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Nucleic-acid dependent conformational changes in CRISPR-Cas9 revealed by site-directed spin labeling

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Abstract

In a type II Clustered-Regularly-Interspersed-Short-Palindromic-Repeats (CRISPR) system, RNAs derived from the CRISPR locus complex with the CRISPR-associated (Cas) protein Cas9 to form an RNA-guided nuclease that cleaves double-stranded DNAs at specific sites. In recent years, the CRISPR-Cas9 system has been successfully adapted for genome engineering in a wide range of organisms. Studies have indicated that a series of conformational changes in Cas9, coordinated by the RNA and the target DNA, direct the protein into its active conformation, yet, details on these conformational changes, as well as their roles in the mechanism of function of Cas9, remain to be elucidated. Here, nucleic-acid dependent conformational changes in *Streptococcus pyogenes* Cas9 (*Spy*Cas9) were investigated using the method of site-directed spin labeling (SDSL). Single nitroxide spin labels were attached, one at a time, at one of the two native cysteine residues (Cys80 and Cys574) of *Spy*Cas9, and the spin-labeled proteins were shown to maintain its function. X-band continuous-wave electron paramagnetic resonance spectra of the nitroxide attached at Cys80 revealed conformational changes of *Spy*Cas9 that are consistent with a large scale domain re-arrangement upon binding to its RNA partner. The results demonstrate the use of SDSL to monitor conformational changes in CRISPR-Cas9, which will provide key information for understanding the mechanism of CRISPR function.

Keywords

CRISPR; Cas9; Spin-labeling; EPR; Conformational change

Introduction

Bacteria and archaea employ CRISPR-Cas (Clustered Regularly Interspaced Short Palindromic Repeats – CRISPR associated protein) as an adaptive immune system to combat invading viruses and other mobile genetic elements (1–3). CRISPR-Cas systems, which are now classified into five major types (4), share a number of key common mechanistic features (1,3,5) in that they: (i) provide adaptive immunity through three distinct stages: spacer acquisition, CRISPR-Cas expression, and interference; and (ii) use small RNAs encoded at

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the CRISPR locus (crRNAs) to guide the sequence-specific detection and silencing of foreign nucleic acids. However, different types of CRISPR-Cas systems also exhibit an amazing degree of diversity, particularly in crRNA biogenesis and interference mechanisms (1,3,5). Investigation into CRISPR-Cas systems provides insights on how nature tailors intricate protein-nucleic acid interaction networks to achieve function, and has led to recent drastic technological breakthroughs in genome engineering.

Of particular interest for genome engineering is a type II CRISPR-Cas system. In the naturally occurring system, a single protein, Cas9 (Figure 1A), forms a complex with two small non-coding RNAs, the crRNA and the tracrRNA (trans-activating crRNA) (6,7). This complex acts as an RNA-guided nuclease to cleave double-stranded DNA at sites determined by the base-pairing complementarity between a 5' segment of the crRNA and the target protospacer DNA, as well as a short protospacer-adjacent-motif (PAM) region within the target DNA (6,7) (Figure 1B). The discovery that the tracrRNA-crRNA can be substituted by a single guide RNA (sgRNA) (Figure 1B) has ushered in the era of using programmable Cas9 for creating site-specific double-strand DNA break at desired genome sites in a large number of eukaryotic systems (6,8,9), leading to a revolution in genome engineering (10,11).

A key question in the study of CRISPR-Cas9 lies in understanding the mechanism by which it targets the correct DNA for cleavage (5). A detailed understanding of this process is critical for further developing this powerful genome editing tool. A combination of biochemical and structural studies, the majority of which focus on the *Streptococcus pyogenes* Cas9 (*SpyCas9*), has led to a proposed mechanism in which multiple large scale conformational changes, guided and coordinated by the RNA as well as the DNA, provide the key determinants to discriminate the correct versus the non-correct target (12–19). However, details on these conformational changes, as well as their roles in the mechanism of function of Cas9, remain to be elucidated (5).

Work reported here used the technique of site-directed spin labeling (SDSL) to monitor conformation changes of *SpyCas9* upon interacting with its nucleic acid partners. SDSL monitors site-specifically attached stable radicals (e.g., nitroxide spin labels) using electron paramagnetic resonance (EPR) spectroscopy, and provides structural (e.g., distance constraints) and dynamic (e.g., motions at the labeling site) information on the parent molecule (20,21). SDSL can be applied to study high-molecular-weight systems under physiological conditions, and avoids a number of issues faced by crystallography (e.g., preparation of diffractable crystals, interference from lattice packing) and NMR (e.g., molecule size limitation). SDSL has matured as a method for investigating proteins (20,22,23) and membranes (24,25), and has been applied to study nucleic acids and, to a more limited extent, protein-nucleic acid complexes (21,26–30).

In this work, a nitroxide label, designated as R5p (Figure 1C), was attached to *SpyCas9* variants containing a single cysteine residue. X-band continuous-wave (cw-) EPR spectrum, which reports on rotational motion of the nitroxide in the 0.5 – 20 ns regime, was obtained for the *apo* protein as well as for the protein-nucleic acid complexes. Analyses of the observed spectral variations revealed conformational changes of *SpyCas9* that are consistent

with large scale domain rearrangements reported in the recent crystal structures (12,13,15–17). The studies established the use of SDSL to investigate structure and dynamics of Cas9 as it is activated by the RNA partner and searches for the correct target, which will aid understanding of the mechanism of CRISPR-Cas9 function.

Materials and Methods

Protein mutagenesis and expression

SpyCas9 expression plasmid, pMJ806, was purchased from Addgene, Inc. (plasmid # 39312, <https://www.addgene.org/39312/>). This pET based expression plasmid was generated by the Doudna laboratory (6) by inserting *SpyCas9* gene encoding 1368 residues from *S. pyogenes* SF370 through ligation-independent cloning (LIC) to yield His₆-MBP-TEV-*SpyCas9*. Single cysteine mutants (referred to as 80C and 574C) and a mutant without cysteine (referred to as Cys-less) were prepared by Quick Change site-directed mutagenesis (31). The correctness of the gene was confirmed by DNA sequencing. Protein purification followed previously reported procedures (6). Briefly, the plasmids were transformed into BL21 Rosetta 2 (DE3) (EMD Millipore). Single colony was inoculated into 2xYT medium starter culture of 100 mL with Kanamycin and Chloramphenicol and overnight starter culture was inoculated into four 1L 2xYT medium at 1:100 dilution next day. The culture was grown at 37 °C for 3–4 hours to reach A₆₀₀ OD of 0.8–1.0, after which the temperature was dropped to 18 °C. Induction was done with 0.2 mM IPTG overnight. The cell pellet was re-suspended in lysis buffer (20 mM Tris pH 8.0, 500 mM NaCl, and 10 mM Imidazole) and frozen in –80 °C. Frozen pellets were thawed and supplemented with protease inhibitors (PMSF and Benzamidine 1 mM, pepstatin and leupeptin 1 µg/mL) and lysed with sonication. Clarified lysate was loaded onto HisTrap FF Crude 5 mL column and washed thoroughly with the lysis buffer before eluting with a linear gradient from 10 mM to 500 mM Imidazole. The fractions were checked for purity on 7.5% SDS-PAGE and the right fractions were pooled. The His₆-MBP tag was removed using Tobacco Etch Virus (TEV) protease while simultaneously dialyzing against the ion-exchange buffer A (20 mM HEPES pH 7.5, 150 mM KCl, 1 mM DTT, 10% glycerol). After TEV cleavage, protein was loaded on to HiTrap SP HP 5 mL column and eluted with a linear gradient of 150 mM – 1M KCl. Fractions were checked on the SDS-PAGE for purity, and correct fractions were pooled and concentrated with Amicon Ultra-15 30,000 MWCO concentrator (EMD Millipore) to 5 mL, then loaded onto HiPrep Sephacryl S-300 size exclusion chromatography column equilibrated with 20 mM HEPES pH 7.5, 150 mM KCl and 1 mM tris(2-carboxyethyl)phosphine (TCEP). Eluted protein was concentrated and flash-frozen in liquid nitrogen and stored at –80°C until further use.

Preparation of nucleic acids

The sgRNA has a sequence of 5' -
GUGAUAAGUGGAAUGCCAUGGUUUUAGAGCUAGAA
 AUAGCAAGUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGA
 GUCGGU GCUU-3', with the guide-segment underlined. The sgRNA was synthesized using T7 *in vitro* transcription following reported procedures (6). Transcription products were purified using denaturing polyacrylamide gel electrophoresis (PAGE), and

concentrations of the sgRNA were determined from absorbance at 260 nm using an extinction coefficient of $980,000 \text{ M}^{-1} \text{ cm}^{-1}$.

The target DNA duplex was assembled from two individual strands, with the sequence of the “c-strand” (the target strand complementary to the guide-segment of sgRNA) being 5′-GCTCAATTTTGACAGCCCCACATGGCATTCCACTTATCACTGGCATCCTTCCACTC-3′ (bold: PAM; underlined: protospacer); and the sequence of the “n-strand” (the non-target strand) being: 5′-GAGTGGAAGGATGCCAGTGATAAGTGGAATGCCATGTGGGCTGTCAAAATTGAGC-3′ (bold: PAM). The DNA strands were generated by solid-phase chemical synthesis (Integrated DNA Technologies, Inc., Coralville, Iowa). The individual strands were purified using denaturing PAGE, and DNA concentrations were determined based on absorbance at 260 nm using extinction coefficients of $495,800 \text{ M}^{-1} \text{ cm}^{-1}$ and $554,700 \text{ M}^{-1} \text{ cm}^{-1}$, respectively, for the c- and n-strand. To assemble the target DNA duplex, appropriate amount of the c- and n-strands were mixed in an equimolar ratio, heated at 95°C for 1 minute, cooled to room temperature for 1 minute, then incubated in the annealing buffer (50 mM Tris pH 7.5 and 100 mM NaCl) at either room temperature for a minimum of 1 hour or at 4°C overnight.

DNA cleavage assay

To monitor DNA cleavage by *SpyCas9*, an individual c- or n-strand strand was first radiolabeled following a previously reported protocol (32). A typical 10-μl radiolabeling reaction contained 1 M DNA, 1 μL ^{32}P γ-ATP (MP Biomedicals), 10 units T4 polynucleotide kinase (PNK, New England BioLabs M0201), and 1X PNK Buffer (New England BioLabs). The mixture was incubated at 37°C for 30 minutes, then the T4 PNK was heat deactivated by incubation at 65°C for 20 minutes. Following PNK inactivation, an equimolar amount of the complementary DNA strand was added. The mixture was transferred to the annealing buffer and incubated at room temperature for 1 hour to allow for duplex formation. The sample was then passed through a 10,000 molecular weight cutoff (MWCO) centrifugal filter (Amicon Ultra UFC501024) to remove the majority of free ^{32}P γ-ATP.

Cleavage of the target DNA duplex was monitored under single turnover conditions using a pre-assembled *SpyCas9*/sgRNA complex. To form the *SpyCas9*/sgRNA complex, appropriate amount of sgRNA was pre-folded by heating at 95 °C for 1 minute, cooling to room temperature for 1 minute, then adding to buffer R (20 mM Tris pH 7.5, 150 mM KCl, 5 mM MgCl_2 , 5% (v/v) glycerol, and 5 mM TCEP). Appropriate amount of *SpyCas9*, also in buffer R, was then added to this sgRNA solution, and the mixture was incubated at room temperature for 30 minutes.

The cleavage reactions were carried out in buffer R, and were initiated by adding an appropriate amount of the DNA duplex (prepared as described above in “Preparation of nucleic acids” and doped with appropriate amount of the ^{32}P -labeled duplex) to the pre-assembled *SpyCas9*/sgRNA complex. The reaction mixtures were incubated at 37°C for 30 minutes, then stopped with the addition of urea and EDTA to final concentrations of 6 M and 2 mM, respectively. The products were resolved with 10% denaturing PAGE. Gels were

dried and quantified by autoradiography using a Bio-Rad Personal Molecular Imager and QuantityOne software.

Spin labeling of SpyCas9 proteins

A typical labeling reaction (~ 1 mL) contained 20 mM Tris-HCl (pH 7.5), 200 mM KCl, 5% (v/v) glycerol, 5 mM MgCl₂, 0.5 mM TCEP, approximately 10 μM protein, and 10 molar excess of the R5p precursor (3-iodomethyl-1-oxyl-2,2,5,5-tetramethylpyrroline) prepared as previously described (33,34). The reaction mixture was incubated with consistent and smooth rocking at 4°C in dark overnight. After incubation, un-reacted precursor was removed by dialysis against a buffer containing 20 mM Tris-HCl (pH 7.5), 200 mM KCl, 5 mM MgCl₂, and 5% (v/v) glycerol. After dialysis, the protein was concentrated to the desired volume using an Amicon Ultra-15 30,000 MWCO concentrator. The protein concentration was determined using a bicinchoninic acid assay (BCA assay) (35). In controls, non-specific labeling of R5p was assessed using a Cas9 protein with both cysteines mutated to serines (“Cys-less”), and was shown to be < 10%.

Continuous-wave EPR spectroscopy

Spectra were obtained on samples in buffer R and containing approximately 20–80 M of R5p-labeled *SpyCas9*. Binary complexes of *SpyCas9* and sgRNA were assembled with a protein/RNA ratio of 1:2; and ternary complexes of *SpyCas9*, sgRNA, and DNA were assembled with a protein/RNA/DNA ratio of 1:2:2. Note that while *SpyCas9* is known to bind sgRNA and target DNA duplex in a 1:1:1 ratio (6), excess nucleic acids were used in these measurements to ensure complete binding of the spin-labeled proteins. The complexes were assembled in buffer R following procedures described above in the “DNA cleavage assay”.

EPR samples of *apo SpyCas9*, binary and ternary complexes (approximately 5 μL each) were loaded into a borosilicate glass capillary (0.64mm ID × 0.84mm OD, Fiber Optic Center, Inc.) sealed at one end. X-band EPR spectra were acquired at room temperature on a Bruker EMX spectrometer using an ER4119HS cavity. The incident microwave power was 2 mW, and the field modulation was 2–3 G at a frequency of 100 kHz. Each spectrum was acquired with 512 points, corresponding to a spectral range of 100 G. Each reported spectrum was averaged from a total of 64 scans, with the total acquisition time being approximately 48 minutes. The averaged spectrum was corrected for background and baseline, and normalized to a fixed number of total spins following reported procedures (36). Signals from a small number of unreacted or detached spin labels (< 5%), if present, were subtracted using a reference spectrum measured with the R5p precursor under the same conditions.

Results and Discussion

Spin labeling of SpyCas9

This study utilized the cysteines present natively in *SpyCas9* at positions 80 and 574 to monitor nucleic-acid mediated protein conformational changes. *SpyCas9* with a single cysteine was created by mutating one of two natively occurring cysteine residues to serine.

The resulting single cysteine mutants, designated as 80C (i.e., Cys754 mutated to serine) and 574C (i.e., Cys80 mutated to serine) were expressed and purified to homogeneity similar to native *SpyCas9* (Figure 2A). The mutant proteins were then reacted with a thio-reactive precursor, 3-iodomethyl-1-oxyl-2,2,5,5-tetramethylpyrroline, generating a protein with an attached nitroxide side-chain (R5p, Figure 1C). Accordingly, the labeled proteins were designated as 80R5p (with the R5p attached at residue 80) and 574R5p (with R5p attached to residue 574). Note that instead of the more commonly used disulfide linkage (37), in this work the nitroxide pyrroline ring was connected to the protein side-chain with a thio-ether bond, which is stable in the presence of the reducing agent (i.e., TCEP) used *in vitro* to maintain activities of most of the nucleic-acid binding proteins including *SpyCas9*.

In vitro DNA cleavage assays were performed in order to assess perturbation of *SpyCas9* folding and activities due to the presence of R5p (Figure 2B–E). Cleavage activities were monitored under single turnover conditions using a target DNA duplex with a 20-base-pair protospacer embedded at the center, and an 82-nucleotide (-nt) sgRNA containing a 20-nt single-stranded segment fully complementary to the protospacer (see Materials and Methods). For the n-strand (Figure 1B), the wild-type *SpyCas9*/sgRNA complex cleaved the 55-nt precursor DNA at multiple sites, resulting in two fast-migrating bands (estimated to be 29–33 nt) (Figure 2B), consistent with previous reports (6). Importantly, the single-cysteine variant (Figure 2B) and the R5p labeled proteins (Figures 2B & 2C) gave the same cleavage pattern as that of the wild-type, and the degree of cleavage was nearly identical. Similar results were observed for the c-strand, where the 55-nucleotide precursor DNA was cleaved at a single site yielding an approximately 22-nt ³²P-labeled product (Figures 2D and 2E). Overall, the data indicate that at residues 80 and 574, the attachment of the R5p label did not affected folding and activities of *SpyCas9*. This is consistent with the fact that these two sites lay at the surface of the nucleic-acid/protein complex (12,15,17,19) and are non-essential for Cas9 function (12).

The R5p label at residue 80 revealed RNA-dependent conformational changes of *SpyCas9*

Upon confirming the activities of the R5p labeled proteins, cw-EPR spectra were obtained to assess *SpyCas9* conformation. With 80R5p, the folded *apo* protein gave a broad spectrum (Figure 3A): the center line showed an observable splitting; and the low-field and high-field manifolds displayed clear peaks, with the measured effective hyperfine splitting (2A_{eff}) being 70.5 G. These features arise from incomplete averaging of the anisotropic nitroxide magnetic tensors, and indicate a limited mobility of the R5p label. The 80R5p lineshape is typical of that observed at a “tertiary contact” or “buried” site of a folded protein (38). Indeed, in the recently reported crystal structure of the *apo* *SpyCas9* (13), Cys80, which is part of the long Arginine rich Bridge-Helix (BH, Figure 1A), is in close contact with the Helical-III domain and only partially exposed to solvent (Figure 3A). This is completely consistent with the low-mobility feature of the R5p reported in the cw-EPR spectrum.

Importantly, upon binding of the sgRNA, a clear change was observed in the 80R5p spectrum (Figure 3B). The center-line became narrower without observable splitting, and the bump at the low-field manifold largely disappeared. The degree of spectral changes was found to depend on the amount of sgRNA added (data not shown). These spectral changes

report a clear increase of R5p mobility, suggesting that sgRNA binding results in protein conformational changes that relieve the crowded environment at residue 80. Examining the crystal structure of the sgRNA bound *Spy*Cas9 showed that the Helical-III domain undergoes a movement of $> 100 \text{ \AA}$ away from the Bridge-Helix (17). Consequently, residue 80 becomes completely exposed to solvent (Figure 3B), which nicely accounts for the observed increased mobility of R5p upon sgRNA binding. We also note that with excess sgRNA added, a small bump at the low-field region remains visible (Figure 3B, marked by the dashed line), which may indicate a small fraction of inactive protein.

Furthermore, a spectrum was obtained for the ternary complex of 80R5p bound with sgRNA and the target DNA duplex (Figure 3C). The resulting spectrum was very similar to that measured from the RNA-bound state, indicating no further change at the local environment at residue 80 when the Cas9/RNA complex engages the target DNA. This again is consistent with the crystal structure of the ternary *Spy*Cas9/RNA/DNA complex (12,15), which shows residue 80 is exposed to solvent and there is little difference at the region surrounding that site upon binding to the target DNA.

The R5p label at residue 574 showed limited sensitivity to *Spy*Cas9 conformational changes

In the *apo* protein, the 574R5p spectrum is clearly distinct from that of the 80R5p. The 574R5p spectrum showed a narrow center line, with the low-field manifold displaying a closely-spaced splitting (Figure 4A). These features are prototypic of the “helical surface” sites (38), at which the nitroxide undergoes restricted anisotropic rotations due to attachment to the protein backbone, yet the pyrroline ring has little direct contact with the protein. In the *apo* *Spy*Cas9 crystal structure (13), the segment encompassing 574 is located at the tip of the Helical-III domain and is expected to be exposed to solvent, which is consistent with the high-mobility characteristics of 574R5p revealed by the EPR spectrum. However, note that no density was reported in the crystal structure for the segment spanning residues 568 to 586, yet the 574R5p spectrum is clearly distinct from those at the very flexible “loop” sites (38), which are expected to show three un-even sharp lines with no splitting at the low-field manifold. Therefore, the EPR spectrum suggests that in solution the 568–586 segment may not be completely disordered, but instead folds into certain secondary structures.

In contrast to 80R5p, no spectral changes were observed with 574R5p when *Spy*Cas9 was complexed with either sgRNA or bound to the target DNA (Figures 4B and 4C). This indicates nucleic-acid induced conformational changes present little alteration to the local environment at residue 574. This is largely consistent with the crystal structures. In both structures of the binary *Spy*Cas9/RNA complex (17) and ternary *Spy*Cas9/RNA/DNA complexes (12,15), residue 574 is located at a structured segment at the tip of the Helical-III domain, and is completely exposed to solvent (Figures 4B and 4C). As such, although nucleic acid binding causes a large scale motion of the Helical-III domain away from the Bridge-Helix (17), little change occurs at the immediate surrounding of the R5p attached at 574, resulting in the lack of variation in the EPR spectra. Overall, the cw-EPR spectra of 574R5p by themselves have little sensitivity to the *Spy*Cas9 conformational changes,

although the label could be used in other detection schemes, for example, as part of the nitroxide pairs for measuring distances within the Cas9 complexes.

SDSL serves as an informative tool for probing Cas9 structure, dynamics, and function

Data presented above demonstrate that cw-EPR spectra measured from singly-attached R5p nitroxides can detect conformational changes of *Spy*Cas9 as it interacts with its RNA partner and DNA target. Current studies of Cas9 indicate that conformational changes directed by RNA activation and target DNA interaction(s) are the key determinants of the mechanism by which Cas9 discriminates between correct and incorrect targets (18). As such, it is essential to identify conformational states of the Cas9 complex, as well as the kinetics and pathways of transitions between these states. A host of biophysical methods, including X-ray crystallography (12,13,15–17,19), electron microscopy (13,19), fluorescence spectroscopy (18) and imaging (14), atomic force microscopy (39), and single-molecule magnetic tweezers (40), have been applied to study the Cas9 system. The use of spin-labeling provides a complementary approach to examine structure and dynamics of the Cas9, as demonstrated by results presented here.

Work reported here is only a first step in the exploration of the large number of available spin labeling techniques to study the CRISPR-Cas system. For example, in addition to the native cysteine sites, nitroxide labels can be placed at other protein sites, as well as at sites along the target DNA and/or the partner RNA (26). In particular, nitroxide scanning, in which cw-EPR lineshapes of nitroxides scanned along a segment of the protein are collectively analyzed (37), can be used to gain information regarding the topological fold of Cas9 complexes in solution. This may be extremely fruitful in revealing conformational states of Cas9. Furthermore, while only the use of single label was described in this work, nitroxide pairs can be placed into the Cas9 system to yield distances between specific residues in the complex, which provide direct structural constraints. As nitroxides are small and more intimately coupled to the parent molecule, inter-nitroxide distances can be measured more precisely and can be explicitly correlated to the native structure. This is advantageous in revealing details on Cas9 conformations, as well as transitions between different states during Cas9 function.

In summary, data presented here demonstrate the feasibility of using SDSL to monitor Cas9 conformational changes. Combined with other biophysical methods, the information gained from SDSL will greatly accelerate investigation of Cas9 structure, dynamics, and function.

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Figure 1.

CRISPR-Cas9 system for spin-labeling studies. (A) Domain organization of *SpyCas9*. The domains are represented as the following: RuvC and HNH are the endonuclease (NUC) domains that cleave non-target (n) and target (c) strands of the DNA, respectively; BH is the bridge helix that is arginine rich; HD (helical domain) I, II, III are alpha-helical rich recognition (REC) domains; and PI is the PAM Interacting domain that is essential in discriminating self from the non-self DNA. Asterisks (*) indicate the two native cysteine residues for attaching the spin label. (B) A schematic of the Cas9 ternary complex. (C) Chemical structure of the R5p label.

Figure 2.

Biochemical characterizations of *SpyCas9* protein function. Panel (A) shows an example of SDS PAGE of the purified proteins, panels (B) – (E) show results from DNA cleavage measurements with the ^{32}P label at either the n-strand [panels (B) & (C)] or the c-strand [panels (D) & (E)]. As shown, the DNA cleavage measurements were carried out at a DNA/sgRNA/*SpyCas9* ratio of 1/3.5/5.0, with the DNA duplex concentration set at 100 nM. Excesses of Cas9 protein over sgRNA were used in order to ensure complete binding of the sgRNA. Neither free Cas9 nor sgRNA is capable of binding or cleaving the target DNA (6), the excess of these species did not interfere with the results reported.

Figure 3.

X-band cw-EPR spectra of R5p labeled at residue 80 of *SpyCas9*. In each panel, shown on the left are the EPR spectra, with the spectral width being 100 G and dash lines marking the respective low- and high-field features for comparison. Asterisks (*) mark an ultra-sharp spectral feature that most likely arises from incomplete subtraction of the unreacted or detached spin labels. Shown on the right are the corresponding crystal structures, with the protein domains color-coded following the scheme used in Figure 1A, and when present, the RNA in pink and the DNA in Cyan. The Cys80 residue is indicated by the red arrow and shown in the CPK mode. (A) The *apo* state. The corresponding structure (PDB entry 4CMP (13)) shows that Cys80 is largely buried by the Helical-III domain. (B) The sgRNA-bound state. The corresponding structure (PDB entry 4ZT9 (17)) shows Cys80 is completely exposed to solvent. (C) The sgRNA/DNA bound state. The corresponding structure (PDB

entry 4UN3 (15)) shows that Cys80 is completely exposed to solvent, and the local environment around Cys80 is identical to that of the sgRNA bound state.

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Figure 4.

X-band cw-EPR spectra of R5p labeled residue C574 of *SpyCas9*. In each panel the spectra are shown on the left. The corresponding crystal structures are shown on the right, with the same color coding scheme as that used in Figure 3. The Cys574 residue (when present in the structure) is indicated by the red arrow and shown in the CPK mode. (A) The *apo* state. The red circle marks the expected location of residue 574, the density of which is not observed in the corresponding crystal structure (PDB entry 4CMP (13)). (B) The sgRNA bound state. The corresponding structure is from PDB entry 4ZT9 (17). (C) The sgRNA/DNA bound state. The corresponding structure is from PDB entry 4UN3 (15).