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Site-specific tagging proteins via a rigid, stable and short thiolether tether for paramagnetic spectroscopic analysis

Yin Yang1, Jin-Tao Wang2, Ying-Ying Pei and Xun-Cheng Su

Increasing the stability of protein bioconjugates and improving the resolution of protein complexes is important for spectroscopic analysis in structural biology. The reaction of phenylsulfonated pyridine derivative and protein thiol generates a stable, rigid and short thiolether tether, which is valuable in high-resolution spectroscopic measurements.

Site-specific labeling proteins with paramagnetic or fluorescent tags provide a powerful technique in determining the structures, interactions and dynamics of proteins and protein-ligand complexes by paramagnetic nuclear magnetic resonance (NMR), electron paramagnetic resonance (EPR), and fluorescent resonance energy transfer (FRET). Many efforts have been made towards high-yield selectivity in site-specific labeling of proteins with a paramagnetic or fluorescent tag including disulfide bond or thiolether bond formation, or via click-chemistry between non-natural amino acid and functional tags. The flexibility of the functional tag in protein bioconjugate averages the paramagnetic effects or delocalizes the paramagnetic/fluorescent center, resulting in deteriorated resolution in the structures determined by these spectroscopic methods. Therefore, restriction of the paramagnetic or fluorescent tag is beneficial in improving structural resolution determined by means of paramagnetic NMR, EPR and FRET. Several strategies have been proposed to increase the rigidity of the tag including coordination of protein to the paramagnetic center, increasing the size of tag, and optimizing the ligation site in a protein. However, an efficient method in tagging a protein is of high importance for high-resolution spectroscopic analysis especially in situ or in vivo in structural biology. We herein present a new way of site-specific tagging proteins with a rigid, stable and short thiolether ether via the reaction of phenylsulfonated pyridine derivatives and protein thiol (Scheme 1). The high performance of these new paramagnetic tags in rigidity and stability has been evaluated by paramagnetic NMR spectroscopy.

Phenylsulfone moiety is a valuable building block in organic synthesis, however, it has not been considered as a good leaving group in mild reaction conditions. To explore the reactivity of 4-phenylsulfonyl pyridine tags to a free thiol in aqueous solution, we synthesized two lanthanide tags, L1 (4-(phenylsulfonyl)-pyridine-2,6-dicarboxylic acid, 4PS-DPA) and L2 (4-phenylsulfonyl-(pyridin-2,6-diy)bismethylenenitrilo-tetrakis(acetic acid), 4PS-PyMTA) (Fig. 1, details in synthesis of L1 and L2 are provided in the supporting information). L1 and L2 are the phenylsulfonated derivatives of DPA and PyMTA, respectively. We show that phenylsulfonyl group at the 4-position of pyridine is a thiol-specific reaction reagent in protein bioconjugations via the formation of thiolether bond (Scheme 1), with a similar mechanism to a recent report of Juila-Kocienki like reagents with free thiols. The reaction of L1 and L-cysteine was first analysed by high resolution NMR spectroscopy in D2O at pD 7.5 with a proton frequency of 600 MHz. Significant chemical shift perturbations were observed for the resonances of 10 mM L1 after addition of 30 mM L-cysteine. By analysis of 2D NOESY, TOCSY, 13C-HSQC and 13C-HMBC spectra recorded for the reaction mixture and starting materials, we confirmed thiol ether bond was formed between L1 and L-cysteine, and phenylsulfinate was produced during the reaction (Fig. S1-S5). Notably, the nucelophilic attack to phenyl ring by releasing 4-sulfanatopyridine-2,6-dicarboxylate was not observed. Incubation of 4-(phenylsulfonyl) pyridine moiety with other amino acids containing potential nucleophiles including hydroxyl and amine groups (up to 48 hours) produced no chemical shift perturbations, suggesting the phenylsulfonated pyridine is highly thiol-specific (Figure S6-S12).

Scheme 1 Site-specific tagging proteins in a short, rigid and stable manner via thiol-ether bond formation between 4-(phenylsulfonyl) pyridine moiety and protein thiol.

Fig. 1 Lanthanide binding tags of pyridine derivatives containing a phenylsulfone or vinyl group.
The high reactivity and chemoselectivity of 4-phenylsulfonyl pyridine moiety to free thiols as demonstrated above suggests the 4-phenylsulfonate pyridine can be a versatile building block in protein modifications. As the chemical environment for individual residues in structured biomolecules may affect the chemical reactivity and selectivity in protein bioconjugations, we thus undertook the selectivity assay of microenvironment effects on the reactions of phenylsulfonated pyridine derivatives towards protein thiols.\(^{15}\) The chemical reaction process was monitored by \(^{15}\)N-HSQC spectra for a mixture of 1.0 mM \(^{15}\)N-labelled G47C mutant of human ubiquitin and 5.0 mM L1 in 20 mM Tris buffer at pH 7.6 and 298 K. The reaction was completed within 10 hours. The chemical shift perturbations showed that the residues with large chemical shift changes were those vicinal to the ligation site of G47C, and no chemical modifications of Met 1, K6, K11, K27, K29, K33, K48 and K63 residing in different structural environments were determined in \(^{15}\)N-HSQC spectrum (Fig. 2). This was further demonstrated by addition of Gd\(^{3+}\) ion into the solution of G47C-L1 conjugate, which was purified after a small desalting PD10 column. Gd\(^{3+}\) is the strongest paramagnetic metal ion, and it generates very strong paramagnetic effects (PREs) on the vicinal nuclear spins close to its binding area (Fig. S13).\(^{1ac}\)

These results clearly show that phenylsulfonated pyridine moiety can be used as a thiol-specific reaction reagent in site-specific labelling of proteins.

Since L1 has only three coordinating atoms for lanthanide ion, its protein conjugate requires the coordination of protein to immobilize the lanthanide ion. L2, a ligand with seven coordinating sites and very high-binding-affinity for lanthanide ion, was explored in the subsequent study. Quantitative ligation of G47C/R72A/R74A triple mutant of human ubiquitin with L2 was achieved by incubating the protein with a five-fold excess of L2 at room temperature and pH 7.6 for 16 hours. Low-molecular weight compounds were subsequently separated from the ligated product by ion-exchange chromatography (yield > 85%). Small chemical shift perturbations close to G47C were observed after ligation with L2 (Fig. 3). The chemical shift mapping indicates only G47C was modified in the protein-L2 conjugate (also in Fig. S14). The resulting ligation product formally resembles the previously described ubiquitin G47C-4vinylPyMTA complex,\(^{1b}\) except that two methylene groups are absent. Titration of G47C/R72A/R74A-L2 with paramagnetic lanthanide ions produced very larger chemical shift changes in the \(^{15}\)N-HSQC spectra (Fig. 4 and Fig. S15) in comparison with those obtained with G47C-4vinylPyMTA.\(^{5b}\) Despite the very short thiolether tether in the protein construct, only a single paramagnetic species was observed as the molar ratio of lanthanide to protein was increased from 0.2 to 1.2. Large PCSs of up to -0.6 ppm were observed even in the presence of the weakly paramagnetic lanthanide Yb\(^{3+}\). Four sets of PCSs were measured for Tb\(^{3+}\), Dy\(^{3+}\), Tm\(^{3+}\), and Yb\(^{3+}\), and the respective \(\Delta\chi\)-tensor parameters were determined using the program Numbat\(^{1b}\) to fit the crystal structure of ubiquitin\(^{11}\) (Table 1). Good correlations between the experimental and backcalculated PCSs were determined (Fig. S16). The metal position was found at a distance of 8.66 Å to carbon atom C\(^\alpha\) of G47 (Fig. 5).

As show in Table 1, the \(\Delta\chi\)-tensors determined in G47C/R72A/R74A-L2 are significantly larger than those obtained with G47C-4vinylPyMTA.\(^{5b}\) It indicates that the short thiolether tether in protein-L2 conjugate is sufficiently rigid to restrict the paramagnetic tag. Both L2 and 4vinylPyMTA in reaction with protein generate a highly stable thiolether tether, but L2, 4PS-PyMTA, is a more rigid paramagnetic tag. The magnetic susceptibility anisotropies
generated by protein-L2 complex are comparable with a number of rigid lanthanide binding tags like 4MMDPA, TAHA, LBT and 4MTDA, but are generally smaller than DOTA-tags that are also varied to the ligation site. The reaction of phenylsulfonated pyridine derivative and protein has several advantageous features: generation of a rigid and stable thiolether tether between protein and functional tag, which is resistant to the reducing reagents, is simple; and it doesn’t need to optimize the ligation site in protein. Moreover, 4PS-PyMTA is relatively small but has high binding affinity for lanthanide ion. As PyMTA has higher affinity for lanthanide ion than EDTA, the lanthanide complex of protein-L2 conjugate can be readily removed by addition of EDTA.

Fig. 4 Superimposition of 15N-HSQC spectra of uniformly 15N-labeled G47C/R72A/R74A ubiquitin derivatized with L2 at G47C in the presence of diamagnetic Y
d+ and paramagnetic Dy+ (red) and Tb+ (black), respectively. The molar ratio of lanthanide to protein was about 1.2:1. The spectra were recorded at 298K and pH 6.4 with a 1H NMR frequency of 600 MHz in 20 mM MES buffer.

Table 1. Δχ4-tensor parameters of ubiquitin G47C/R72A/R74A-L2 complexed with Tb+, Dy+, Tm+, and Yb+, respectively.[a]

<table>
<thead>
<tr>
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<th>Tb+</th>
<th>Dy+</th>
<th>Tm+</th>
<th>Yb+</th>
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<tbody>
<tr>
<td>Δχ4</td>
<td>-21.5 (3.4)</td>
<td>-24.5 (4.3)</td>
<td>16.6 (1.7)</td>
<td>6.7 (0.9)</td>
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<tr>
<td>Δχ2</td>
<td>-5.1 (0.9)</td>
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<td>3.9 (1.1)</td>
<td>1.4 (0.4)</td>
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<tr>
<td>α</td>
<td>91.4</td>
<td>91.2</td>
<td>91.4</td>
<td>91.6</td>
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<tr>
<td>β</td>
<td>122.2</td>
<td>121.7</td>
<td>123.2</td>
<td>121.2</td>
</tr>
<tr>
<td>γ</td>
<td>113.9</td>
<td>109.9</td>
<td>116.8</td>
<td>112.8</td>
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[a] The tensor parameters are in units of 10−32 m3. The tensor parameters determined for the corresponding lanthanide complexes of ubiquitin G47C-4vinylPyMTA are shown in brackets for comparison.

Table 2. Comparison of alignment tensor and Δχ4-tensor parameters of ubiquitin G47C/R72A/R74A-L2 complexed with Tb+, Dy+, Tm+, and Yb+, respectively.[a]

<table>
<thead>
<tr>
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</table>

[a] All RDCs for protein backbone amide group were measured at 25 °C and 600 MHz 1H NMR frequency. Alignment tensors determined by RDCs of backbone amides in regularly structural segments. Δχ4 and Δχ2 were obtained from Δχ4 and Δχ2 values in Table 1 by using equation (1)

Using Module program, the alignment tensors were determined by simulation the backbone amide RDCs of regularly structured residues to the protein structure (PDB code: 1UBI). Notably, the alignment tensors determined by RDCs are significantly smaller than those back-calculated from PCSs based on Δχ4-tensor using equation (1)

\[
A_{\alpha,\beta} = \frac{B_i^2}{15kT\mu_0} \Delta \chi_{\alpha,\beta} (1)
\]

where \(A_{\alpha,\beta}\) are the axial and rhombic components of the alignment tensor, \(\Delta \chi_{\alpha,\beta}\) the axial and rhombic components of magnetic susceptibility anisotropy tensor, respectively (Table 2 and Fig. S17). The different sensitivities of PCSs and RDCs to protein local mobility, first reported in the K79A mutant of cytochrome c in an alkaline condition, have been found in many lanthanide loaded proteins.7 In the case of ubiquitin G47C-L2 conjugate, the much smaller RDCs are probably not only caused by the local mobility of protein. The residual mobility of the tag and non-specific transient contact of the tag with protein surface are also likely to average RDCs. It should be pointed out that the hydrophobic patch formed by residues Leu8, Ile44 and Val70 in ubiquitin dominates most interactions of ubiquitin with its binding partners. Since G47C sits close to the hydrophobic hot spot, any non-specific contact of L2 with these hydrophobic residues will decrease the RDCs greatly. The less sensitivity of PCSs to protein local mobility and probably also residual fluctuations of tag than RDCs offers additional advantages in
structural biology by paramagnetic NMR spectroscopy. The PCS defined rigidity of protein-L2 conjugate provides the possibility of high-resolution distance measurements by double electron-electron resonance (DEER) and FRET methods, which can be extended to high-molecule-weight systems.

In conclusion, we report a new way of generating a stable, short, and rigid thioether bridge between the target protein and functional tag, which is also resistant to reducing reagents, for paramagnetic NMR spectroscopy analysis. 4PS-PyMTA is a small and high-affinity lanthanide binding tag, and the protein stability in protein-tag conjugate for the study of DEER, FRET (manuscript in preparation). With the high demand of simplicity and rapidity of protein ion binding tags including DOTA-derivatives or fluorescent tags defined rigidity of protein-L2 conjugate provides the possibility of high-resolution distance measurements by double electron-electron resonance (DEER) and FRET analysis. The sulfonated pyridine moiety can also be engineered into the established paramagnetic moiety will find broad applications in structural biology and cell biology.

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Notes and references

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1-10 Y and JT contributed equally in this work.

† Electronic Supplementary Information (ESI) available: [details of any supplementary information available should be included here].


The reaction of phenylsulfonated pyridine derivative and protein thiol is suitable for high-resolution spectroscopic analysis by generation of a rigid, stable and short thioether tether.