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Site-Specific Labeling of Proteins with a Chemically Stable, High-Affinity Tag for Protein Study

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Abstract: Site-specific labeling of proteins with paramagnetic lanthanides offers unique opportunities by virtue of NMR spectroscopy in structural biology. In particular, these paramagnetic data, generated by the anisotropic paramagnetism including pseudocontact shifts (PCS), residual dipolar couplings (RDC), and paramagnetic relaxation enhancement (PRE), are highly valuable in structure determination and mobility studies of proteins and protein–ligand complexes. Herein, we present a new way to label proteins in a site-specific manner with a high-affinity and chemically stable tag, 4-vinyl(pyridine-2,6-diyl)bismethylenenitrilo tetrakis-(acetic acid) (4VPyMTA), through thiol alkylation. Its performance has been demonstrated in G47C and E64C mutants of human ubiquitin both in vitro and in a crowded environment. In comparison with the published tags, 4VPyMTA has several interesting features: 1) it has a very high binding affinity for lanthanides (higher than EDTA), 2) there is no heterogeneity in complexes with lanthanides, 3) the derivatized protein is stable and potentially applicable to the in situ analysis of proteins.

Keywords: alkylation · conjugation · lanthanides · NMR spectroscopy · protein modifications

Introduction

Lanthanide ions are chemically similar but have very different magnetic and optical properties. Paramagnetic lanthanides with anisotropic paramagnetism provide a wealth of structural restraints including pseudocontact shifts (PCS), paramagnetic relaxation enhancement (PRE), and residual dipolar couplings (RDCs) of biomolecules.[1] These paramagnetic effects offer great advantages in the study of structures and dynamics of proteins and protein–ligand complexes.[2]

PCS are usually described by

$$PCS = \frac{1}{12\pi r^3}[\Delta\chi_{ax}(3\cos^2\theta - 1) + 1.5\Delta\chi_{rh}\sin^2\theta\cos 2\phi$$

in which r, θ, and ϕ are the polar coordinates of the nuclear spin with respect to the principal axes of the $\Delta\chi$ tensor. $\Delta\chi_{ax}$ and $\Delta\chi_{rh}$ are the axial and rhombic components of the $\Delta\chi$ tensor, respectively. It is evident that PCS are unique structural restraints containing both distances and angular orientations of nuclear spins relative to the paramagnetic center.

Because most proteins do not bind lanthanides specifically, generation of these paramagnetic data are usually achieved through site-specific labeling of proteins with lanthanide-binding ligands.[3] Recently, much effort has been directed towards tagging proteins with lanthanides, including attaching a chemically synthesized ligand through one or two disulfide bonds,[4] insertion of a lanthanide-binding peptide into a protein,[5] and non-covalently but specifically binding a protein with a lanthanide complex.[6] Fusion of a lanthanide-binding peptide into a protein avoids chemical modification of the protein and disulfide bond formation between the protein and tag, but suitable insertion sites in a protein are rare and limited. The specific interaction between a lanthanide complex and a protein has been shown to be an attractive way of obtaining such paramagnetic data, but engineering a specific binding motif capable of recognizing the lanthanide complex is needed for many proteins.[6b] To date, disulfide-bond tethers between the protein and the chemically synthesized ligand is still the most common way to tag proteins due to the unique reactivity of the thiol group and the flexibility of engineering the ligation site in a protein. However, the instability of the disulfide-bond tether precludes the subsequent use of -S–S- derivatives in the presence of reducing agents and in situ conditions.

In designing a lanthanide-binding tag, several issues have to be taken into account: 1) the affinity for lanthanide metal ions, 2) rigidity, 3) chirality, 4) size, and 5) side-effects on the protein. Lanthanide metal ions have eight to nine coordination sites and a suitable tag needs to possess multiple coordinating atoms to form a stable complex with lanthanide. In addition, generation of the enantiomer in coordination with lanthanide should be avoided so that a single paramagnetic species is present in the NMR spectrum. Because a low-

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binding-affinity tag does not capture the lanthanide tightly, a certain amount of free paramagnetic ion will be present, which can result in nonspecific PRE or additional binding to the protein. Side-effects on proteins may also be caused by nonspecific interactions between the tag and the protein, especially for tags with high molecular weight. 4-mercaptomethyldipicolinic acid (4MMDPA),[7a] 3-mercaptodipicolinic acid (3MDPA),[7b] 4-mercaptodipicolinic acid (4MDPA),[7c] nitrilotriacetic acid (NTA),[4f] and 4-vinyl dipicolinic acid (4-vinylDPA)[8] have been shown to have minimum perturbation on proteins due to their small sizes, but the insufficient coordinating numbers of these tags require the coordination of one acidic amino acid close to the ligation site in proteins.

To overcome the above limitations, we developed an approach through which site-specific labeling of proteins could be achieved with a high-affinity lanthanide-binding tag in a chemically stable manner.[8] We first synthesized an interesting lanthanide-binding tag, 4-vinyl(pyridine-2,6-diyl)bismethylenenitrilo tetrakis(acetic acid) (4VPyMTA; Figure 1).

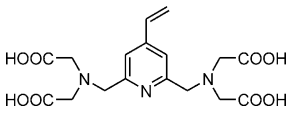


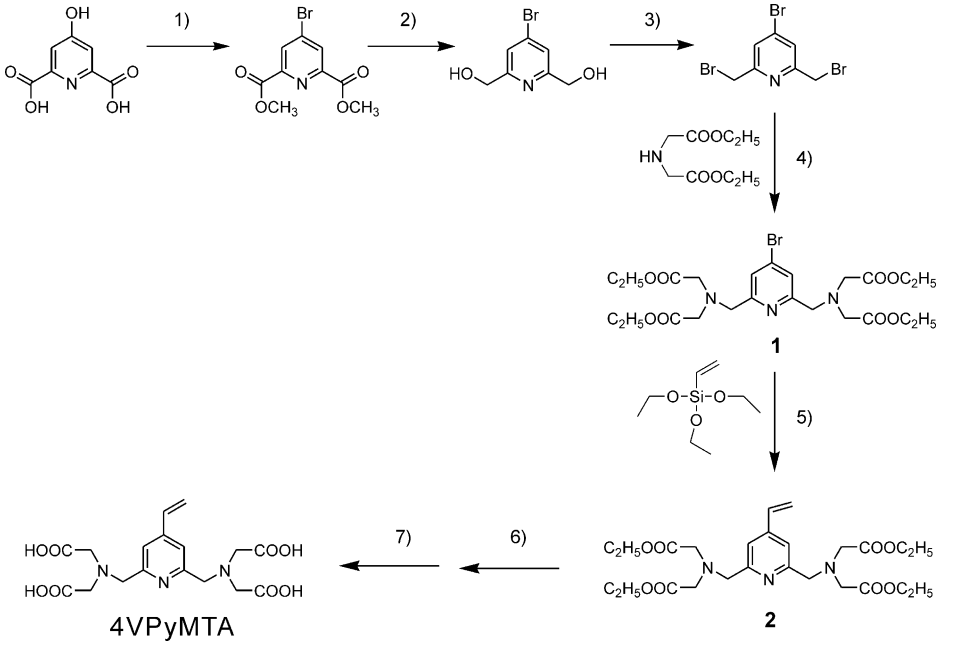
Figure 1. Chemically stable and high affinity lanthanide tag, 4VPyMTA.

4VPyMTA contains one vinyl group that reacts with SH of cysteine and a common metal-chelator, pyridine-2, 6-diyl-bismethylenenitrilo tetrakis (acetic acid), which has been widely used in luminescence and MRI studies[9] and also has very high affinity for lanthanide metal ions ($K_a > 10^{18}\,M^{-1}$).[9c] Two G47C and E64C mutants of human ubiquitin were successfully labeled with 4VPyMTA through thiol alkylation reactions. Both constructs are stable in the presence of reducing reagents and present high-quality NMR spectra. Sizable PCS were produced in paramagnetic samples. In addition, no heterogeneity was observed in the paramagnetic coordinated protein samples. It has been shown that the protein–4VPyMTA construct is an ideal system for the study of protein stability and self-assembly processes under in situ conditions.

Results

Synthesis of 4VPyMTA: Starting from chelidamic acid, seven steps were required to obtain 4VPyMTA with a total yield of approximately 11% (Scheme 1). In comparison with the synthesis of published tags,[3,4] large quantities of 4VPyMTA is practically achievable. It is noteworthy to point out that only a trace amount of 4VPyMTA was obtained from the fluorine-free cross-coupling between **1** and triethoxyvinylsilane in the presence of $Pd(OAc)_2$ and NaOH in aqueous solution,[10] which is probably due to the inactivation of palladium in coordination with the hydrolyzed **1** in basic aqueous solution.

Tagging reaction and NMR assignments: The reaction of 4VPyMTA and L-cysteine was studied by 1D ^{1}H NMR spectroscopy at 298 K; the NMR spectral changes are shown in Figure 2. The peak intensity of 1.0 mM 4VPyMTA in 20 mM phosphate at pH 7.5 was gradually decreased after addition of 3.0 mM L-cysteine, and the reaction was completed within six hours. Notably, no line broadening effects were observed in the NMR spectra of the mixture of 4VPyMTA and L-cysteine, suggesting that no radicals were produced in the reaction (Figure 2). In contrast, no NMR spectral changes of 4VPyMTA were detected upon incubation of 1.0 mM 4VPyMTA with 6.0 mM L-lysine or L-methionine for 24 h under the same conditions (Figure S1), indicating that the amino group of lysine and the N-terminal methionine do not react with 4VPyMTA. The reaction of 4VPyMTA and cysteine is specific for the sulfhydryl group. In the case of 4VPyMTA and ubiquitin mutants, the reaction rate was slower than with free cysteine. At pH 7.8, thiol alkylation was monitored by ^{15}N-HSQC spectroscopic analysis and the cross peaks corresponding to the free protein completely disappeared after 24 h, implying that the tag was quantitatively attached to the protein both for G47C and E64C (Figure S2 in the Supporting Information).



Scheme 1. Synthesis of 4VPyMTA. Reagents and conditions: 1) PBr_5, CH_3OH; 2) $NaBH_4$, EtOH; 3) PBr_3, $CHCl_3$; 4) K_2CO_3, CH_3CN; 5) TBAF, $Pd(OAc)_2$, PPh_3, DMF; 6) NaOH, H_2O, EtOH; 7) Dowex W50H.

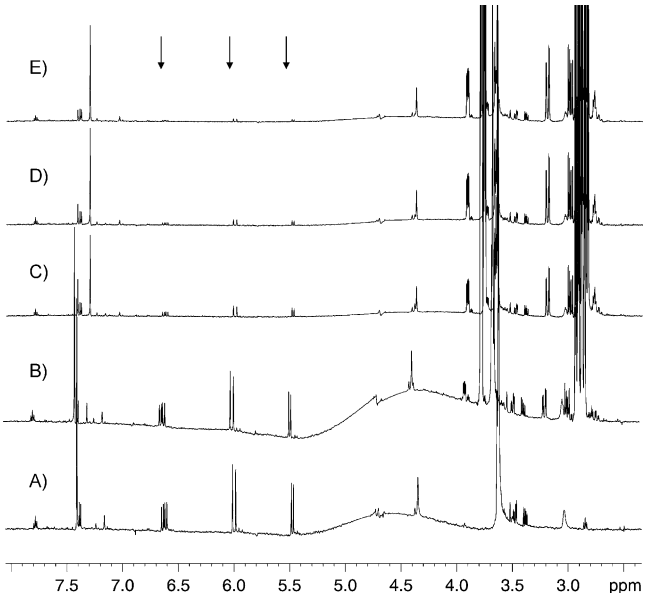


Figure 2. Reaction of 4VPyMTA and L-cysteine in 20 mM phosphate buffer at pH 7.5, which was monitored by 1D ^{1}H NMR spectroscopic analysis. A) 1.0 mM 4VPyMTA; B) 1.0 mM 4VPyMTA mixed with 3.0 mM L-cysteine; C) incubation of the mixture B at room temperature for two hours; D) as in C for 3.5 h; E) as in C for 5 h. The NMR spectra were recorded at 298 K with a proton frequency of 600 MHz; the arrows depict the intensity changes of vinyl protons in 4VPyMTA during the reaction.

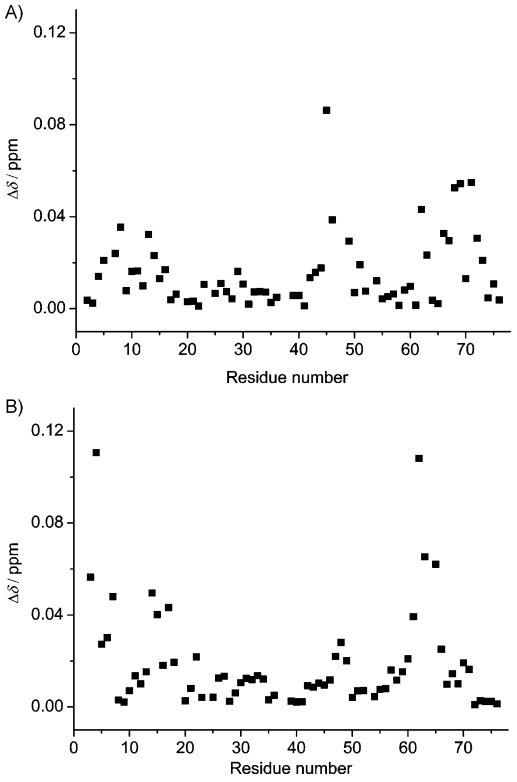


Figure 3. Chemical shift differences between ubiquitin and ubiquitin–4VPyMTA derivatives; the chemical shifts of protein backbone amides were calculated as $\Delta\delta=((\Delta\delta_H)^2+(\Delta\delta_N/10)^2)^{1/2}$. A) G47C and G47C-4VPyMTA; B) E64C and E64C-4VPyMTA.

The protein backbone amides were assigned on the basis of 3D ^{15}N-NOESY-HSQC and ^{15}N-HSQC spectra. Chemical shift changes in 4VPyMTA-derivatized G47C and E64C ubiquitins were limited to the amides in the vicinity of G47C and E64C, respectively. The sizes of chemical shift changes were small and most changes were within 0.05 ppm, as shown in Figure 3, indicating that the conjugation of 4VPyMTA to protein has negligible perturbation on protein structure.

The ^{15}N-HSQC cross-peaks of the paramagnetic samples were assigned on the basis of assignments of the diamagnetic spectrum, following the chemical shift changes of an amide group. With respect to the paramagnetic center, the ^{1}H and ^{15}N spins of one amide group are close, and they have similar chemical shift changes. As a consequence, a number of well-resolved paramagnetic peaks could readily be assigned. For additional assignments, the 3D structure of ubiquitin was used in comparison with the back-calculated PCS in the paramagnetic ^{15}N-HSQC spectrum.

PCS measurements and $\Delta\chi$-tensors: Significant PCS were observed in the complexes of 4VPyMTA-derivatized G47C and E64C ubiquitin with paramagnetic lanthanides Tb^{3+}, Dy^{3+}, Tm^{3+}, and Yb^{3+} (Table 1). All the paramagnetic metal ions produced high quality NMR spectra (Figure 4, Figure S3, and Figure S4). For each backbone amide, a single cross-peak was observed in the presence of diamagnetic Y^{3+} or any other paramagnetic ion. No heterogeneity of protein conformation exchange in the lanthanide loaded protein samples was detected.

Table 1. The $\Delta\chi$-tensor parameters of ubiquitin-4VPyMTA complexed with Tb^{3+}, Dy^{3+}, Tm^{3+}, and Yb^{3+}, respectively.[a]

		Tb^{3+}	Dy^{3+}	Tm^{3+}	Yb^{3+}
G47C-4VPyMTA	$\Delta\chi_{ax}$	3.4	4.3	1.7	0.9
	$\Delta\chi_{rh}$	0.9	2.3	1.1	0.4
E64C-4VPyMTA	$\Delta\chi_{ax}$	3.8	4.4	−2.3	−1.2
	$\Delta\chi_{rh}$	1.2	2.1	−0.5	−0.2

[a] The tensor parameters are in units of 10^{-32} m^3.

PCS were measured in the samples containing 1:1 mixtures of diamagnetic (Y^{3+}) and paramagnetic (Tb^{3+}, Dy^{3+}, Tm^{3+}, or Yb^{3+}) lanthanides. The PCS measured for the ubiquitin–4VPyMTA complexes with Tb^{3+}, Dy^{3+}, Tm^{3+}, and Yb^{3+} were used to determine the $\Delta\chi$-tensors of the respective lanthanides. Four sets of PCS were applied in simultaneous fit of the tensors with the crystal structure of ubiquitin. The calculated $\Delta\chi$-tensor parameters are listed in Table 1. Similar magnitudes of $\Delta\chi$-tensors for a paramagnetic ion were observed in 4VPyMTA-tagged G47C and E64C proteins.

To verify the simulation of $\Delta\chi$-tensors, Figure 5 depicts the plot of experimental and calculated PCS. Good correlations between the experimental PCS and back-calculated data are observed, which are in good agreement for the G47C and E64C constructs, respectively.

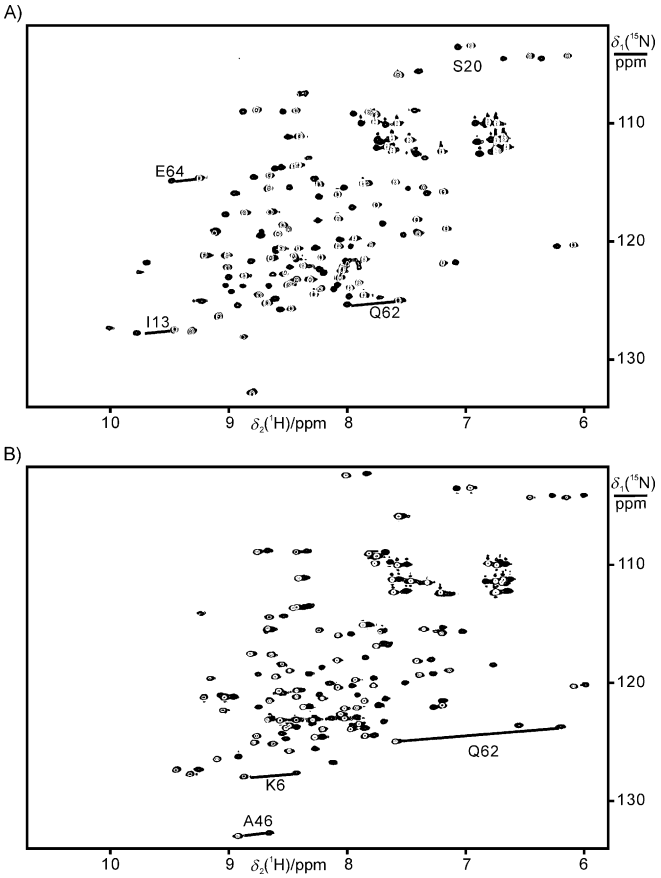


Figure 4. A) Superposition of ^{15}N-HSQC spectra of 0.10 mM uniformly ^{15}N-labeled G47C-4VPyMTA in the absence (grey) and presence of a 1:1 mixture of Y^{3+} and Dy^{3+} (black). B) Superposition of ^{15}N-HSQC spectra of 0.10 mM uniformly ^{15}N-labeled E64C-4VPyMTA in the absence (grey) and presence of a 1:1 mixture of Y^{3+} and Dy^{3+} (black). The ratio of lanthanides to protein was about 1:1.

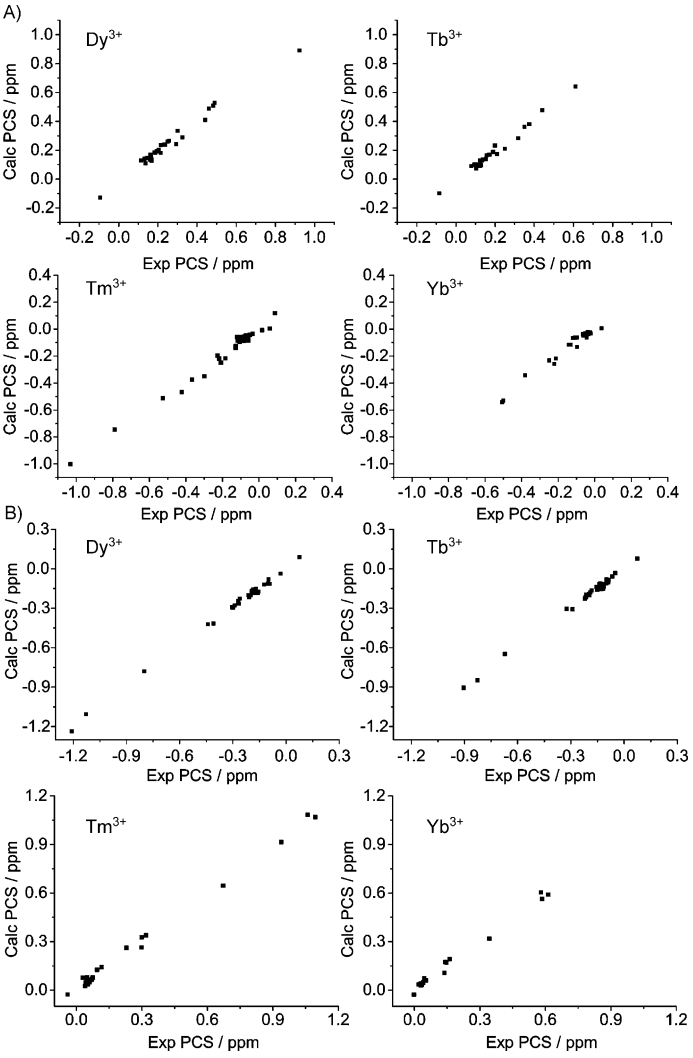


Figure 5. Correlations of the back-calculated PCS (Calc PCS) plotted against the experimentally measured PCS (Exp PCS). A) Ubiquitin G47C-4VPyMTA; B) ubiquitin E64C-4VPyMTA.

Stability of 4VPyMTA with lanthanides: To assess the thermostability of 4VPyMTA-derivatized proteins with lanthanide complexes, we titrated ethylenediaminetetraacetic acid (EDTA), which is a widely used high-affinity chelating ligand for lanthanides, into a solution of ubiquitin–4VPyMTA and lanthanide complex. At [EDTA]/[protein-4VPyMTA-Dy^{3+}] molar ratios up to 2:1, no significant changes were observed in the ^{15}N-HSQC spectrum of ubiquitin–4VPyMTA and lanthanide complex (Figure S5), suggesting that the protein–4VPyMTA has a higher binding affinity than EDTA.

Analysis of ubiquitin–4VPyMTA in crowded conditions: The protein stability of 0.1 mM ^{15}N-G47C-PyMTA was investigated by using 2.0 mM hen white lysozyme (HEWL) and 2.0 mM bovine serum albumin (BSA) as the crowder, respectively. Paramagnetic metal ions were titrated into the above protein solution and ^{15}N-HSQC spectra were recorded (Figure 6). In the presence of HEWL and BSA, the protein construct was stable. Addition of paramagnetic lanthanide produced similar PCS to those shown in Figure 4 and Figure S3.

Discussion

We demonstrated a new approach to site-specific labeling of proteins with a lanthanide-binding tag that is not only chemically stable but also has high affinity for lanthanides. The tagged protein and lanthanide complex generates a single species so that the protein sample produces a clean and high-quality NMR spectrum. Thiol alkylation, especially the thiol-ene reaction, has been well-studied in material science,[11] but few examples have been applied in protein chemistry,[9,12] of which maleimide derivatives were mostly used in the site-specific labeling of proteins with radicals for EPR and fluorescence studies.[12,13] However, the reaction of maleimide with cysteine produces a new chiral center, resulting in two cross-peaks in the NMR spectrum for the paramagnetic lanthanide-bound protein.[8] Compared with holoacetamide tags, which are also widely used in protein conjugations,[14] the reaction rate of 4VPyMTA with cysteine is slower but more selective in many cases. Holoacetamide

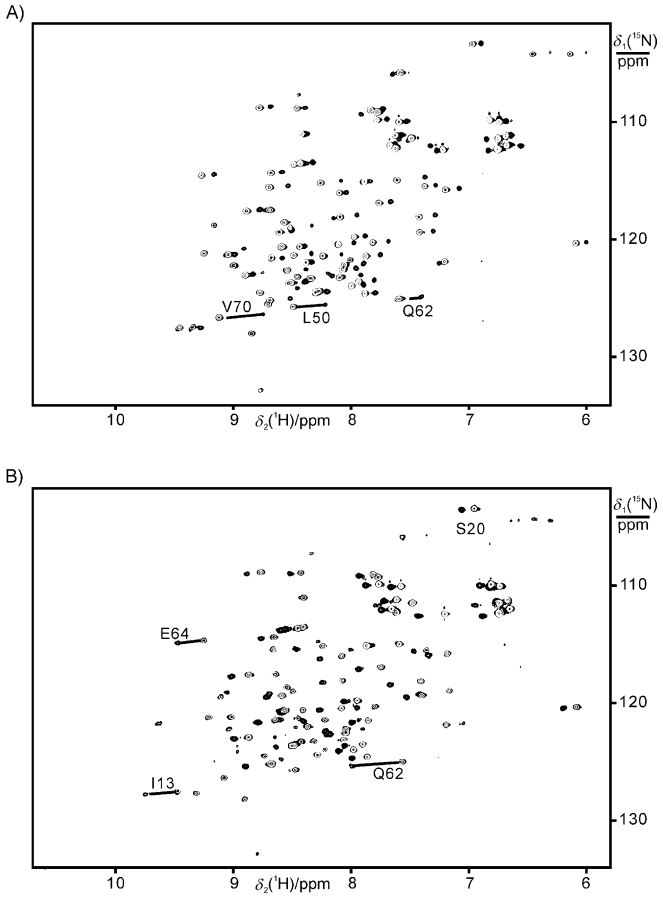


Figure 6. A) Superposition of ^{15}N-HSQC spectra of 0.10 mM uniformly ^{15}N-labeled G47C-4VPyMTA and 2.0 mM HEWL in the absence (grey) and presence of a 0.05 mM Tm^{3+} (black). B) Superposition of ^{15}N-HSQC spectra of 0.10 mM uniformly ^{15}N-labeled G47C-4VPyMTA and 2.0 mM BSA in the absence (grey) and presence of 0.05 mM Dy^{3+} (black).

tends to react with the amino groups of proteins at a slower rate than with the free thiol group.

Only one paramagnetic species was observed in the sample of 4VPyMTA-derivatized protein. Below pH 8.2, there were no detectable side-products generated by 4VPyMTA and the amino group of the N-terminal methionine or the side chain of lysine in the protein. In addition, generation of radicals due to the irradiation applied in polymer science for thiol-ene addition,[11] which is harmful to protein and DNA, was also avoided in the present study. The reaction of 4VPyMTA and cysteine shows no radicals produced during the reaction because the presence of radicals would generate strong PRE effects on the NMR signals. In addition, the reactivity of 4VPyMTA depends on the position of cysteine in proteins, and 4VPyMTA only reacts with the solvent-exposed cysteine residues. No thiol alkylation was observed for Sortase A (SrtA) from *Staphylococcus aureus*, a cysteine protein enzyme that has an active cysteine that is not solvent exposed (data not shown). In comparison with the previously reported DPA[7,8] and NTA[4f] tags, which need to be immobilized with coordination of protein, 4VPyMTA has several advantages: first, the thioether tether between protein and tag is more stable than disulfide bonds under harsh conditions, and is also resistant to the reducing reagents; second, 4VPyMTA has seven coordination numbers and forms highly stable lanthanide complexes[9c] and also prevents the generation of enantiomers in complex with lanthanide; third, 4VPyMTA only reacts with the solvent-exposed cysteine and not with buried cysteine residues within the proteins. It is noteworthy to point out that the site-specific labeling of proteins through thiol alkylation reaction or disulfide bond formation is not practical for proteins that have multiple solvent-exposed cysteines or one solvent-exposed cysteine that is crucial to protein function. In these cases, insertion of a lanthanide-binding peptide into the protein sequence[5] or the design of binding sites for paramagnetic metal complex[6] is preferable.

Ubiquitin plays a central role in the regulation of many cellular processes through its ability to bind a large number of proteins specifically. Structural analysis of ubiquitin protein complexes has shown that G47 and E64 are located in a flexible region covered by the active sites of protein when ubiquitin binds to its partners. The difficulty of locating paramagnetic metal ions by fitting of $\Delta\chi$-tensors lies in both the size and sign of PCS. The uniform sign of PCS generated by each paramagnetic ion means it is hard to assign a metal position precisely even when multiple sets of PCS are used.[7b] In Figure 5, Dy^{3+}, Tb^{3+}, Tm^{3+}, and Yb^{3+} all generate both positive and negative PCS in ubiquitin. Indeed, the position of the paramagnetic ion has been precisely determined by simultaneous fit of four sets of PCS to the protein structure. Figure 7 depicts the calculated metal position with four sets of paramagnetic ions; the distances of metal ion to the amide protons of G47 and E64 are 7.0 and 4.5 Å, respectively.

Sizable PCS and sufficient numbers of PCS are equally important in structure determination and mobility studies of proteins. A rigid tag gives large PCS but can lead to strong

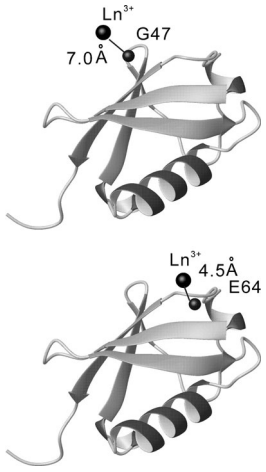


Figure 7. Ribbon drawing of ubiquitin showing the metal ion position determined by fitting the PCSs of the ubiquitin-4VPyMTA and lanthanide complexes to the ubiquitin structure (PDB code: 1ubi), and the respective distances to the amide proton of G47 and E64.

broadening effects on protein residues. Bulky tags usually generate significant PCS,[15] but the dynamic exchange between the tag and target protein tends to result in strong PRE effects, especially in μs–ms motion. Because G47 and E64 are located in a flexible part of ubiquitin, the mobility of the loop averages the size of PCS, resulting in smaller magnitude of $\Delta\chi$-tensors compared with DPA[7,8] and NTA[4f] tags, as well as DOTA-derivatives,[4e,15a–c] peptide tags,[4d,g,15d] and TAHA-like tags.[16] Nevertheless, a large number of residues still experience profound PCS. Remarkably, only a few residues are broadened even for strong anisotropic paramagnetic ions such as Tb^{3+} and Dy^{3+} (Figure 4). The lower magnitude of $\Delta\chi$-tensors is beneficial to the more observable residues in the paramagnetic bound protein samples.

The ubiquitin–4VPyMTA construct is stable against the commonly used crowders, HEWL and BSA, in mimicking the cellular environment.[17] In particular, the construct is stable in the presence of 2.0 mM BSA, which contains 35 cysteines and also binds lanthanide strongly.[18] The 4VPyMTA conjugated protein constructs thus offer great opportunities to investigate protein stability and self-assembly under in situ conditions by paramagnetic NMR spectroscopic analysis.

Conclusion

We have demonstrated an interesting lanthanide-binding tag, 4VPyMTA, that can be simply incorporated into proteins through thiolalkylation reactions. Because of its high binding affinity for lanthanides and chemical stability, it is anticipated that 4VPyMTA will become a very useful tag in site-specific labeling of proteins with lanthanides for structural biology studies.

Experimental Section

Synthesis of 4VPyMTA: The synthesis of 4VPyMTA is shown in Scheme 1.

4-Bromo-2,6-bis[*N*,*N*′-bis(ethyoxycaronylmethyl)aminomethyl]pyridine (1): Starting from chelidamic acid, **1** was synthesized by following a similar procedure to that reported previously.[19]

4-Vinyl-2,6-bis[*N*,*N*′-bis(ethyoxycaronylmethyl)aminomethyl]pyridine (2): Similar to the previous report,[20] **1** (2.0 g, 3.5 mmol) was dissolved in DMF (60 mL), and then triethoxyvinylsilane (1.5 mL, 7 mmol) and TBAF (tetrabutylammonium fluoride; 13.5 mL, 13.5 mmol) in THF stock, $Pd(OAc)_2$ (40 mg, 2.8 mmol), and PPh_3 (140 mg, 5 mmol) were added stepwise under argon protection. The resulting solution was stirred at 70 °C for 6 h and then cooled to room temperature. The solution was diluted with water (300 mL), the mixture was extracted with ethyl acetate (2×60 mL), and the combined organic phases were washed with brine, dried with anhydrous sodium sulfate, filtered, and evaporated under reduced pressure. The resulting yellowish oil was purified by chromatography on silica (petroleum ether (b.p. 60–90 °C)/ethyl acetate 1:1.5) to give the product as a yellowish oil (0.95 g, 52 %). ^{1}H NMR (400 MHz, $CDCl_3$, 298 K): δ = 7.48 (s, 2H), 6.69 (dd, J = 17.7, 11.0 Hz, 1H), 6.03 (d, J = 17.7 Hz, 1H), 5.45 (d, J = 11.0 Hz, 1H), 3.74 (s, 4H), 3.53 (q, J = 7.08 Hz, 8H), 3.10 (s, 8H), 1.06 ppm (t, J = 7.24 Hz, 12H).

4-Vinyl(pyridin-2,6-diyl)bismethylenenitrilo tetrakis(acetic acid) (4VPyMTA): Compound **2** (0.9 g) was first mixed with ethanol (5 mL) and H_2O (5 mL), and then 2.0 M NaOH (5 mL) was added into the above mixture. The resulting solution was stirred at room temperature overnight. Dowex H^+-ion-exchange resin (10.0 g) was added and the solution was filtered when the pH of the suspension was decreased to 3. The solution was evaporated under reduced pressure and the solid was suspended in acetone (10 mL) and filtered to give the product (0.62 g, 84 %) as a white solid. ^{1}H NMR (400 MHz, 298 K, D_2O): δ = 7.40 (s, 2H), 6.66 (dd, J = 18.8, 11.3 Hz, 1H), 6.00 (d, J = 18.8 Hz, 1H), 5.43 (d, J = 11.3 Hz, 1H), 3.72 (s, 4H), 3.07 ppm (s, 8H). ESI-MS: m/z calcd for $C_{17}H_{21}N_3O_8$: 394.13 $[M-H]^-$; found: 394.20.

Construct design and expression of ubiquitin G47C and E64C: The single-point mutants G47C and E64C of human ubiquitin were prepared in a pET3a vector by PCR-mediated site-directed mutagenesis using the primers 5′-AGGCTGATCTTTGCTTGTAAACAGCTGGAAGATGGGC-3′ and 5′-TCTTCCAGCTGTTTACAAGCAAAGATCAGCCTCTGCTGG-3′ for G47C (GGA > TGT), and primers 5′-CTACAACATCCAGAAATGCTCCACCCTGCACCTGGTAC-3′ and 5′-GTGCAGGGTGGAGCATTTCTGGATGTTGTAGTCAGAC-3′ for E64C (GAG > TGC), respectively.

The coding region in all plasmids was confirmed by DNA sequencing. The correct plasmid was transformed into the *E. coli* strain Rosetta (DE3) (Novagen).

Uniformly ^{15}N-labeled protein was expressed by growing the cells with high-density methods.[21] The target protein was purified from the soluble fraction of cell lysate by ammonium sulfate precipitation, followed by chromatography on DEAE columns (GE Healthcare Biosciences) and G50 (GE Healthcare Biosciences) gel filtration. 30 mg of protein was usually obtained from 250 mL media.

Tagging reactions: A ten-fold excess of 4VPyMTA in a 100 mM stock in water was added to a solution of 0.30 mM ^{15}N-labeled protein in 2.0 mL, 20 mM Tris and 0.30 mM TCEP at pH 7.8. The mixture was adjusted to pH 7.8 with 1.0 M NaOH and the protein solution was incubated at room temperature for about 24 h. Excess tag was removed with a PD10 column and the sample was concentrated with a Millipore ultrafilter to a final protein concentration of about 1.0 mM. The overall yield of purified ligation product was usually above 80 %.

NMR spectra: All 2D and 3D NMR experiments were performed at 298 K in 20 mM MES buffer (pH 6.5) with a ^{1}H NMR frequency of 600 MHz with a Bruker AV600 NMR spectrometer equipped with a QXI probe. 3D NOESY-^{15}N-HSQC spectra (100 ms mixing time, total recording time 36 h) were recorded with a 0.80 mM solution of diamagnetic ^{15}N-ubiquitin G47C and E64C in 90 % H_2O/10 % D_2O, respectively. All ^{15}N-HSQC spectra in the presence of diamagnetic and paramagnetic lanthanide ions (molar ratio 1:1) were recorded in 0.10 mM protein solution. PCS were measured from ^{15}N-HSQC spectra as differences in ^{1}H chemical shifts between samples with paramagnetic lanthanide and diamagnetic Y^{3+}. In situ NMR spectroscopic analysis was performed with 0.10 mM ^{15}N-ubiquitin-4VPyMTA in the presence of 2.0 mM hen egg white lysozyme (HEWL) and 2.0 mM bovine serum albumin (BSA) as the crowder, respectively, and 20 mM MES at pH 6.5.

Fitting of the $\Delta\chi$-tensors: The $\Delta\chi$-tensor parameters were determined by using the Numbat program.[22] Only PCS data from residues located in regions of well-defined secondary structure were included in the fits to the crystal structure of ubiquitin (pdb code: 1ubi).[23] In particular, no data from the flexible loops and the flexible C-terminal residues were used.

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