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Enlarging the Panoply of Site-Directed Spin Labeling Electron Paramagnetic Resonance (SDSL-EPR): Sensitive and Selective Spin-Labeling of Tyrosine Using an Isoindoline-Based Nitroxide

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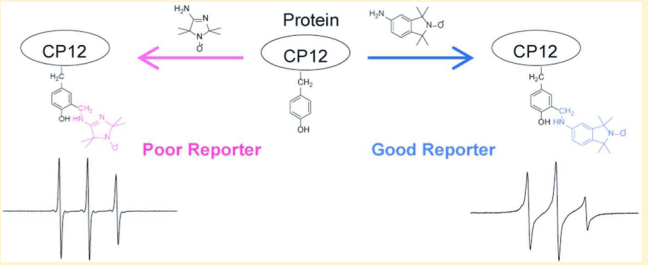
Supporting Information

ABSTRACT: Site-directed spin labeling (SDSL) combined with electron paramagnetic resonance (EPR) spectroscopy has emerged as a powerful approach to study structure and dynamics in proteins. One limitation of this approach is the fact that classical spin labels are functionalized to be grafted on natural or site-directed mutagenesis generated cysteine residues. Despite the widespread success of cysteine-based modification strategies, the technique becomes unsuitable when cysteine residues play a functional or structural role in the protein under study. To overcome this limitation, we propose an isoindoline-based nitroxide to selectively target tyrosine residues using a Mannich type reaction, the feasibility of which has been demonstrated in a previous study. This nitroxide has been synthesized and successfully grafted successively on *p*-cresol, a small tetrapeptide and a model protein: a small chloroplastic protein CP12 having functional cysteines and a single tyrosine. Studying the association of the labeled CP12 with its partner protein, we showed that the isoindoline-based nitroxide is a good reporter to reveal changes in its local environment contrary to the previous study where the label was poorly sensitive to probe structural changes. The successful targeting of tyrosine residues with the isoindoline-based nitroxide thus offers a highly promising approach, complementary to the classical cysteine-SDSL one, which significantly enlarges the field of applications of the technique for probing protein dynamics.



INTRODUCTION

For the past decade, chemists and biologists have begun to share a common interest in developing methods aiming at studying biomolecules in their native settings. Proteins, with their numerous side-chain functionalities, complex secondary and tertiary structures, and diverse biological functions, were early favorites for chemical modification. Classical protein bioconjugation selectively targets the functionalities present in the side-chains of the canonical amino acids. Among those, cysteine and lysine residues are the most commonly modified ones. The thiol group of cysteine can undergo disulfide exchange to form mixed disulfides as well as alkylation with alkyl halides or Michael addition with α,β-unsaturated carbonyl compounds to yield thioethers.[1] Lysine residues can be modified through amide, sulfonamide, urea, and thiourea formation with *N*-hydroxysuccinimide-activated esters, sulfonyl chlorides, isocyanates, and isothiocyanates.[2] In comparison, the remaining 18 proteogenic amino acids have been minimally exploited for residue-selective modification. Site-directed spin labeling (SDSL) has become a powerful biophysical tool to study structure and dynamics of proteins that are not readily amenable to X-ray crystallography.[3−7] This approach requires site-directed mutagenesis to replace the residue of interest with a cysteine and all reactive native cysteine residues with a suitable substitute. The remaining cysteine residue is then labeled with a sulfhydryl selective nitroxide reagent (such as 1-oxy-2,2,5,5-tetramethyl-δ3-pyrroline-3-methyl)-methanethiosulphonate (MTSL), nitroxide **1**, Chart 1) to introduce a paramagnetic reporter of the local environment. The motional dynamics of the spin label, reflected in the electron paramagnetic resonance (EPR) spectral line shape, has been shown to correlate with the general feature of the protein fold.[8]

This approach has been extensively used to monitor conformational changes arising from either protein−substrate, protein−ligand, or protein−protein interactions.[3,9−11] It has been widely applied to study the architectural characteristics of membrane proteins.[6,7] It has been also used to map folding events in intrinsically disordered proteins (IDP),[5,12−14] which are proteins devoid of a stable secondary and tertiary structure under physiological conditions.[15−18]

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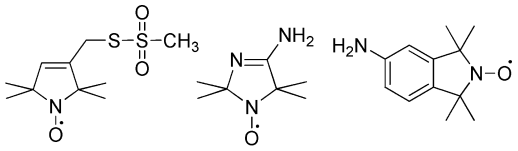
Chart 1. Nitroxide Spin Labels[a]



1 2 3

[a]Nitroxide **1**, (1-oxy-2,2,5,5-tetramethyl-δ3-pyrroline-3-methyl)-methanethiosulphonate (MTSL); **2**, 4-amino-2,2,5,5-tetramethyl-3-imidazoline-yloxyl; **3**, 5-amino-1,1,3,3-tetramethyl-isoindolin-2-yloxyl referred to as "Nox" radical.

Despite the widespread success of cysteine-based labeling strategies, the technique is however unsuitable when cysteine residues play a role in the function (involved for example in active sites) or in the structure (disulfide bridges, binding of metal cofactors) of the protein. To address these limitations, we are currently developing strategies for targeting other residues than cysteines. Among the various residues targeted for spin labeling, we focused our efforts on tyrosine residues because of their low occurrence in disordered proteins for which SDSL-EPR is a technique of choice for structural characterization. Among several reactions recently proposed to selectively target specific residues,[19] we used a three-component Mannich-type reaction developed in the group of M. B. Francis[20] to demonstrate the feasibility of targeting tyrosine on a model protein using a commercial nitroxide, **2**.[21] The model protein was a small chloroplastic protein from the green alga *Chlamydomonas reinhardtii*, called CP12 (∼11 kDa including the His-tag), known to play a role in the formation of a complex with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and phosphoribulokinase (PRK), two key enzymes involved in the Calvin cycle.[15,22,23] CP12 is constituted of about 80 amino acids and has two pairs of cysteine residues, able to form disulfide bridges under oxidizing conditions (C23−C31 and C66−C75). Although disulfide bridges can favor the formation of some structural elements, CP12 is a flexible protein sharing some physicochemical properties with intrinsically disordered proteins (IDPs).[24] CP12 has a unique tyrosine (Y78) located in the C-terminus region (Table 1) that has been modeled as a flexible region[25] and that has been shown to keep a high flexibility upon binding to GAPDH.[26,27]

Even if the grafting with nitroxide **2** has been shown to be specific and efficient for selectively labeling tyrosine residues, only small spectral changes arose between the EPR spectra of the bound and free nitroxide. This result suggested that this label was weakly sensitive in probing protein structure and dynamics modifications. To overcome this limitation, we synthetized the isoindoline-based nitroxide **3**, hereafter referred as "Nox", inspired by the synthesis reported by Griffiths et al.[28] This nitroxide is characterized by a planar fused ring system[29] that is expected to restrict its motion. In addition, isoindoline-based nitroxides show an excellent thermal and chemical stability in a wide variety of chemical environments.[30] Beyond showing that the isoindoline based nitroxide **3** (Nox) is able to selectively target tyrosine on proteins, the main purpose of this work is to demonstrate that Nox is able to reflect protein structure and dynamics.

We proceeded step-by-step to fully explore and characterize the spin labeling reaction of Nox and its ability to give information on its environment. We first reacted the nitroxide with *p*-cresol, a small molecule mimicking tyrosine, and then Nox was reacted with the tetra-peptide V**Y**ED corresponding to the CP12 C-terminus, and full-length CP12, chosen as a model protein. Finally, labeled CP12 has been studied in association with its partner protein GAPDH to evaluate the ability of Nox to be a good reporter for structural changes.

EXPERIMENTAL SECTION

Chemicals. Unless otherwise noted, all chemicals and solvents were of analytical grade and used without further purification.

Synthesis of Nox and Its Diamagnetic Analogue. **3** and **5** were prepared according to published procedures.[28,31] Synthesis and full characterization are given in Supporting Information.

CP12 Purification. Recombinant CP12 of *C. reinhardtii* with its histidine tag was purified to apparent homogeneity as previously described.[32] CP12 protein was dialyzed against 50 mM Tris and 100 mM NaCl (pH 8) and stored at −20 °C.

ESI-MS Spectrometry. Electrospray ionization mass spectrometry (ESI-MS) analyses were performed using a mass spectrometer 3200 QTRAP (Applied Biosystems SCIEX, Concord, ON, Canada) equipped with a pneumatically assisted electrospray ionization source. The samples were dissolved in acetonitrile and then diluted (dilution factor 1/10) in a methanolic solution of ammonium acetate (3 mM). The sample solution was infused in the ionization source at a 10 μL min^{-1} flow rate. Ionization was performed in positive and negative mode. The mass spectrum was obtained using a quadrupole mass analyzer.

Table 1. CP12 Sequence and Peptide Mass Fingerprint Results

CP12 Sequence	
GHHHHHHHHHHSSGHIEGRHM[a]	
$_{1}$SGQPAVDLNK$_{11}$KVQDAVKEAE$_{21}$DACAKGTSAD$_{31}$CAVAWDTVEE	
$_{41}$LSAAVSHKKD$_{51}$AVKADVTLTD $_{61}$PLEAF**C**KDAP	
$_{71}$DADE**C**RV**Y**ED	
Fragments Detected after Proteolytic Digestion[b]	
$_{77}$V**Y**ED*	labeled
HM $_{1}$SGQPAVDLNK	n.l
$_{49}$KD $_{51}$AVKADVTLTD $_{61}$PLEAF**C**K	n.l.
$_{54}$ADVTLTD $_{61}$PLEAF**C**KDAP $_{71}$DADE**C**R	n.l.
$_{26}$GTSAD $_{31}$**C**AVA**W**DTVEE $_{41}$LSAAVSHK	n.l.
$_{50}$D $_{51}$AVKADVTLTD$_{61}$PLEAF**C**KDAPDADE**C**RV**Y**ED*	labeled
$_{12}$VQDAVKEAE $_{21}$DA**C**AKGTSAD $_{31}$**C**AVA**W**DTVEE $_{41}$LSAAVSHK	n.l.
$_{18}$EAE $_{21}$DA**C**AKGTSAD $_{31}$**C**AVA**W**DTVEE $_{41}$LSAAVSHKKDAVK	n.l.

[a]His-tag. [b]n.l. for nonlabeled.

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Labeling Procedures. *(1) Labeling of p-cresol* **4**. *p*-Cresol **4** (0.38 mmol, 40.5 mg) and label **5** (0.08 mmol, 18.0 mg) were dissolved in H_2O (500 μL). Formaldehyde (0.08 mmol, 2.4 mg, 50 μL of a solution 1.5 M in water) was subsequently added to the solution. The mixture was stirred under air, at pH = 7, at RT for 16 h. After removal of the solvent, the solid was dissolved in the suitable buffer for ESI mass analysis and in $CDCl_3$ for NMR analysis. *(2) Spin labeling of VYED peptide.* To a mixture of Nox **3** (6.4 μmol, 1.3 mg) and VYED peptide (6.4 μmol, 3.3 mg) in phosphate buffer/ACN 50:50 is added a formaldehyde solution in phosphate buffer (64 μmol, 1.9 mg, 42 μL of a solution 1.5 M). The heterogeneous reaction mixture is stirred overnight at RT. For the purification of the reaction mixture see Supporting Information. *(3) Spin labeling of CP12 protein.* A three-component Mannich-type reaction was performed as described[21] on a mixture of CP12 (0.02 μmol, 9.0 μL of a solution 2.6 mM in phosphate buffer) in solution in 100 mM phosphate buffer at pH 6.5, formaldehyde (2.0 μmol, 0.06 mg, 4.4 μL of a solution 0.5 M in phosphate buffer) and 5-amino-1,1,3,3-tetramethyl-isoindolin-2-yloxyl **3** (0.6 μmol, 0.12 mg, 2.5 μL of a solution 0.24 M in ACN) in a molar excess of 1:100:30. The mixture was stirred during 16 h at room temperature under air. The excess of unreacted spin label was first removed by gel filtration (PD10 desalting column) and then by several rounds of spin concentration (Amicon Ultra-2 mL, MCWO 3 kDa, prewashed with TRIS buffer 10 mM, pH = 8.0 to remove glycerol) into 2 mL of TRIS buffer 10 mM, pH = 8.0, to a final volume of approximately 200 μL. The concentration of the labeled proteins was evaluated by double integration of the EPR signal recorded under nonsaturating conditions and comparison with that given by a MTSL standard solution. The labeling quantification was obtained by measuring the protein concentration (using the Bio-Rad reagent protein assay)[33] and by calculating the spin concentration from the double integral of EPR spectroscopy signals; an average yield of 40% (±10%) has been obtained on a series of 20 experiments.

MALDI-ToF MS Spectrometry. *1. Determination of Global Mass of Labeled and Unlabeled CP12.* Samples of 20−30 pmol of CP12 (unlabeled) and labeled CP12 were prepared by dilution in 10 μL of 0.1% trifluo-oacetic acid (TFA) in water (v/v) before being spotted onto a MALDI target plate (1 μL) and added saturated solution of matrix α-cyano-4-hydroxycinnamic acid (1 μL) of 70% acetonitrile in water, 0.1% TFA (v/v). The global mass was measured on the MALDI-ToF mass spectrometer Microflex II from Bruker Daltonics (Deutschland) in the range from 2000 to 20000 Da and in a linear and positive mode. External mass calibration was performed on the averaged $[M+H]^+$ from the Protein Calibrant I (Bruker Daltonics).

2. Peptide Mass Fingerprint after Trypsin Digestion. Samples of 60−100 pmol were digested by trypsin at a ratio enzyme to substrate of 1/50 (w/w) (Sigma, St Louis, MO, USA) for 4 h at 37 °C. The digested solutions were then acidified by 1 μL of 12.5% trifluoroacetic acid (TFA) in water (v/v), vacuum-dried, and then dissolved in 0.1% TFA in water (v/v) before being spotted onto a MALDI target plate (1 μL) and added saturated solution of matrix α-cyano-4-hydroxycinnamic acid (1 μL) of 70% acetonitrile in water, 0.1% TFA (v/v). Tryptic peptides were analyzed on the MALDI-ToF mass spectrometer Microflex II from Bruker Daltonics in the range from 600 to 5000 Da. Data acquisition was operated in positive and reflectron mode. External mass calibration was performed on the monoisotopic $[M+H]^+$ from the peptide calibration standard (Bruker Daltonics). A peak list was generated by a peptide mass fingerprint (PMF) method from the FlexAnalysis software and manually checked. The experimentally measured peptide masses were compared with the theoretical tryptic peptides calculated from the sequence of CP12, with variable modifications of the cysteine residues in reduced (S- or -SH) or oxidized (−S-S-, −2 Da) states, of tryptophane and tyrosine by the spin label **3** [Mannich bridge structure increased mass by 217 Da (nitroxide −NO•) or 218 (reduced state of nitroxide −NOH) or benzoxazine structure increased mass by 229 Da (oxidated state of nitroxide −NO•) or 230 (reduced state of nitroxide −NOH)].

Circular Dichroism. CD spectra were recorded on a Jasco 815 CD spectrometer using 2 mm thick quartz cells in 10 mM TRIS (pH 8.0) at 20 °C. CD spectra were measured from 250 to 190 nm, at 20 nm/min and were averaged from five scans. Mean ellipticity values per residue ($[\theta]$) were calculated as $[\theta]_{mrw,\lambda} = MRW \times \theta_\lambda / (10 \times d \times c)$, where MRW is the mean residue weight, θ_λ is the observed ellipticity (in deg) at wavelength λ, d is the pathlength (0.1 cm), and c is the protein concentration expressed in g/mL. Protein concentrations of 0.10 mg/mL were used. CD spectra were recorded for labeled CP12 and unlabeled CP12 as control. Increasing amount of trifluoroethanol (TFE) was used as a secondary structure stabilizer for both labeled and unlabeled CP12.

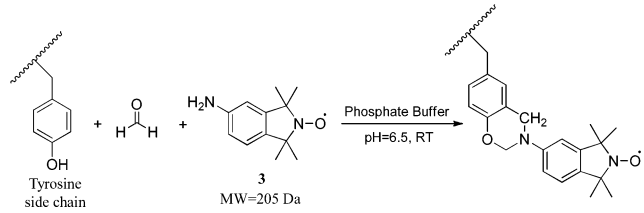
EPR Spectroscopy. EPR spectra were recorded at room temperature on an ESP 300E Bruker spectrometer equipped with an ELEXSYS Super High Sensitivity resonator operating at X-band (9.9 GHz). Samples were injected in a quartz capillary, whose sensible volume was 40 μL. The microwave power was 10 mW, the magnetic field modulation amplitude was optimized to avoid overmodulation of the signal (typically 0.1 mT), and the frequency modulation was 100 kHz.

EPR spectra were simulated using EasySpin software (chili function)[34] in order to determine the rotational correlation time τ_c for each condition.

RESULTS AND DISCUSSION

Small Molecules As Models of Tyrosine Side Chain. The Mannich-type reaction (Scheme 1) enables tyrosine modification via imine formation from formaldehyde and aniline derivatives in aqueous solution. The pH range of [5.5−7.0] is appropriate in order to prevent nonspecific reactions of other amino groups with formaldehyde. In light of the reported weak stability of imine in aqueous media,[35] a model study on *p*-cresol was performed (Figure 1A), before investigating the Mannich reaction on proteins. In order to facilitate the NMR characterization of the reaction products, the labeling reaction on *p*-cresol was performed using the alkoxyamine **5**, a diamagnetic analogue of nitroxyde **3**, easily obtained by trapping the methyl radicals generated by a Fenton reaction in the presence of DMSO (Supporting Information). According to Mannich reaction, mixing *p*-cresol **4**, formaldehyde, and **5** likely proceeds through the addition of the amino group to formaldehyde to form a Schiff base derivative; this

Scheme 1. Bioconjugation Pathway



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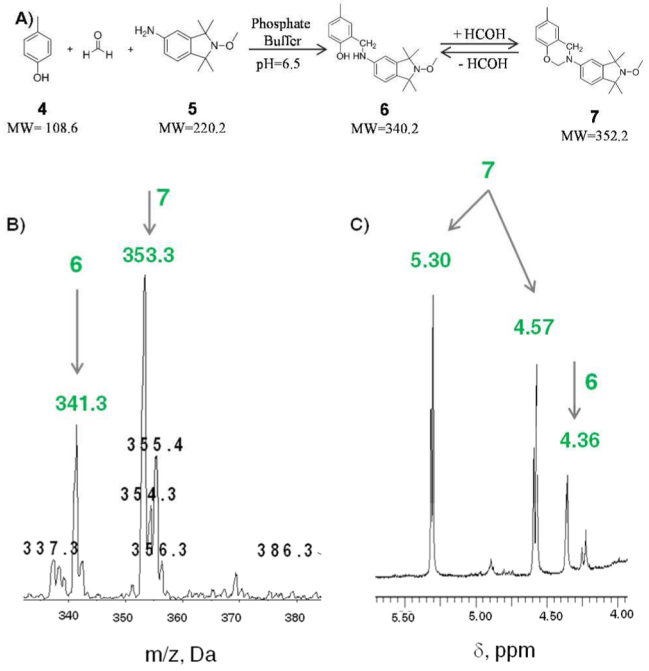

Figure 1. (A) The Mannich type reaction performed with *p*-cresol **4** and alkoxyamine **5** characterized by (B) ESI mass spectrometry (positive ion mode) analysis and (C) ^{1}H NMR (400 MHz, $CDCl_3$).

intermediate then reacts with the *ortho* position of the phenol to yield **6**, the Mannich bridge structure (Figure 1A). In the presence of an excess of formaldehyde and a long time of incubation, **6** gives benzoxazine **7**. In aqueous solution, the benzoxazine ring is labile, and it can easily open releasing a molecule of formaldehyde and yielding the Mannich bridge structure **6**. The ESI MS spectrum of the reaction mixture revealed the formation of two products having m/z = 341.3 Da and m/z = 353.3 Da corresponding to the Mannich bridge and benzoxazine structures, respectively (Figure 1B). NMR spectroscopy was used to confirm the formation of the two products detected by ESI mass spectrometry. ^{1}H NMR analysis (Figure 1C) revealed three peaks ascribed to the methylenic protons typical of **6** and **7**. The peak at δ = 4.36 ppm (2H) is attributed to the benzylic methylene in tyrosine analogue **6** (Figure 1A) and peaks at δ = 4.57 (2H) and δ = 5.30 (2H) ppm to the benzylic methylene and the benzoxazine methylene respectively, corresponding to tyrosine analogue **7** (Figure 1A).

A similar labeling procedure (see Experimental Section) was carried out with Nox and a synthetic tetra-peptide VYED corresponding to the last four amino acids of the model protein CP12. The mass analysis confirmed the labeling of the tetrapeptide (Figure 2 and Figures S1−3) with the formation of both Mannich products, i.e., the Mannich bridge **8** and the benzoxazine **9** structures. Before EPR analysis, the reaction products were separated by reverse-phase chromatography. By comparing the spectrum of free Nox in solution and after labeling reaction with the tetrapeptide, a slight but significant modification of the spectrum was observed, indicating a slower motion of the nitroxide when bound VYED (Figure S4), thus confirming the grafting of Nox.

Site-Directed Spin Labeling on Chloroplast Protein (CP12). The CP12 protein having a single tyrosine and two disulfide bridges was chosen as a model in order to evaluate the potential of Nox to be grafted on tyrosine residues of proteins.

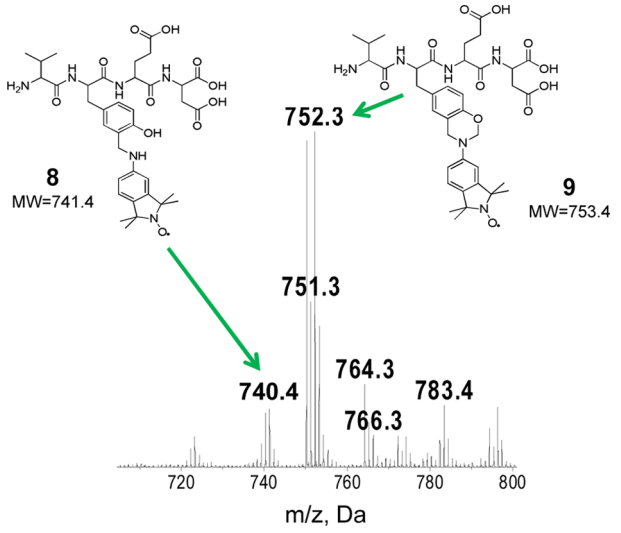

Figure 2. ESI mass spectrometry (negative ion mode) analysis of the three-component Mannich type reaction on VYED peptide with Nox.

After the labeling procedure, mass analysis was performed. Figure 3 shows the superimposition of the MALDI-ToF mass

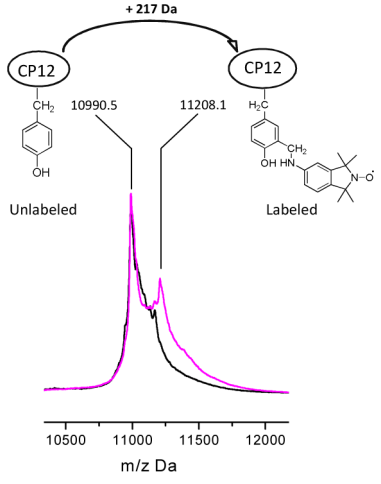

Figure 3. MALDI-ToF analysis: superimposed spectra of unlabeled (10990.5 Da, black) and labeled CP12 (11208.1 Da, magenta).

spectra obtained for unlabeled and Nox labeled CP12. Because of the large mass increment of 217 Da expected from the grafting of Nox, the MALDI-ToF analysis clearly shows that only one molecule of Nox is bound to the protein yielding a Mannich bridge adduct (**6**, Figure 1A). The absence of the benzoxazine form (**7**, Figure 1A) can be accounted for by the low pH required for MALDI experiments; a low pH favors the opening of the benzoxazine ring leading to the Mannich bridge adduct.

The reaction selectivity for tyrosine residues was demonstrated by a proteolytic digestion of a sample of the labeled protein. Labeled and unlabeled (for control) CP12 were digested with trypsin without preliminary reduction−alkylation of the two disulfide bridges (C23−C31 and C66−C75) to avoid any unspecific and supplementary reactions. Analysis of the samples by MALDI-ToF mass indicated the presence of different peptides (100% of recovering, Table 1 and SI), two of which containing the tyrosine residue and bearing the expected mass increment for the grafted spin label (+217 Da) corresponding to the Mannich bridge form. One of these fragments

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has been assigned to the C-terminal VYED and the other one to the C-terminal 31 amino acids. The possibility that Nox could be grafted on cysteine residues was rejected as they are engaged in disulfide bridges in oxidized CP12. It has been shown that after a long reaction time, the Mannich-type reaction can modify tryptophan residues.[20] Modification of the unique tryptophan residue at position 35 in CP12 was not detected under our experimental conditions (Table 1). From these analyses, we concluded that Nox was grafted on the unique tyrosine residue of CP12.

Influence of the Spin Label on Protein Structure. An important point was to assess whether the bioconjugation method and the introduction of the covalently bound nitroxide radical could affect the overall secondary structure of CP12. 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: the two characteristic minima at 222 and 208 nm confirm that unlabeled and labeled proteins have the same α-helical content (Figure 4). The structural

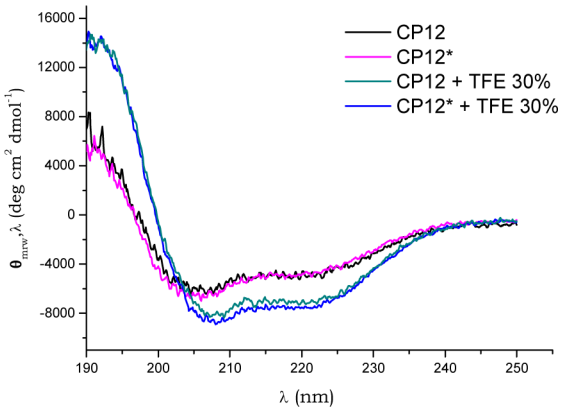

Figure 4. CD spectra of the oxidized CP12 in the absence (black) and in the presence of TFE 30% (green), labeled CP12 in the absence (magenta) and in the presence of TFE 30% (blue). CP12* stands for labeled CP12 with Nox.

properties of the spin labeled protein were also analyzed in the presence of 30% 2,2,2-trifluoroethanol (TFE),[36] used as secondary-element stabilizer.[13] The addition of increasing amounts of TFE triggers the same increase of the α-helical content in both labeled and unlabeled proteins (Figure 4 and data not shown). This result indicated that 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 fully disordered protein.[36] Altogether these experiments showed that, in spite of the use of a high concentration of formaldehyde and a long incubation time at room temperature, the labeled CP12 kept its structural characteristics.

EPR Analysis. We have recently reported the use of the commercial nitroxide **2** as spin label to modify tyrosine in CP12.[21] Although a successful selective binding of **2** to CP12 was observed, an unexpected high mobility of this nitroxide was evidenced by the three sharp lines in the EPR spectrum exhibited by the labeled protein (Figure 5A). Beyond the fact that Tyr 78 is located in a disordered region of CP12, it has been shown that the distance between the protein backbone and the nitroxide moiety also strongly affects the EPR spectral shape, the mobility of the label increasing with its distance from the backbone.[37] Compared to the usual MTSL nitroxide **1** grafted on cysteine, nitroxide **2** is more distant from the backbone, explaining its weak sensitivity to reflect the structural properties of

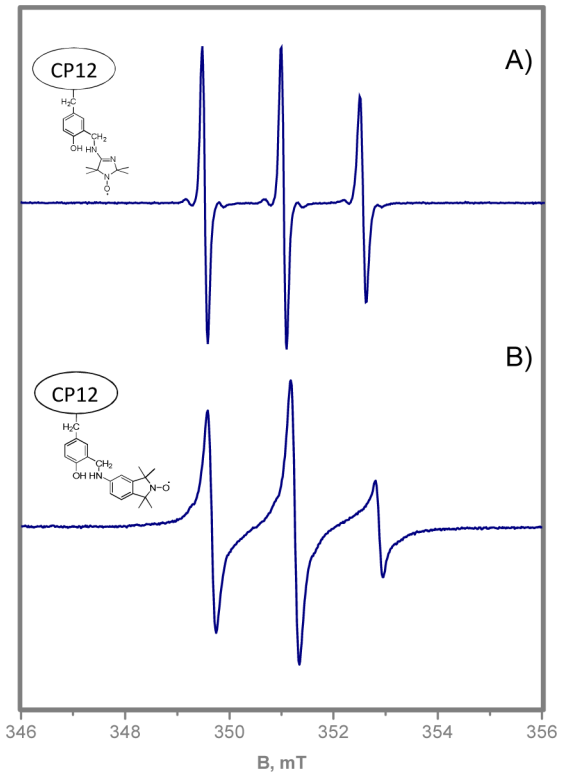

Figure 5. EPR spectra recorded at 298 K: (A) CP12 labeled with nitroxide **2**.[21] (B) CP12 labeled with Nox.

the protein. The main purpose of this study was to synthetize a label more suitable for tyrosine SDSL, in particular, a nitroxide able to balance the poor sensitivity observed with spin label **2**. The Nox radical was chosen according to its particular geometry with respect to its planar fused ring structure. Remarkably, the labeling of the same residue of the model protein (Tyr78 on CP12) leads to an important spectral change compared to the change observed with nitroxide **2** (Figure 5). The spectral shape of Nox bound to CP12 clearly reflects the decreasing mobility of the label. The fact that the mobility of Nox is considerably more reduced than that of nitroxide **2** led us to hypothesize that the size and geometry of the fused rings in Nox induce more hindered rotations compared to nitroxide **2**. Furthermore, a decrease in Nox motions was expected on the basis of the cation-π interaction arising from the noncovalent binding between the negative electrostatic potential of the aromatic ring of Nox and arginine and lysine cations in the vicinity.[38] The difference in the chemical structure and geometry of the two labels thus determines the higher ability of Nox in probing protein dynamics.

Simulation of the EPR spectrum of Nox grafted at position 78 of CP12 was performed to characterize the dynamics of the spin label (Figure 6A and Table 2). The best fit was obtained with two major components: a rapid-motion component with τ_c = 0.65 ns and a slow-motion one with τ_c = 3.3 ns contributing in proportions of 43 and 53%, respectively (the remaining 4% corresponds to the free label). These two components might be attributed to different modes of interaction of the spin label with its local protein environment as already observed.[39] It might also be assigned to the two products arising from the Mannich-type reaction: the benzoxazine and the Mannich-bridge forms.

EPR spectrum of labeled CP12 was also recorded in the presence of 30% sucrose (w/v), a condition where the contribution of protein rotation to the EPR spectral line shape is canceled.[40,41] A significant decrease in the mobility of the label was observed

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Table 2. EPR Spectra Simulation Results[a]

		CP12*		CP12* + sucrose		CP12* + GAPDH	
	free label	fast	slow	fast	slow	fast	slow
τ_c (ns)	0.070 ± 0.002	0.65 ± 0.03	3.3 ± 0.2	1.3 ± 0.1	4.2 ± 0.2	0.93 ± 0.04	5.6 ± 0.4
%	4−5	43	53	32	63	43	53

[a]Rotational correlation times τ_c for EPR signals displayed in Figure 6. CP12* stands for labeled CP12 with Nox. % corresponds to the proportion of the simulated component.

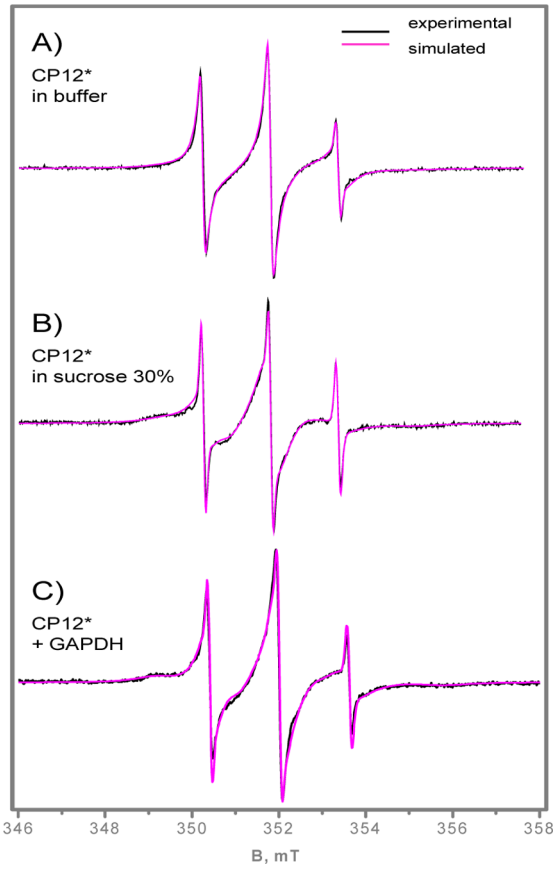

Figure 6. EPR spectra recorded at 298 K (black) superimposed with simulated spectra (magenta): (A) CP12* in Tris buffer pH = 8; (B) CP12* in the presence of 30% of sucrose; (C) CP12* in the presence of equimolar GAPDH. CP12*stands for labeled CP12 with Nox.

(Figure 6B, Table 2) for both components. This decrease of mobility is comparable to the one observed on other disordered proteins labeled with classical spin labels such as MTSL.[13]

Finally, we studied the interaction between the labeled CP12 and its partner protein GAPDH constituting the first step of the supramolecular complex.[42] Addition of an equimolar ratio of GAPDH to labeled CP12 induced a clear reduction in the radical mobility (Figure 6C), comparable to the one observed in the presence of sucrose. This result agrees with the fact that the contribution of the global rotation of the complex in the motion of the label is negligible due its high molecular mass (171 kDa). This experiment clearly demonstrated the ability of Nox grafted on tyrosine to report protein−protein interaction.

CONCLUSION

In this work, we reported a detailed and full characterization of the Mannich bioconjugation reaction using an isoindoline-based nitroxide radical. Thanks to our step-by-step approach of the labeling process going from small molecules to the full-length protein, we demonstrated the selectivity of the spin label for tyrosine residues. Moreover, we showed that Nox is a better reporter of structural changes in proteins compared to nitroxide **2**. Targeting tyrosine with such a label offers an alternative approach, complementary to the classical cysteine SDSL approach. Beyond enlarging the panoply of SDSL-EPR approaches, the results presented here are also of potential interest for SDSL combined with paramagnetic relaxation enhancements (PRE) experiments.[43,44] The perspective of this work will be to systematically investigate the relationship between nitroxide side-chain mobility and protein structural elements such as in secondary and/or tertiary folds.

ASSOCIATED CONTENT

Supporting Information

Synthesis, characterization data, full experimental procedures, and supporting figures are available. 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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ABBREVIATIONS

SDSL, site-directed spin labeling; EPR, electron paramagnetic resonance; CP12, chloroplast protein 12; ESI, electrospray ionization; 3-maleimido-proxyl, 3-maleimido-2,2,5,5-tetramethyl-1-pyrrolidinyloxyl; MALDI, matrix assisted laser desorption/ionization; ToF, time of flight; CD, circular dichroism

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