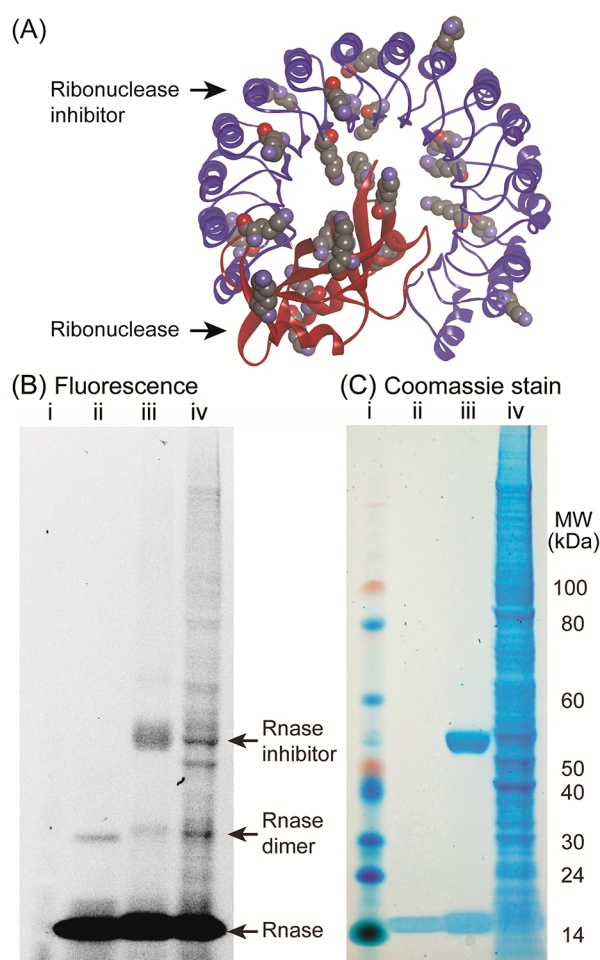

Figure 6. (A) X-ray structure of bovine Rnase A (red) bound to porcine Rnase inhibitor (blue, PDB ID 1DFJ) illustrating the proximity of lysine residues (CPK models) in the complex. (B–C) SDS-PAGE gel imaged by fluorescence (B) followed by staining of all proteins in the same gel with coomassie (C). (Lane i) protein MW marker. (ii) Pyronin-conjugated bovine Rnase A alone. (iii) Reaction of pyronin-conjugated bovine Rnase A (20 μM) with recombinant human Rnase inhibitor (20 μM) for 18 h at 37 °C. (iv) Reaction of pyronin-conjugated bovine Rnase A (20 μM) with a crude HeLa cell extract for 18 h at 37 °C. The arrows show specific proteins identified by comparison with authentic standards.

lysines in the complex. This close proximity enforced by the protein–protein interaction explains the highly selective reactivity of residues in Fragments A, D, and E that are homologous to SpA Lys-246.

To examine whether this approach might allow detection of protein–protein interactions in complex biological matrices such as a cell extract, we conjugated bovine ribonuclease (Rnase) to bromopyronin **5**. Rnase binds tightly to Rnase inhibitor, and a structure of a complex formed by these proteins is shown in Figure 6.[21] This fluorescent Rnase conjugate was added both to purified human ribonuclease inhibitor and to a crude extract of human HeLa cells, to promote transfer of the fluorophore to interacting proteins. Although the low solubility of Rnase limited the extent of labeling to 0.3–0.6 fluorophores per protein, reducing the sensitivity of the assay in this case, analysis by SDS-PAGE revealed transfer of the fluorophore to both recombinant human Rnase inhibitor and endogenous human Rnase inhibitor in HeLa extracts (Figure 6). These

proteins, and oligomeric forms of the Rnase conjugate,[22] were identified by comigration with authentic standards. Comparison of the limited number of fluorescent bands with the abundance of total proteins stained by coomassie blue in the cell extract demonstrated that fluorinated pyronin conjugates can exhibit high selectivity for the detection of specific interacting proteins.

We demonstrated that fluorinated pyronin fluorophores offer unique tools for detection of interactions between biomolecules. In a buffered aqueous solution (pH 7.4), amino derivatives of fluorinated pyronins can be highly stable, even in the presence of a large excess of other soluble amines. However, the high effective concentration that results from approximation of lysine residues at protein–protein interfaces enables transfer of these fluorophores between proteins, providing a means to identify interacting partners. Given that lysine is one of the most common amino acids on protein surfaces,[23] and these residues are often in close proximity at protein–protein interfaces, this method may be useful for the discovery of novel interactions and factors that affect these interactions.

ASSOCIATED CONTENT

Ⓢ Supporting Information

Experimental methods, compound characterization data, spectroscopic data, and additional control experiments. 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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