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Characterization of Interactions Between Proteins Using Site-Directed Spin Labeling and Electron Paramagnetic Resonance Spectroscopy

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Abstract

Site-directed spin-labeling and the analysis of proteins by electron paramagnetic resonance spectroscopy provides a powerful tool for identifying sites of contact within protein complexes at the resolution of aminoacyl side chains. Here we describe the method as we have used it to study interactions of proteins involved in export via the Sec secretory system in *Escherichia coli*. The method is amendable to the study of most protein interactions.

Keywords

Site-directed spin labeling; electron paramagnetic resonance spectroscopy; EPR; spin-labeled protein

1. Introduction

1.1. The System Under Study

The secretion of proteins from their site of synthesis through a biological membrane involves crucial interactions among numerous proteins. Early studies of the Sec, or general secretory, system in *Escherichia coli* identified all of the proteinaceous components of that system. Over the last three decades sufficient knowledge concerning the interactions among components has accumulated so that the pathway taken by a precursor polypeptide destined for export can be described (for a review see Ref. 1). The passage through the membrane is provided by the translocon comprising a SecYEG core and the accessory proteins SecD, SecE, and YajC. The Sec system cannot translocate stably folded proteins. Therefore, soluble chaperones must capture precursors before they fold. SecB is such a chaperone that binds promiscuously to many species of unfolded polypeptides. With a polypeptide ligand bound, SecB interacts specifically with SecA, which itself has affinity for SecY. In this way a precursor to be secreted is delivered to the translocation channel. SecA, an ATPase, is the motor of the translocon. It undergoes cycles of binding and hydrolysis of ATP thereby providing energy that is transduced into movement of the polypeptide through the channel.

Our earlier work focused on the interactions among the components of the system. We identified and characterized complexes between SecB and unfolded polypeptides, as well as between SecB and SecA. This work utilized several techniques including size-exclusion

column chromatography (2), sedimentation velocity analytical centrifugation (3), and Fourier transform ion cyclotron resonance mass spectrometry (4). Static light scatter in line with size-exclusion chromatography allowed the determination of the stoichiometry of components within a complex (5). Isothermal titration calorimetry was used to determine the thermodynamic parameters (K_d and ΔH) governing the interactions (6, 7).

Having established a basic understanding of the complexes, we have shifted our focus to the determination of contact sites between binding partners at the resolution of aminoacyl residues (8–10). In this chapter we describe the technique we have used, site-directed spin-labeling and electron paramagnetic resonance spectroscopy (EPR). We have introduced spin probes into both SecB and SecA for these studies. The method is described in general terms so that it can be applied to any protein of interest.

1.2. Principles of EPR

We begin with a brief discussion of the theory of EPR spectroscopy (for a review *see* Ref. 11). Molecules absorb energy when incident electromagnetic radiation has an energy equal to the difference in energy between two states. The energy absorbed causes a transition from the lower energy state to the higher state. In EPR spectroscopy the energy differences studied are due to the interaction of unpaired electrons with an applied magnetic field. When the magnetic moment of the unpaired electron aligns parallel with the magnetic field it is in the lowest energy state ($-1/2$ spin state) and when it is antiparallel to the applied field it is in the highest energy state ($+1/2$ spin state). The difference in the energy states is proportional to the strength of the applied field; thus, both spin states have the same energy in the absence of an applied magnetic field and their energy levels diverge linearly as the field is increased (Fig. 11.1). Resonant absorption occurs if $E = h\nu$, where E is the energy difference, h is Planck's constant, and ν is the frequency of the incident radiation.

An unpaired electron is sensitive to its local environment and experiences the local magnetic fields produced by the magnetic moments of nuclei in close proximity. The field from a nucleus (Fig. 11.1, $B_{\text{nitroxide}}$) will either add to or subtract from the applied magnetic field (Fig. 11.1, B_0) depending on the orientation of the nuclear dipole as illustrated by the arrows in Fig. 11.1. This interaction between the unpaired electron and a nitrogen nucleus (in the case of a nitroxide spin label) gives rise to splitting such that instead of a single line the absorption spectrum contains three lines.

During an experiment the applied magnetic field is slowly swept through a range of field values that include the field for resonance of the electron. For studies employing nitroxide probes we use an X-band microwave bridge with a frequency of 9.75 GHz and sweep a magnetic field of 100 gauss centered at a field for resonance of 3356 gauss. The resonance signal is amplified by encoding it in such a way that it becomes distinguishable from background noise. Modulation coils placed on both sides of the magnet generate a small magnetic field that imposes oscillation, commonly two or three gauss (chosen by the operator), on the applied field. The signal, resulting from absorption of energy at resonance, oscillates at the same frequency as the modulating field. This oscillating signal is selectively amplified whereas all other changes in microwave intensity are ignored as background noise. The modulation of the field is constant through the sweep and the instrument records a

signal that is proportional to the change in amplitude of the oscillating intensity during a single modulation cycle. Thus, the signal is recorded as a first-derivative of the absorption (Fig. 11.2).

The energy of microwave radiation is too weak to break chemical bonds; therefore, the technique is nondestructive to biological molecules and one can study interactions among proteins in their native state. In addition, there is no size limitation so one can examine large protein complexes. However, most proteins do not contain unpaired electrons; thus, a paramagnetic probe, the spin label, must be introduced.

1.3. The Approach

The approach involves construction of a collection of variants of the protein of interest each containing a single cysteine side chain so that a spin label can be introduced at a specific site using sulfhydryl chemistry. The reagent used in this study (1-oxy-2,2,5,5 tetramethyl pyrroline-3-methyl)-methanethiosulfonate and the nitroxide side chain it generates are shown in Fig. 11.3. This is the reagent of choice for several reasons: (1) it derivatizes the protein through a disulfide bond making it highly specific for cysteine thus eliminating the possibility of labeling other aminoacyl residues by less specific reactions, (2) the chemistry is rapid and for the proteins we have labeled to date (>100 variants of SecB and SecA) the available cysteines are quantitatively labeled, (3) the nitroxide side chain generated has been extensively studied by W. L. Hubbell and his colleagues (12–15) and a large body of knowledge is available that aids in interpretation of the data.

The approach is amenable to any protein, including membrane proteins (15), provided that the protein can be engineered so that the only cysteine accessible for reaction with the nitroxide reagent is that introduced at the site of interest. If the protein contains native cysteine residues, ideally they should be substituted by another amino acid. However, in one protein we study, SecB, two of the four native cysteines, C76 and C113, could not be replaced without disrupting the structure. Fortunately, neither of these cysteines showed reactivity with the reagent and so our base protein in those studies had only two of the four native cysteines removed. All four of the native cysteines in SecA were successfully changed to serine without perturbing the protein.

In our studies we have mapped the surface of contact between proteins by using changes in line shape of a spin-labeled protein that occur when the protein forms a complex with a binding partner. The shape of an EPR spectrum contains information about the mobility of the nitroxide on a nanosecond time scale (11, 16). The motion of the nitroxide has its origin in rotation around bonds within the nitroxide side chain as well as in local backbone fluctuations. As the nitroxide goes from highly mobile to constrained or immobile, two features of the spectrum, readily seen by visual inspection, change. First, the central line width broadens (Fig. 11.4, H_{pp}) which is also seen as a decrease in intensity of the central line since all spectra are normalized. Second, the overall spectral breadth increases; that is, the total intensity is spread over a wider range of the magnetic field. For spectra reflecting slow motional states the hyperfine extrema are well-resolved and the separation of the extrema can be used to measure the spectral breadth (Fig. 11.4, A'_{zz}).

The solid traces in Fig. 11.5 show nitroxides at several different positions in the chaperone SecB to illustrate the diversity of line shapes that will be observed depending on the location of the substituted amino acid within the protein structure. The line shape of the spectrum displayed in Fig. 11.5A is characteristic of a highly constrained residue that makes tertiary contacts with other structural elements within the protein, Fig. 11.5B illustrates a position exposed on the surface of a helix, and Fig. 11.5C shows a very mobile residue in a region of little tertiary structure. If within a complex the nitroxide probe makes contact with residues on a binding partner, either another protein or a small ligand, the mobility of the nitroxide will be constrained. When SecB is in complex with an unfolded polypeptide even the most immobile of these residues (L126, SecB) shows changes that are indicative of further constraint (Fig. 11.5A, compare the solid trace with the dotted trace). The central line amplitude drops and intensity moves out which is most clearly seen at the low field side (indicated by arrow). Constraints are also seen on residues Q144 and T149 when complexes with unfolded polypeptide or with SecA are formed.

In addition to constraints of mobility of a nitroxide, increased mobility will be observed if within a complex changes in conformation occur that result in breaking interactions of a side chain with neighboring structural elements. Figure 11.6 shows two positions on the surface of helices that become more mobile when the spin-labeled protein, SecA, binds to the membrane-bound translocon, SecYEG. Mobilization results in movement of intensity from the extrema toward the center line. The amplitude of the center line is also increased.

1.4. Considerations

Identification of the interface of contact between two proteins within a complex requires that enough residues be surveyed to cover a considerable surface of the protein under investigation. Interpretation depends on the emergence of patterns. Constraint of a single residue is likely to indicate a contact, but it is possible that contact at a distance from the nitroxide results in a conformational change that in turn constrains a residue that lies outside of the binding surface. In our study of SecB in complex with unfolded polypeptide ligands we examined 49% of the entire surface.

Observation of constraints allows one to define sites of contact and observation of mobilization such as we observed in SecA in complex with SecYEG and SecB (10) are likely to reflect conformational changes that release side chains from contacts that exist in the protein in the absence of a binding partner. It is important to find sites which show no change to define regions on the surface that are not involved in binding. In this respect when no change is observed in line shape it is crucial to be certain that substitution by the nitroxide has not inactivated the protein of interest. Each spin-labeled variant should be assayed for formation of a complex. In our studies all complexes of interest between soluble proteins could be demonstrated by size-exclusion column chromatography. SecA binds the membrane-bound translocon SecYEG and since column chromatography cannot be used to assess binding to vesicles we used a biological assay, i.e., the stimulation of SecA ATPase activity by binding to vesicles to make certain all SecA species were active (10). If simple assays of activity or complex formation are not available one should minimally check that the protein remains folded. This can usually be done by comparing the position of elution

during size-exclusion chromatography of the protein before and after substitution with the nitroxide.

In order for line shapes to be interpreted in terms of local backbone fluctuations and internal motion of the nitroxide side chains, the molecular tumbling of the protein must be slow relative to the timescale of EPR spectroscopy. If the radius of hydration of the protein is known, the timescale of tumbling (τ_c) can be calculated as follows:

$$\tau_c = \frac{1}{6 D_r}; D_r = \frac{kT}{8\pi\eta a^3},$$

where D_r is the rotational diffusion coefficient, k is Boltzmann's constant, T is temperature, η is the viscosity of the solvent, and a is the radius of hydration of the protein. Proteins of molar mass 40 kDa or greater will tumble sufficiently slowly. Proteins of lower mass can be studied by addition of sucrose to the solution to 30%. This will increase the viscosity 2.75-fold relative to water and thereby slow tumbling accordingly.

2. Materials

2.1. Mutagenesis to Introduce a Single Accessible Cysteine

In this section we provide a detailed description of the mutagenesis procedure that we have used to create our single-cysteine variants. We do not describe purification of the proteins since the procedure of choice will depend on the species of protein under study.

1. Double-stranded DNA (dsDNA) template: a plasmid containing the gene for the protein of interest isolated from a *dam*⁺ *Escherichia coli* strain (*dam* encodes DNA adenine methylase, which methylates DNA duplexes on adenine in GATC sequences) suspended in either 10 mM Tris (HCl) pH 8.0 to 8.5 or H₂O at a concentration of 10 ng/μL.
2. *PfuUltra II* Fusion HS DNA polymerase and 10 X PCR reaction buffer (Stratagene).
3. *DpnI* endonuclease 10 units/μL (Fermentas).
4. dNTP mixture containing 10 mM of each of the deoxynucleotides, dATP, dCTP, dGTP, and dTTP (see Note 1)
5. Thermal cycler for PCR.
6. Competent cells for transformation.
7. Growth media and antibiotics appropriate for the bacterial strain used.
8. Thin-walled PCR tubes.
9. Sterile pipette tips and sterile 1.5 mL microcentrifuge tubes.

¹If the solution of deoxynucleotides is prepared by the investigator and not obtained from a kit, the pH must be adjusted to between pH 7.5 and pH 8.0 to neutralize the acid of the phosphate groups.

10. Sterile 15 mL Falcon tubes.
11. Desk-top microcentrifuge.
12. Plasmid preparation kit (Qiagen).
13. DNA sequencing primer to sequence region of mutagenesis.
14. Reducing agent: Dithiothreitol (DTT) or tris-(2-carboxyethyl) phosphine hydrochloride (TCEP, Molecular Probes).

2.2. Labeling of Cysteine Variants with Nitroxide Reagent

1. The nitroxide spin label reagent: (1-oxy-2,2,5,5 tetram-ethyl pyrroline-3-methyl)-methanethiosulfonate (Toronto Research Chemicals, Inc.).
2. Acetonitrile, HPLC grade.
3. Lyophilizer or centrifugal vacuum evaporator (Labconco).
4. Nap 10 column (Amersham Biosciences).
5. Buffer A: 10 mM Hepes (HAc), 300 mM KOAc, pH 7.0.
6. Centrifugal concentrator: Nanosep 30 (Pall Life Science) or Centricon 30 (Millipore).
7. Buffer B: 10 mM Hepes (HAc), 300 mM KOAc, 5 mM Mg(Ac)₂, pH 7.0.

2.3. EPR Measurements

1. Spectrometer: Bruker EMX X-band spectrometer with a high sensitivity resonator. To work at temperatures other than room temperature it needs to be equipped with a variable temperature accessory.
2. Liquid nitrogen for temperature regulation.
3. Capillaries from Fiber Optic Center, Inc. (*see* Note 2). Synthetic silica capillaries: 0.6 mm I.D. × 0.84 mm O.D., Supracil Cat. No. CV 6084S. These are used for gathering data. Glass capillaries: 0.6 mm I.D. × 0.84 mm O.D., Cat. No. CV 6084. These are used during isolation of the spin-labeled protein. Synthetic silica capillaries: 1 mm I.D. × 1.2 mm O.D., Cat. No. CV 1012S. These are used for making an adaptor to hold the sample capillary in the resonator.
4. Torch, natural gas, oxygen to seal capillaries at one end.
5. Critoseal, Cat. No. 8889-215003, from Oxford Labware.
6. Optional for making the capillary holder: Teflon heat shrink tubing (PTFE/FEP tube 0.06 inch ID, Part No. SMDT-060-24, Small Parts, Inc.) and a heat gun to shrink the tubing to fit tightly around the 1 mm capillary.

²Synthetic silica capillaries are used for collection of data because they have no background EPR signal. For routine surveys of fractions eluted from the Nap10 column (*see* step 6 of **Section 3.2.3**) quartz capillaries can be used. The background signal is often barely detectable. The price difference is considerable (\$3.32 per silica capillary versus \$0.26 for quartz).

7. Eppendorf gel loader tips, 20 μ L, and Gilson 20 μ L micropipettor to fill the capillaries.
8. Benchtop low speed centrifuge which holds tubes at least 11–13 cm long so that the capillaries can be centrifuged to force solution to the bottom.
9. Software appropriate for data analysis: Labview programs written by Christian Altenbach, Jules Stein Eye Institute, Department of Chemistry and Biochemistry, UCLA. An alternative is the WinEPR software supplied by Bruker. Software for generating figures comparing spectra: Origin (OriginLab).

3. Methods

3.1. Mutagenesis to Introduce a Single Accessible Cysteine

1. Design two complementary mutagenic primers, one priming the upper strand and the other priming the lower strand of the dsDNA template (30 to 40 bases), with the desired mutation near the middle and a stretch of 15 to 18 unmodified bases on each side that are a perfect match to the template. Both of the primers must contain the desired mutation.
2. Have primers commercially synthesized and suspend at 5 μ M in endonuclease free water.
3. Prepare the reaction mixture: 5 μ L of dsDNA template at a concentration of 10 ng/ μ L; 5 μ L of each of the 5 μ M primers; 5 μ L 10 X PCR reaction buffer; 1 μ L of 10 mM dNTP mixture; endonuclease-free H₂O to a final volume of 49 μ L. Add 1 μ L of *PfuUltra II* Fusion HS DNA polymerase (2.5 units/ μ L) last and mix gently. If you do not have a heated lid on your thermocycler, overlay the reaction with a drop of mineral oil.
4. Run the reactions using the following parameters:
 - a. 95°C for 2 minutes to denature;
 - b. Use a three step cycle: (1) 95°C 30 seconds, (2) 55°C 30 seconds, and (3) 68°C 15 seconds per kilobase of template plasmid.

If a single base change was used to introduce the cysteine run 14 cycles.
If two or three bases were changed, run 16 cycles.
 - c. 68°C for 3 minutes; 4°C hold.
5. Place the sample on ice briefly after removing it from the thermal cycler. The sample must be below 37°C before proceeding to the next step.
6. Add 1 μ L of the *Dpn I* endonuclease (10 units/ μ L) to the sample, gently mix and incubate the sample for 1 hour at 37°C. The reaction is carried out in the PCR reaction buffer so no changes to the buffer condition are needed. The buffer supplied with the *DpnI* enzyme when purchased is not used.
7. All subsequent steps are to be carried out under sterile conditions.

8. Thaw competent cells on ice. Add 50 to 100 μ L of competent cells to a 15 mL round-bottom Falcon tube. Add 1 to 5 μ L of the *Dpn* I-treated DNA. Incubate on ice for 30 minutes to transform the cells.
9. Heat pulse the transformation mixture for 2 minutes at 42°C, then place it on ice for 2 minutes.
10. Add 0.5 mL of growth medium appropriate for the strain used, WITHOUT ANTIBIOTICS, to the Falcon tube containing the heat-treated competent cells and incubate with shaking for 1 hour at 37°C.
11. Transfer cells to a sterile 1.5 mL microcentrifuge tube and centrifuge for 1 minute at maximum speed in a desktop microcentrifuge. Remove all but approximately 50 μ L of supernatant. Suspend the cells in the remaining 50 μ L of supernatant and plate the entire volume onto an agar plate with the growth medium and ANTIBIOTICS appropriate for the strain used.
12. Incubate the plate at 37°C overnight or until distinct colonies are visible.
13. Select individual, isolated colonies from the overnight plate and grow separate cultures of 4 mL from each colony.
14. Isolate plasmid DNA from each culture following the instructions included with the plasmid preparation kit used.
15. Determine the absorbance of each plasmid preparation at wavelength 260 nm. Calculate the DNA concentration using a conversion factor of absorbance of 1 is equivalent to 50 μ g/mL of dsDNA.
16. Have each plasmid DNA preparation sequenced commercially following the guidelines provided by the supplier of the service for the amount of sample and primers required.
17. After your DNA sequence has been verified as correct, purify the protein by the method of choice for the species under study. During purification keep the solutions reduced at all times using 2 mM DTT or 2 mM TCEP (*see* Note 3).

3.2. Labeling of the Cysteine Variants with the Nitroxide Reagent

3.2.1. Preparation of the Nitroxide Reagent

1. Purchase the reagent in vials of 10 mg and suspend the entire amount in 380 μ L of acetonitrile to give a concentration of 100 mM nitroxide reagent.
2. Dispense the solution in portions of 20 μ L into 0.65 mL Eppendorf tubes and take to dryness by lyophilization or using a centrifugal vacuum evaporator (Labconco).
3. Store the reagent in the dark at -80°C .

³During protein purification all solutions must be kept reduced to prevent oxidation of the cysteines. Since oxidation to sulfinic, sulfinic, and sulfonic acid is irreversible, it is crucial to keep solutions reduced from the start. We disrupt our cells using a French Press. Sonication introduces air and additionally causes local heating near the probe.

4. As needed add 20 μ L of acetonitrile to a tube to give a solution of 100 mM nitroxide reagent. The solution can be used for several experiments and frozen between usages.

3.2.2. Removal of Reducing Agent and Exchange into Labeling Buffer—If

possible one should start with 10 mg of protein so there is sufficient protein labeled not only for EPR studies but also for assays of activity (see **Section 1.4.**). If the protein is only available in limited quantities, smaller amounts can be labeled. We have never labeled less than 3 mg, but besides needing a good recovery so that the final sample will contain a high concentration of spin there should be no difficulty.

1. Equilibrate a Nap10 column with 15 mL of Buffer A.
2. Apply the protein to be spin labeled to the top of the column in a maximum volume of 1.0 mL. If the protein is contained in less than 1 mL do not dilute it (see Note 4), rather apply the sample and then follow with Buffer A to give a total volume of 1.0 mL. Allow this volume to flow through the column and discard it.
3. Elute the protein by addition of 1.2 mL of Buffer A. If the sample was applied in 1 mL, it will be completely recovered in 1.5 mL. However, you should not collect more than 1.2 mL so that all reducing agent is removed.

3.2.3. Substitution of Cysteine with the Nitroxide Reagent

1. If necessary, concentrate the sample using a Centricon 30 so that it is in 1 mL or less to facilitate removal of the free spin in the subsequent step no. 4 (see below).
2. Determine the concentration of the protein to be labeled by reading the absorbance at wavelength 280 nm. If the sequence of the protein is known, an extinction coefficient (ϵ) can be calculated using *ProtParam* (<http://us.expasy.org>). The concentration should be expressed in terms of molarity (i.e., not mg/mL) so that one can calculate the amount of reagent to add. Add the reagent in a 3-fold molar excess (see Note 5) to the accessible cysteine in each polypeptide chain. Care must be taken to keep the concentration of acetonitrile below 2% to avoid denaturation of the protein.
3. Incubate the protein and reagent together on ice in the dark for 2–3 hours.
4. Remove the free reagent by passage of the spin-labeled protein over a Nap10 column, equilibrated in Buffer B (see Note 6).

⁴The smaller the volume of the sample applied, the sharper the peak of elution is for the protein. Therefore, most of the protein can be recovered in 1.2 mL, well-separated from the included volume that contains the low molecular weight reducing agents.

⁵This approach is applicable to proteins that contain cysteine in addition to the target cysteine as long as the target is more accessible so that it can be preferentially labeled. When other cysteines are in the protein species we use a 1.3-fold molar excess to the target cysteine and incubate on ice in the dark for 1 hour.

⁶Nitroxide free in solution gives three very sharp lines. The sharp signal overlaps the line shape of the spin-labeled protein making it difficult to see changes in the line shape. The Labview software from Christian Altenbach provides a means to subtract free spin up to approximately 5%. Figure 11.7 shows the appearance of free spin in a sample stored at 7°C for 4 months. Since the free spin has very sharp lines and a nitroxide on a protein has a much broader spectrum, a small percentage of free spin dominates the line shape.

5. As before, see step 2 of **Section 3.2.2**, apply the protein to the top of the column. If the volume of the protein solution is less than 1 mL then Buffer B is added after the protein solution enters the column so that the total volume applied is 1 mL. Discard the initial 1 mL of volume that comes through the column.
6. Apply 1.2 mL Buffer B to the top of the column and collect the eluent in 4–5 drop fractions (approximately 0.15 mL).
7. Analyze each fraction by EPR (*see Section 3.3*). Determine which fractions to pool in order to maximize recovery of the labeled protein without including any free spin (*see Note 7*). The free spin elutes in the later fractions.
8. Determine the concentration of the spin-labeled protein by reading the absorbance at 280 nm and using the extinction coefficient as described in step 2 (*see above*).
9. Concentrate the spin-labeled protein using a Nanosep 30 centrifugal concentrator, if necessary. Generally, a sample containing 60 μ M spin gives an excellent signal (*see Note 8*).

3.3. Acquiring Data and Subsequent Analysis

1. Prepare capillaries for data collection. All capillaries are sealed at one end by a brief heating with an oxygen gas torch (*see Note 9*). Make an adaptor to hold the 0.6 mm I.D. capillary in the resonator by sealing one end of a 1 mm I.D. capillary. This capillary is too small to be held in the smallest collet that is supplied with the Bruker resonator. It must be further modified to increase the diameter. This can be simply done using parafilm or tape. To make a more robust adaptor one can insert the top of the capillary into a short stretch of Teflon heat shrink tubing and then use a heat gun to shrink it to fit tightly around the capillary. This capillary will now be held tightly in a small collet supplied with the resonator.
2. Fill the sealed capillary using a gel loader tip which will fit into the top of the 0.6 mm capillary. Slowly dispense 5–6 μ L of the solution while withdrawing the tip. This leaves the solution at the top of the capillary. It is forced to the bottom by a brief spin in a bench top centrifuge using a test tube that is slightly longer than the capillary (10 cm) as an adaptor. If the sample is available in sufficient quantities, it is simpler to use unsealed capillaries. Immerse the tip of an open capillary into a solution allowing it to fill by capillary action. Insert the filled capillary into the Critoseal container to introduce a plug at the end.

⁷When removing the free label we work in the cold room to suppress the release of the nitroxide. We find that substitution at some positions is susceptible to release of the nitroxide.

⁸When the 0.6 mm diameter capillaries are inserted into the resonator only a volume of 5 to 6 μ L at the bottom of the tube is in the path of the microwave radiation. Thus loading more volume does not increase the signal. The spectrometer records the total spin in the element of volume irradiated. It does not matter if it is uniformly dispersed.

⁹Caution must be taken so that the sealed end does not have a ball on it that prevents insertion into the 1 mm capillary serving as a holder. The tube should be heated as briefly as possible. After cooling, capillaries can be checked under a low power dissection microscope to be sure they are sealed. One should also test each sealed capillary by inserting it into a 1 mm capillary to be certain that it fits before using them with sample. If necessary, samples can be removed from the capillary by inserting the open end into a centrifuge tube and applying a brief spin, or the capillary can be broken off and a Hamilton syringe used to withdraw the sample.

3. Compare a series of samples containing the spin-labeled protein that are prepared in the same buffer conditions. For example, in experiments with unfolded polypeptide ligands and SecB we dilute the ligand from denaturant; thus, denaturant must be added to the same final concentration to the sample with SecB alone (*see* Note 10).
4. Scan over a field of 100 gauss (1 gauss = 10^{-4} Tesla) centered at 3356 gauss using an incident power of 20 mW (attenuation 10 dB).
5. We routinely gather 15 scans; the ratio of the signal intensity to the noise increases as the square of the number of scans. Whereas four scans give a 2-fold improvement relative to two scans and 16 scans give a 2-fold improvement over four scans, it is not practical to improve the signal by gathering more than 15 scans since a 2-fold increase in the signal to noise would require 225 scans.
6. Choose a field modulation appropriate for the spectrum. Set the frequency of modulation to 100 kHz and set the field of modulation to one gauss. Acquire an initial spectrum (one scan will do) and measure the center line width (H_{pp}). Increase the field of modulation to improve the signal-to-noise ratio, but do not exceed the width of the center line. We usually modulate at two gauss. Scans must be gathered at the same modulation in order to be compared. However, it is not necessary to gather the same number of scans for comparison since the data are normalized during analyses.
7. Set a baseline and normalize each spectrum using WinEPR or the Labview programs.
8. Compile the data into a scientific plotting and analysis software program such as Origin. Compare the spectral line shape of a given spin-labeled variant with the line shapes of the protein in complex with the binding partners to be tested. Also compare spectra to determine changes in line shape that result from addition of components to the solution, such as denaturant.
9. Visual inspection is used to determine whether changes in line shape represent a constraint or a mobilization of the side chain as described in **Section 1.3**.

References

1. Papanikou E, Karamanou S, Economou A. Bacterial protein secretion through the translocase nanomachine. *Nat Rev Microbiol.* 2007; 5:839–851. [PubMed: 17938627]
2. Randall LL, Topping TB, Hardy SJS. No specific recognition of leader peptide by SecB, a chaperone involved in protein export. *Science.* 1990; 248:860–863. [PubMed: 2188362]
3. Randall LL, Crane JM, Liu G, Hardy SJS. Sites of interaction between SecA and the chaperone SecB, two proteins involved in export. *Protein Sci.* 2004; 13:1124–1133. [PubMed: 15010547]

¹⁰SecB binds polypeptides in an unfolded state with no affinity for stably folded proteins. Therefore, the unfolded ligand must be diluted from denaturant directly into a solution containing SecB to allow formation of a complex. For this reason in our studies of SecB complexed with unfolded polypeptides, there is a low level of denaturant present. The denaturant is not necessary for any other reason.

4. Bruce JE, Smith VF, Liu C, Randall LL, Smith RD. The observation of chaperone-ligand noncovalent complexes with electrospray ionization mass spectrometry. *Protein Sci.* 1998; 7:1180–1185. [PubMed: 9605322]
5. Randall LL, Crane JM, Lilly AA, Liu G, Mao C, Patel CN, Hardy SJ. Asymmetric binding between SecA and SecB two symmetric proteins: implications for function in export. *J Mol Biol.* 2005; 348:479–489. [PubMed: 15811382]
6. Randall LL, Hardy SJS, Topping TB, Smith VF, Bruce JE, Smith RD. The interaction between the chaperone SecB and its ligands: evidence for multiple subsites for binding. *Protein Sci.* 1998; 7:2384–2390. [PubMed: 9828004]
7. Patel CN, Smith VF, Randall LL. Characterization of three areas of interactions stabilizing complexes between SecA and SecB, two proteins involved in protein export. *Protein Sci.* 2006; 15:1379–1386. [PubMed: 16731972]
8. Crane JM, Mao C, Lilly AA, Smith VF, Suo Y, Hubbell WL, Randall LL. Mapping of the docking of SecA onto the chaperone SecB by site-directed spin labeling: insight into the mechanism of ligand transfer during protein export. *J Mol Biol.* 2005; 353:295–307. [PubMed: 16169560]
9. Crane JM, Suo Y, Lilly AA, Mao C, Hubbell WL, Randall LL. Sites of interaction of a precursor polypeptide on the export chaperone SecB mapped by site-directed spin labeling. *J Mol Biol.* 2006; 363:63–74. [PubMed: 16962134]
10. Cooper DB, Smith VF, Crane JM, Roth HC, Lilly AA, Randall LL. SecA, the motor of the secretion machine, binds diverse partners on one interactive surface. *J Mol Biol.* 2008; 382:74–87. [PubMed: 18602400]
11. Fajer, PG. *Encyclopedia of Analytical Chemistry*. Meyers, RA., editor. John Wiley & Sons Ltd; London: 2000. p. 5725–61.
12. McHaourab HS, Lietzow MA, Hideg K, Hubbell WL. Motion of spin-labeled side chains in T4 lysozyme. Correlation with protein structure and dynamics. *Biochemistry.* 1996; 35:7692–7704. [PubMed: 8672470]
13. Hubbell WL, Gross A, Langen R, Lietzow MA. Recent advances in site-directed spin labeling of proteins. *Curr Opin Struct Biol.* 1998; 8:649–656. [PubMed: 9818271]
14. Columbus L, Kalai T, Jeko J, Hideg K, Hubbell WL. Molecular motion of spin labeled side chains in alpha-helices: analysis by variation of side chain structure. *Biochemistry.* 2001; 40:3828–3846. [PubMed: 11300763]
15. Fanucci GE, Cafiso DS. Recent advances and applications of site-directed spin labeling. *Curr Opin Struct Biol.* 2006; 16:644–653. [PubMed: 16949813]
16. Schneider, D., Freed, J. *Spin Labeling: Theory and Application, Biological Magnetic Resonance*. Berliner, L., Reuben, J., editors. Vol. 8. Plenum; New York: 1989.

Fig. 11.1.
Dependence of the energy difference between spin states as a function of the applied magnetic field.

Fig. 11.2.
An absorption curve (*upper*) and its derivative (*lower*), as seen in EPR.

Fig. 11.3.
Reaction of the methanethiosulfonate spin label to give the nitroxide-derivatized cysteine side chain.

Fig. 11.4. Parameters reflecting mobility. The peak-to-peak width of the central resonance line (H_{pp}) is measured as indicated and is equivalent to the peak width at half-height of an absorbance spectrum. The spectral breadth ($2A'_{zz}$) is the distance between the outermost hyperfine extrema. The spectrum used to illustrate these parameters is that of residue L126 of SecB.

Fig. 11.5. Spectra of constrained residues with the spin label on the SecB residue indicated. (A) SecBL126, alone (*solid trace*) and in complex with unfolded polypeptide ligand (*dotted trace*); (B) SecBQ144, alone (*solid trace*) and in complex with unfolded polypeptide ligand (*dotted trace*); and (C) SecBT149, alone (*solid trace*) and in complex with SecA (*dotted trace*).

Fig. 11.6. Spectra of mobilized residues. *Solid traces* are spin-labeled SecA alone; *dotted traces* are spin-labeled SecA in complex with lipids. All spectra were gathered at 27°C. (A) Spin-labeled on residue R602 on SecA; (B) spin-labeled on residue R642 of SecA.

Fig. 11.7.

Effect of free spin on line shape. SecB was spin-labeled on residue Q33. A sample containing 160 μM spin was loaded into a capillary and the open end sealed with Critoseal. The capillary was stored at 7°C and scanned periodically. The *arrows* show the positions of the three lines that represent the free spin. (A) Scanned within a week of labeling, (B) after 1 month at 7°C, and (C) after 4 months at 7°C.