Protein Labeling

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# Tyrosine-Targeted Spin Labeling and EPR Spectroscopy: An Alternative Strategy for Studying Structural Transitions in Proteins**

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Site-directed spin labeling (SDSL) combined with electron paramagnetic resonance (EPR) spectroscopy is a powerful technique for studying structural transitions in proteins,[1] especially flexible or disordered proteins.[2] The conventional use of this technique is based on insertion of a paramagnetic label (nitroxide) at a cysteine residue, most often introduced by site-directed mutagenesis. However, although cysteine residues are rare in proteins, they frequently have functional roles and are involved in structural elements such as disulfide bridges or in the binding of metal cofactors.[3]

Such a difficulty was recently encountered in the study of a small and flexible chloroplast protein CP12 from the green alga *Chlamydomonas reinhardtii* by spin-labeling EPR spectroscopy. In this organism, CP12 contains four cysteine residues involved in two disulfide bridges in its oxidized state. Although the introduction of spin labels at the two cysteine residues of the C-terminal disulfide bridge enabled us to identify a new role of the partner protein glyceraldehyde 3-phosphate dehydrogenase (GAPDH), it precluded the direct study of the complex formation GAPDH/CP12.[4] To overcome this difficulty, grafting of the nitroxide probe to residues other than cysteines is required. One strategy using a genetically encoded unnatural amino acid has been recently proposed.[5] The incorporation of such unnatural amino acids relies, however, on a rather complex strategy involving an orthogonal tRNA/aminoacyl-tRNA synthetase pair specific for the unnatural amino acid. As such a strategy is difficult to set up, we propose an alternative method consisting of selectively targeting residues other than cysteines with a nitroxide probe.

Bioconjugation of small molecules to protein residues is very challenging, and several reactions have recently been proposed to target specific residues selectively.[6] In particular, efforts have been paid to modify the aromatic amino acid side chains of tryptophan[7] and tyrosine.[8] Among them, a three-component Mannich-type reaction has been developed that allows the modification of tyrosine under mild, biocompatible, and metal-free conditions.[8a] Inspired by these recent studies, we present the selective grafting of a nitroxide probe to tyrosine by using the Mannich-type reaction on CP12, a protein bearing only one natural tyrosine residue. This unique tyrosine residue, located at position 78 in the sequence of a total of 80 amino acids, makes this protein an ideal candidate for demonstrating the feasibility of tyrosine-targeted spin labeling (Figure 1).

The three-component Mannich-type reaction was performed as described by McFarland et al.[8a] on a mixture of

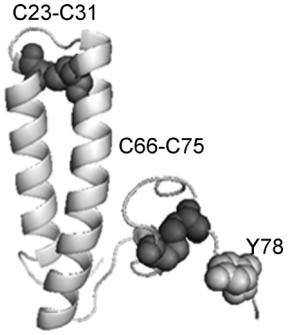


***Figure 1.*** Modeled structure of CP12 in its oxidized state, indicating the position of the tyrosine residue and the two disulfide bridges (from Ref. [9]).

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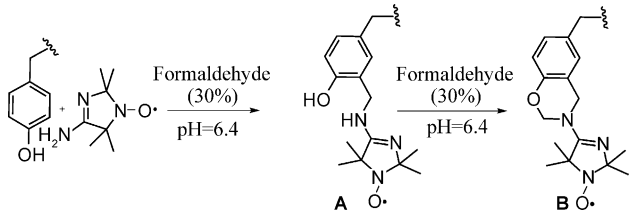
130 μM CP12 in 10 mM phosphate buffer at pH 6.4 with formaldehyde and 4-amino-2,2,5,5-tetramethyl-3-imidazoline-1-yloxy nitroxide in 1250-fold and 10-fold molar excess, respectively (Scheme 1). The mixture was stirred for 16 h at room temperature in air. The excess of unreacted spin label was removed by gel filtration (PD10 column, GE Healthcare). The elution fractions were analyzed both by measuring the protein concentration (using the Bio-Rad reagent protein assay)[10] and by calculating the spin concentration from the double integral of EPR spectroscopy signals, yielding 70(10) % labeling.




***Scheme 1.*** Three-component Mannich-type reaction on tyrosine residues with the two possible products **A** and **B**.

To confirm the site selectivity of the reaction on CP12, the cysteine residues of the labeled protein and a control sample of unlabeled CP12 were reduced by dithiothreitol and alkylated by iodoacetamide (mass increment of 57 Da per cysteine residue), then digested with trypsin. Analysis of the samples by MALDI-ToF MS indicated the presence of different peptides (see the Supporting Information), one of which bore the expected mass increment for the grafted spin probe (180 Da) corresponding to product **B** (Scheme 1). This peptide corresponded to the C-terminal 27 amino acids containing the tyrosine residue, a sequence confirmed by MS/MS data obtained after fragmentation of the intense ion precursor at *m*/*z* 2999.4 (see the Supporting Information). Reduction and alkylation reactions were not complete, and the same C-terminal peptide bearing the spin label was observed as mono- and dicarbamidomethylated forms of cysteines C66 and C75 (Figure 2).

The possibility that the spin probe could be grafted to cysteine residues was rejected, as they do not participate in the reaction except as reduced disulfides,[8a] a requirement that was not satisfied under our experimental conditions during the labeling reaction. Moreover, the detection of the labeled dicarbamidomethylated form of CP12 reinforced this finding. It is known that after a long reaction time, the Mannich-type reaction can modify tryptophan residues.[8a] Modification of the unique tryptophan residue at position 35 in CP12 was not detected, however, under our experimental conditions. From these analyses we concluded that the spin probe was grafted on the unique tyrosine residue of CP12. A mass increment of 168 Da corresponding to the product **A** (Scheme 1) was not found on the peptide mass fingerprint of the labeled CP12. This observation was confirmed by electrospray MS analyses of an esterified tyrosine free amino acid modified by the nitroxide probe, which showed that **B** was the only product of the reaction (see the Supporting Information).

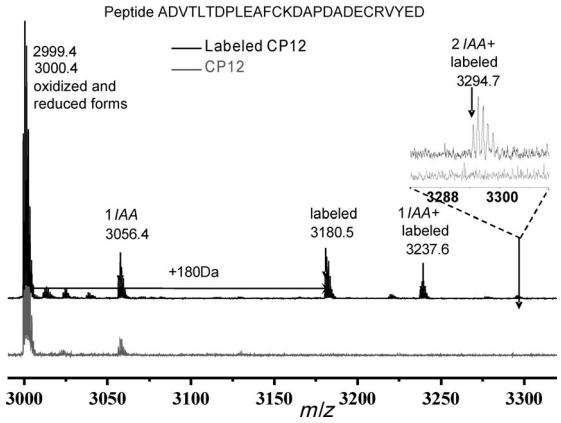



***Figure 2.*** MALDI-ToF mass spectra obtained after tryptic digestion of the reduced and alkylated samples of unlabeled (gray) and labeled CP12 (black). Focus is made in the range of 3000–3320 Da and in the inset in the range of 3282–3310 Da to show the different ions corresponding to the C-terminal peptide of 27 amino acids. *IAA* corresponds to iodoacetamide and means that the cysteine residue is carbamidomethylated.

To check that the experimental conditions of the Mannich-type reaction and the presence of the grafted label did not alter the structural and biochemical characteristics of the CP12, control experiments were performed. The global structure of the labeled protein was checked by circular dichroism studies. The CD spectra of the modified CP12 exhibited similar features as the unmodified one in its oxidized state: it revealed the presence of some α-helical secondary structure elements (Figure 3a), and the use of

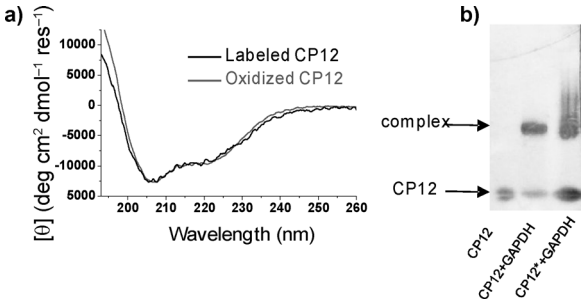



***Figure 3.*** a) CD spectra of the oxidized CP12 and the labeled CP12. b) Western blot analyses of the in vitro reconstitution of the GAPDH/CP12 complex (CP12* means labeled CP12).

trifluoroethanol as secondary-element stabilizer induced the same increase of the α-helical content (see the Supporting Information).[4] Moreover, this result indicated that the disulfide bridges are present in the labeled CP12, as it is known that the reduction of these bridges leads to a CD spectrum typical of a disordered protein.[11] The ability of modified CP12 to form a stable complex with GAPDH was checked by an in vitro reconstitution test (Figure 3b).[12] Altogether these experiments showed that, even using a high concentration of formaldehyde and a long incubation time at room temperature, modified CP12 kept its biochemical and structural characteristics.

When grafted onto proteins, nitroxide spin labels give rise to EPR spectra whose shape reflects the spin-label mobility. The EPR signal of the labeled CP12 was typical of a nitroxide probe having a very high mobility (Figure 4). In this high-

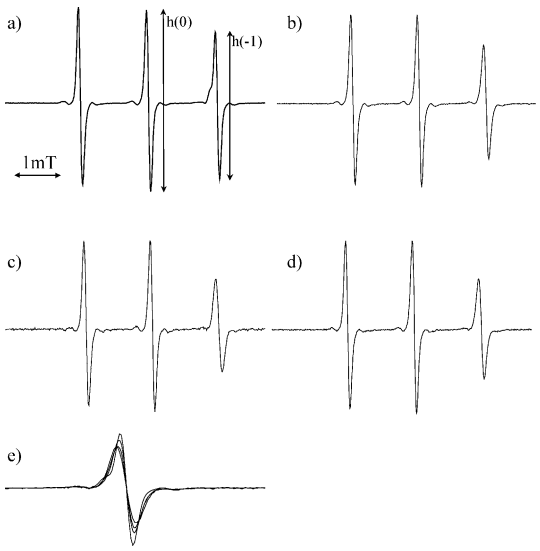



***Figure 4.*** Amplitude-normalized EPR spectra recorded at 296(1) K: a) free label, b) CP12*, c) CP12* in the presence of 30% sucrose, d) CP12* complexed to the partner protein GAPDH, and e) inset of the high-field line (tall to small, (a), (b), (d), (c))

mobility regime, the most sensitive semi-quantitative parameter to describe this spectral change is the ratio of the peak-to-peak amplitude of the high-field line h(−1) over the central line h(0).[1c,13] A significant decrease of this parameter was, however, observed, going from 0.83 for the free nitroxide to 0.67 for the grafted nitroxide (Table 1 and Figure 4). Simulation of the EPR spectra showed an increase of the rotational correlation time $\tau_c$ from 0.060 to 0.125 ns (see Table 1 and the Supporting Information). For such small proteins (8.5 kDa), the contribution of the protein rotation to the EPR spectral line shape is not negligible. To eliminate this contribution, we used 30% sucrose as a viscosity agent that increased the protein rotational correlation time without exerting any influence on the mobility of the nitroxide itself.[14] As expected, a significant decrease of the h(−1)/h(0) ratio was observed, from 0.67 to 0.54, corresponding to an increase of $\tau_c$ from 0.125 to 0.225 ns (Table 1 and Figure 4). The rather high mobility of the grafted probe located at the C-terminal end of the protein confirmed the non-structuration and thus high flexibility of this segment, as proposed by Gardebien et al. on the basis of structural modeling (Figure 1).[9] Mobility of the nitroxide probe and flexibility of its binding site are reflected in the EPR spectral shape, and the type of signal obtained here is not unusual for a standard MTSL (methanethiosulfonate spin label) spin probe either grafted on a small peptide or in a disordered region of a protein.[2b,15,16]

***Table 1:*** Ratio h(−1)/h(0) and correlation times $\tau_c$ for EPR signals displayed in Figure 4.

| | Free label | CP12*[a] | CP12* + sucrose[b] | CP12*GAPDH[c] |
|---|---|---|---|---|
| h(−1)/h(0)[d] | 0.83 | 0.67 | 0.54 | 0.58 |
| $\tau_c$ [ns][e] | 0.060 | 0.125 | 0.225 | 0.180 |

[a] CP12* for labeled CP12. [b] CP12* in the presence of 30% sucrose. [c] CP12* complexed to the partner protein GAPDH. [d] See Figure 4a, error ±0.02. [e] See the Supporting Information, errors ±0.015 ns.

Introduction of the partner protein GAPDH in an equimolar ratio (protein concentration 40 μM) induced a weak but significant modification of the EPR spectral shape; the h(−1)/h(0) ratio decreased from 0.67 in the absence of the partner protein to 0.58 in its presence the partner protein, and $\tau_c$ increased from 0.125 to 0.180 ns (Table 1 and Figure 4). Owing to the high molecular mass of the GAPDH/CP12 complex (greater than 150 kDa), the mobility of the ensemble does not contribute to the EPR spectral shape. Hence the observed decrease of the ratio can be attributed to the cancelation of this contribution rather than a local change in the environment of the probe, in accordance with the similar decrease of mobility observed with sucrose (Figure 4). This result indicates that the C-terminal region where the label is grafted remained highly flexible in the complex and that the tyrosine residue at position 78 is not directly involved in the interaction site between the two partners. This observation is consistent with the proposed interaction region involving the central α helix of CP12 and not the C-terminal end of the protein.[17]

This work exemplifies that a nitroxide probe can be grafted to tyrosine residues by using the Mannich-type electrophilic aromatic substitution reaction. Application of this approach to other proteins is currently under investigation. Beyond the demonstration of feasibility of grafting a nitroxide radical to residues other than cysteine, this approach extends the capabilities of SDSL EPR spectroscopy and opens new perspectives for the study of proteins bearing functional cysteines.

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