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Electron Spin Resonance Spectroscopy Labeling in Peptide and Protein Analysis

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Spin labels are nitroxide derivatives with a stable unpaired electron and a functional group for specific attachment to the protein (covalent or as a ligand). The most popular covalent sites are cysteine residues, which, if necessary, can be introduced into the protein structure using molecular biology techniques.

The physical basis for nearly all ESR applications is the anisotropy of the nitroxide signal and the sensitivity of the ESR spectra to various relaxation pathways. The interaction between an electron of a spin label and an external magnetic field depends on their relative orientations. The splitting and the center of ESR spectra of an oriented sample are used to determine the orientation of labeled domains. For samples with little disorder the orientational sensitivity is better than 1°. The width of the signal is proportional to the orientational disorder, which is used to measure conformational heterogeneity of proteins.

If the spin label reorientates itself on the ESR timescale (nanoseconds) then the spectral anisotropy is averaged. The extent of averaging defines the ESR line shape which is used to determine the rotational rate and anisotropy of motion. The dynamic range of ESR is very broad, rotational correlation times range from $10^{-12}$ to $10^{-7}$ s for conventional ESR and the sensitivity can be extended to slower motions ($10^{-3}$ s) with nonlinear saturation transfer electron spin resonance (STESR). Protein (spin label) mobility is used to follow conformational changes, steric restrictions on the spin label and the formation of large complexes.

Spin labels are also sensitive to the presence of other paramagnetic species. Collisions with water and lipid-soluble relaxing agents provide additional relaxation pathways measured by changes in relaxation times. The probability of these collisions reflects the accessibility of a spin label to the relaxant. The periodic patterns along the polypeptide chain of this accessibility are used to determine the secondary and tertiary structure of proteins. In the presence of another bound spin label or a paramagnetic metal complexed by histidine residues, spectra become broadened by dipolar or exchange interactions. Both mechanisms depend on the distance between the paramagnetic centers. Thus ESR can be used to determine intra- and intermolecular distances. The range of sensitivity is 5–25 Å and there are intensive efforts to increase the upper range to >50 Å. ESR as a spectroscopic ruler is used in protein structure determination and the investigation of macromolecular assembly processes and protein folding.

The foremost limitation of spin labeling ESR is the necessity to modify a protein with a spin probe. In some cases, the spin labels may perturb protein function and therefore cannot be used for spectroscopy. However, even an unsuccessful modification that results in functional
loss identifies functional regions of proteins and as such represents successful “mutational analysis” experiments.

1 INTRODUCTION

A spinning electron orbiting around a nucleus is a magnetic dipole. When placed in an external magnetic field, the dipole aligns parallel or antiparallel with the external field. These two orientations of the magnet represent two energy levels, with the difference in energy levels of the electron spin proportional to the strength of the magnetic field. The electron can be excited from one level (i.e. parallel dipole orientation) to another (antiparallel orientation) by an oscillating magnetic field. The energy of the oscillating field has to match the energy difference between the two levels. For a free electron in a magnetic field with a strength of a few hundred gauss, the frequency range of the exciting field is in the microwave region of the electromagnetic wave spectrum. The resonance between the orbiting electron and the microwave field forms the basis of ESR, also known as electron paramagnetic resonance or electron magnetic resonance.

ESR is commonly used to investigate protein and peptide structure, particularly studies of molecular orientation, protein dynamics and ligand binding. Observation of a resonance requires samples containing an unpaired electron, e.g. transition metals or organic radicals. Proteins and peptides are generally not paramagnetic and therefore require the use of extrinsic probes called spin labels. Spin labels are derivatives of nitroxides, small stable organic radicals, which are covalently attached to protein side chains or to metabolic substrates. In the last decade, the development of site-directed spin labeling (SDSL), which utilizes molecular biology to introduce new labeling sites, has established ESR as a protein structural determination technique. Patterns of side-chain mobility, accessibility to quenchers and the measurement of distances between spin labels have allowed the determination of the secondary, tertiary and quaternary structure of proteins.

This article is focused exclusively on spin labeling applications in protein and peptide biochemistry. The vast literature on metalloproteins, photosynthesis and reactive radicals in biology is not discussed here, and interested readers are directed to the many excellent reviews on these topics.1–6

2 HISTORICAL PERSPECTIVE

The first ESR experiments were performed by Zavoisky at the University of Kazan (Russia) during the Second World War.7 Inspired by the experiments of Gorter8 and Rabbi et al.9 on paramagnetic relaxation and atomic beams, Zavoisky demonstrated resonance between microwaves and the precession of Cu2+ ions in a magnetic field. Resonance was observed as an absorption of microwaves whenever the frequency of the oscillating microwave field was equal to the ion precession frequency.

In the decade following the Second World War, ESR was the domain of physical chemists and physicists, with the first biological applications appearing in the mid-1950s. This early work included structural studies of metalloproteins,10 measurement of free radicals in biological tissues,11 carbonized carbohydrates,12 and X-ray irradiated silk and hair.13 Assenheim provides an excellent review of this early work with intrinsic ESR signals.14

In 1965, McConnell introduced extrinsic spin labels designed to label proteins. Using nitroxide derivatives first synthesized in Russia,15,16 McConnell et al. demonstrated a helix–coil transition of a polylsine peptide.17 Since then, ESR spin labeling has been used to study conformational changes in a number of proteins modified by maleimide nitroxides, which specifically target cysteine residues. However, reliance on the naturally occurring cysteine residue was a severe limitation. The SDSL strategy developed by Hubbell in 1989 employs molecular biology to introduce new cysteines for spin label attachment. The use of SDSL to scan the protein sequence with cysteines has stimulated the resurgence of ESR as a structural biology method.

The methodology of ESR was also undergoing an evolution. In 1957, Feher invented electron–nuclear double resonance (ENDOR) spectroscopy, a combination of both ESR and nuclear magnetic resonance (NMR).18 In which nuclear spin transitions are observed indirectly by monitoring electron spin transitions. A few years later, electron–electron double resonance (ELDOR) spectroscopy was developed by Hyde et al.19 and Benderskii et al.20 which allowed the measurement of spectral diffusion between distinct spin populations. The development of spin-echo instruments by Mims et al.21 introduced time-domain ESR in the 1960s. This was followed by Fourier transform electron spin resonance (FTESR), developed independently in the 1980s by Elia and Freed,22 Dinse et al.23 and Bowman.24 The first spin label applications appeared in 1986 when Gorester and Freed performed two-dimensional (2-D) FTESR experiments to measure spin dynamics.25

ESR moved towards high field (high frequency) with Lebedev et al.’s construction of a 150-GHz spectrometer,26 followed by Freed et al.’s 250-GHz spectrometer, which was based on quasi-optics. The latter instrument was used extensively to investigate spin labels
in biological systems.\textsuperscript{(27)} Ultra-high-field spectrometers operating at 550 GHz now exist and their application to nitroxide labels is only a question of time.\textsuperscript{(28)}

Readers interested in the history of ESR are directed to a series of historic recollections of the ESR pioneers assembled by Eaton et al.\textsuperscript{(29)}

3 SAMPLE PREPARATION

3.1 Nitroxide Spin Labels

Proteins are ESR silent, with the exception of metalloproteins, and must therefore be “labeled” with paramagnetic probes. These probes, or spin labels, are nitroxide derivatives containing an unpaired electron in the $\pi$ orbital of the N–O bond (Figure 1a–c). The nitroxide radical is stable owing to the presence of methyl groups on neighboring carbon atoms. To limit flexibility, the NO group is enclosed in either a six-membered piperidine or a five-membered pyrrole ring. Pyrrole rings with an unsaturated bond are the least flexible.

The unpaired electron in the $\pi$ orbital also interacts with the spin of the nitrogen nucleus, splitting the ESR signal into resonances corresponding to different nitrogen nuclear manifolds. Thus, the number of resonant peaks depends on the nitrogen isotope, three for $^{14}$N and two for $^{15}$N. $^{15}$N labels have the advantage of less spectral dispersion which increases the signal amplitude 1.5-fold in conventional ESR and allows for full spectral coverage in FTESR. Reduction of the nuclear manifolds also simplifies the interpretation of nuclear relaxation and accelerates computer simulations of ESR line shapes. $^{15}$N labels, however, are considerably more expensive than $^{14}$N and only a handful of them are available commercially.

A weaker interaction occurs between the electron spin and the hydrogen nuclei of the ring and methyl groups. Each resonance peak is split by the nuclear spin, but the splittings are unresolved, resulting in a broad peak. The broadening can be removed by the substitution of hydrogen with deuterium which increases the peak height 1.5-fold for Gaussian and 5-fold for Lorentzian lines.

![Figure 1](image)

**Figure 1** Commonly used nitroxides: (a) six-membered piperidine ring; (b) saturated five-membered pyrrole ring; (c) unsaturated pyrrolidine ring.

3.2 Labeled Sites

Nitroxide spin labels are used either covalently as modifiers of selected amino acids or noncovalently as analogs of substrates or enzymatic cofactors. The specificity of the label is conferred by the functional group attached to the nitroxide. For example, maleimide, iodoacetamide, indanedione and $\alpha$-ketone groups attached to the nitroxide moiety target cysteine residues, while lysines are modified by activated esters in Figure 2(a–d). Attachment of the nitroxides by disulfide bonds allows for reversible modification. Reduction of the disulfide bonds with a mild reducing agent yields the unmodified protein. Bifunctional spin labels with two linker groups facilitate attachment to two sites on a protein, reducing probe mobility with respect to the protein. The ability to engineer neighboring attachment sites in a protein using molecular biology is likely to increase the use of bifunctional labels.

The molecular biology revolution has had a profound impact on spin label ESR. The limitations of using naturally occurring binding sites are circumvented by the site-directed spin-labeling method pioneered by Hubbell et al.\textsuperscript{(30)} In SDSL, native cysteines are mutated out and new cysteines are introduced at desired vantage points. The power of this method is best illustrated by cysteine scanning where each residue along the polypeptide chain is changed to a cysteine and labeled with nitroxide.

Noncovalent labels are used in the investigation of active sites, e.g. substrate or cofactor analogs, adenosine triphosphate (ATP) or nicotinamide adenine dinucleotide (NAD) nitroxide adducts (Figure 3a–c). The binding and function of these substrates are often not compromised by the presence of the nitroxide. Both approaches

![Figure 2](image)

**Figure 2** Various spin labels used in covalent modification of proteins: (a) maleimide spin label; (b) methyl thiosulfonate spin label; (c) iodoacetamide spin label; (d) hydroxysuccinamide (lysines).
are combined in photoactivated labels with an additional azido or nitrene groups. The label is guided by a substrate analog moiety to an active site and photoactivation attaches the label covalently to the protein.

3.3 Attachment Rigidity

Spin labels are attached to a protein via one or more single bonds about which the nitroxides can rotate on a sub-nanosecond timescale. In studies of protein orientation and dynamics, such an independent (librational) motion is a major hindrance since it averages the orientational dependence of magnetic tensors – anisotropy. Anisotropy of magnetic tensors is the basis for ESR orientational and motional sensitivity as discussed in sections 5.1 and 5.2. The extent of probe motion is estimated by immobilizing the protein on either glass or ion-exchange beads and comparing spectral parameters such as effective splitting (in conventional ESR) or line-height ratios (in STESR) to their rigid limit values. Alternatively, the protein mobility can be reduced by increasing the medium viscosity, \( \eta \). The observed spectral parameters can then be plotted against \( \eta \) (Perrin plots) and extrapolated to infinite viscosity. If the extrapolated values are lower than the rigid limit of the nitroxide, or if discontinuities exist in the Perrin plots, then it can be concluded that the probe moves independently of the protein.

3.4 Impairment of Function/Structure of Labeled Proteins

Covalent modification of proteins with extrinsic probes carries the danger of damaging the function of the molecule. Certain labels are innocuous at certain sites while others are not. No generalizations can be made. For example, out of 32 spin-labeled cysteine mutants of T4 lysozyme, 11 displayed intact activity and 11 had activity \( \geq 50\% \). Modification of buried residues and residues in tertiary contacts decreased appreciably the enzymatic activity.\(^{31} \) In K+ channels, ion pumping was affected by \(<50\% \) in 46 out of 66 spin-labeled mutants and very few were completely inactivated.\(^{32} \) Therefore, a careful functional characterization is necessary if the objective of the study is the correlation of structure and function.

The overall global structure of a protein is generally less affected than its function by labeling. Nitroxide labels are relatively small and a labeled cysteine is not much larger than a tryptophan residue. Surface labeling has been shown to have little effect on protein folding or stability. However, when buried residues are labeled, side chains and backbone can shift to accommodate the labels and restore packing of the protein core.\(^{33} \)

4 TECHNIQUES AND INSTRUMENTATION

4.1 Continuous Wave Electron Spin Resonance

4.1.1 Theory

The magnetic moment, \( \mu \), of an electron interacts with an external magnetic field just like a compass needle interacts with the earth’s magnetic field. The interaction of electron spin with the field is often referred to as the Zeeman interaction. The energy \( (E) \) of a magnetic dipole \( (\mu) \) in a static magnetic field is given by Equation (1):

\[
E = -\mu H
\]  

(1)
where $H$ is the magnetic field strength. The magnetic moment of an electron is generated by its spin ($S$) (Equation 2):

$$\mu = -g\beta S$$  \hspace{1cm} (2)$$

with $\beta$ denoting the Bohr magneton (intrinsic unit of electron magnetic moment) and $g$ denoting the spectroscopic splitting factor (relates contribution of spin and orbital motion of the electron to its total angular momentum).

Unlike a compass needle, electron spin is quantized. For a single electron, the projection of $S$ on the magnetic field axis $S_z$ can only take values of $\pm 1/2$. Thus the energy levels from Equations (1) and (2) are $E = \pm (1/2)g\beta H$, resulting in an energy gap which increases linearly with the magnetic field, $\Delta E = g\beta H$ (Figure 4). An oscillating magnetic field can flip the electrons from one energy level to the other if its own energy, defined by the oscillating frequency $\nu$, equals the energy gap. Hence, for resonance between the oscillating field (microwave) and the electron spin, the condition in Equation (3) has to be satisfied:

$$\nu = g\beta H$$  \hspace{1cm} (3)$$

The resonance condition can also be obtained by considering a spinning electron moving in an orbit around a nucleus placed in a magnetic field. From classical mechanics, the rate of change of the magnetic moment is proportional to the torque produced by the interaction of the moment and the magnetic field and given by their vector product (Equation 4):

$$\frac{\Delta \mu}{\Delta t} = \mu \otimes \gamma H$$  \hspace{1cm} (4)$$

Substitution of $\gamma$ from Equation (5) into Equation (6) yields the resonant condition $\nu = g\beta H$ stated in Equation (3). Applying an oscillating microwave field of the same frequency as the Larmor frequency cancels the orienting effect of the static magnetic field. The spin then rotates about an axis perpendicular to the static field direction, periodically aligning itself with or against the static field. This is equivalent to dipole ($\mu$) flipping between the two energy levels.

The extent of microwave absorption, which defines the intensity of the ESR signal, is proportional to the difference in spin populations, $N$, between the upper and lower energy states. The ratio of the two populations is determined by the Boltzmann distribution (Equation 7):

$$\frac{N_{+1/2}}{N_{-1/2}} = \exp \left( \frac{-\Delta E}{kT} \right)$$  \hspace{1cm} (7)$$

The difference in spin populations is increased by either increasing the magnetic field $H$ or reducing the temperature $T$. For example, at 0.35 T and room temperature the population difference is 0.1% but it can be increased to 13% by reducing the temperature to 3 K. The difference between the levels decreases with absorption and an efficient relaxation pathway has to exist to restore the Boltzmann equilibrium. Relaxation pathways include the *dipolar spin–spin* relaxation – sharing of energy between electrons or nuclei – and *spin–lattice* relaxation – sharing vibrational modes with the lattice. They are characterized by relaxation times $T_2$ and $T_1$, respectively. Relaxation times are defined as the time interval between initial perturbation and when the deviation from equilibrium decays to $1/e$ of its initial value. The relaxation rates are additive and their sum defines the width (at half-height) of the resonance, $\Gamma$ (Equation 8):

$$\Gamma = \frac{1}{\gamma} \left( \frac{1}{T_2} + \frac{1}{T_1} \right)$$  \hspace{1cm} (8)$$

Figure 4 Energy level diagram of Zeeman and hyperfine interactions for a $^{14}N$nitrooxide ($S = 1/2, I = 1$). The vertical arrows denote ESR transitions with the resulting first-derivative spectrum below.

where $\gamma$ is a magnetogyric ratio (ratio of magnetic and inertia moments) characteristic of a given electron (Equation 5)

$$\gamma = \frac{g\beta}{\hbar}$$  \hspace{1cm} (5)$$

where $\hbar$ is Planck’s constant. The torque will force the magnetic dipole ($\mu$) to precess around the static field at a defined frequency, the *Larmor frequency*, $w$, given by Equation (6):

$$w = \gamma H$$  \hspace{1cm} (6)$$

Faster relaxation (shorter $T_1$ or $T_2$) results in broader line widths. For paramagnetic ions, the strong coupling of spins to lattice (short $T_1$) produces broad lines. Lowering the temperature weakens lattice coupling (increases
magnitudes of the nucleus has both an isotropic and dipolar component. The case of nitroxide labels, it is the nitrogen nucleus which spin number \( m \) the oxygen’s attraction and increases the electron density on nitrogen, increasing the polarity of the medium decreases unpaired electron is located between the oxygen and to the electron spin density on the nucleus. Since the selection rule for spin transitions dictates that the total spin quantum number can only change by 1, the hyperfine interactions lead to \( 2 \) spin quantum number can only change by 1, the hyperfine interactions lead to \( 2 \) transitions (Figure 4). In the case of nitroxide labels, it is the nitrogen nucleus which interacts with the unpaired electron. For \(^{15}\text{N}\) the nuclear spin number \( m_1 \) is 1/2 so that two electron transitions are observed; for \(^{14}\text{N}\), \( I = 1 \) and therefore there are three electron transitions.

The resonance condition of Equation (3) is thus modified to include hyperfine interactions, \( A \) (Equation 9):

\[
\hbar v = g \beta H + m_1 A
\]  (9)

The hyperfine interaction between an electron and the nucleus has both an isotropic and dipolar component. The magnitude of the isotropic splitting, \( a_0 \), is proportional to the electron spin density on the nucleus. Since the unpaired electron is located between the oxygen and nitrogen, increasing the polarity of the medium decreases the oxygen’s attraction and increases the electron density on the nitrogen. Thus, \( a_0 \) is a sensitive measure of the spin environment.

The \( p \sigma \) orbital of an unpaired electron is asymmetric, making the dipolar interactions of the electron and nucleus orientation dependent. For example, hyperfine interactions are stronger when the \( z \)-axis of the orbital is aligned with the magnetic field and weaker when the field is aligned perpendicular. The hyperfine interactions are best described by a second rank tensor, \( A \) (Equation 10):

\[
A = \begin{bmatrix}
A_{xx} & 0 & 0 \\
0 & A_{yy} & 0 \\
0 & 0 & A_{zz}
\end{bmatrix}
\]  (10)

Typical values for nitroxide spin labels are \( A_{xx} \approx A_{yy} \approx 7 \text{ G} \) and \( A_{zz} \approx 35 \text{ G} \). The difference between the \( x \)- and \( y \)-components is small and often the hyperfine tensor is assumed to be axially symmetric.

The Zeeman interaction of the electron spin with the static magnetic field (Equation 1), is also anisotropic. Asymmetry of orbital motion in the \( p \sigma \) orbital results in different contributions of spin and orbital momenta and thus the \( g \)-value can also be described by a tensor (Equation 11):

\[
g = \begin{bmatrix}
g_{xx} & 0 & 0 \\
0 & g_{yy} & 0 \\
0 & 0 & g_{zz}
\end{bmatrix}
\]  (11)

In contrast to the hyperfine tensor, the \( g \)-tensor is rhombic with typical values \( g_{xx} \approx 2.0085, g_{yy} \approx 2.0065 \) and \( g_{zz} \approx 2.0027 \). The asymmetry of the Zeeman and hyperfine interactions defines ESR sensitivity to orientation and to rotational motion.

### 4.1.2 Electron Spin Resonance Spectrometer

A modern ESR instrument consists of three basic units: (a) a microwave bridge and resonator, (b) a variable field magnet and (c) signal amplification circuitry (Figure 5).

Microwaves of the desired frequency are generated by either a klystron or Gunn diode. Their intensity is adjusted by an attenuator and transmitted via a waveguide to the sample chamber/resonator. During resonance, a small amount of microwaves is reflected from the resonator and detected by a Shottky diode. To separate the reflected and incident microwaves, a circulator is placed between the attenuator and resonator. The circulator channels the microwaves in a forward direction: incident microwaves to the resonator and reflected microwaves to the detector. The bridge often contains an additional pathway – a reference arm which taps off a small fraction of the microwaves from the source – which bypasses the resonator and falls on to the detector to ensure its bias for the optimal detection of small intensity changes during resonance.

A static magnetic field is provided by an electromagnet stabilized by a Hall probe. The field is slowly swept by varying the amount of current passing through the electromagnet. In order to decrease microwave noise,
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the resonance signal is encoded by modulating the static field with a small magnetic field generated by modulation coils. The modulation field sweeps periodically through the nitroxide resonance field. Therefore, the changes in microwave absorption due to resonance occur with the modulation frequency. A lock-in amplifier selects and amplifies only the signal which is in phase and in frequency with the modulation field and rejects all other microwave fluctuations as noise. The signal detected using field modulation is proportional to the changes in the microwave intensity during one cycle of modulation, i.e. the signal is the first derivative of the absorption (Figure 6a–b).

The microwave field produced by the klystron is too weak to induce any detectable absorption by the sample. Resonant cavities, loop gap resonators (LGRs) or, more recently, dielectric resonators (DRs) are used to increase the microwave magnetic field at the sample. The cavities rely on the generation of a standing wave pattern of microwaves whose intensity builds up during cavity resonance. The main drawback of cavities is the presence of an electric component of the microwave. The electric component is absorbed by “lossy”, aqueous samples (common in biology) causing sample heating and loss of cavity resonance. To avoid this problem, sample volume is restricted to the nodal planes of the electric field, limiting the usable volume of the cavity and thus resulting in a low filling factor, \( \eta \). Cavities are high-\( Q \) structures \( Q = E_{\text{stored}}/E_{\text{dissipated}} \), storing thousands of times more energy than is dissipated on the walls. High \( Q \) can only be achieved within a narrow frequency bandwidth of stored microwaves as \( Q = \nu/\Delta \nu \). Small changes in the sample, cavity geometry or temperature can all cause frequency shifts and mismatching of the incident microwave with the cavity. Automatic frequency control (AFC) circuitry is employed to track the frequency of the klystron to that of the cavity. However, the AFC feedback response time limits the deadline of signal changes in transient experiments such as stop-flow. In pulse experiments it is necessary to wait until the energy of the perturbing pulse is fully dissipated. This ring-downtime is proportional to \( Q \); thus in the high-\( Q \) structures a longer time has to elapse before a relatively weak spin echo or free induction decay (FID) signal can be collected.

Most of these problems with cavities have been overcome by low-\( Q \) resonators such as an LGR or DR. These resonators condense the magnetic component of the microwave, separating it from the electric component. Lossy samples are no longer heated by the electric component. Small sample volumes and large filling factors offer an additional advantage especially when dealing with genetically engineered proteins which are often purified in picomolar quantities. Furthermore, fast dissipation of energy and the large bandwidths of LGRs and DRs make them suitable for pulsed and transient experiments.

4.1.3 Instrumental Variables Affecting the Electron Spin Resonance Spectrum

Two instrumental parameters influence the line shape of experimental spectra: modulation amplitude and microwave power. The amplitude of the ESR signal initially increases with the modulation amplitude \( (H_m) \) as it approaches the intrinsic line width \( (\Delta H_{pp}) \). Maximum amplitude is attained at \( H_m = 3.5\Delta H_{pp} \) for Lorentzian and at \( H_m = 1.8\Delta H_{pp} \) for Gaussian line shapes. Any further increases in \( H_m \) result in a decrease of the signal. The broadening of \( \Delta H_{pp} \) is observed well before the maximum amplitude of the signal. When \( H_m = \Delta H_{pp} \), the observed Lorentzian and Gaussian widths are 25% and 15% larger, respectively. As a rule of thumb, modulation should be kept at one-fifth of the intrinsic line width when resolution or line width is of importance. The errors in line width are then <1%.

The ESR signal is proportional to the static microwave field, \( H_1 \), and hence to the square root of the microwave power \( (P^{1/2}) \), in the absence of saturation. When the rate of relaxation lags behind the rate of excitation and when the spin populations in the ground and excited states are equalized, the signal saturates and decreases to nil. The amplitude of the ESR signal \( (y) \) for a Lorentzian line shape as a function of the microwave field is given by

![Figure 6 Conventional ESR signals: (a) absorption, \( V_0 \); (b) first derivative \( V_1 \); (c) STESR spectrum, second derivative, 90° out-of-phase display, \( V_2 \).](image-url)
Equation (12):
\[
y = \frac{y_0 H_i}{\sqrt{1 + H_i^2 c_1^2 T_1 T_2^2}}
\]
where \(y_0\) is a field-independent parameter. Hence it is important to keep power levels well below the maximum amplitude whenever spectra are used to quantify the number of spins. Line-width distortions are less pronounced than those due to the modulation field, but at powers giving maximum amplitude, the observed line width increases 1.2 times over the intrinsic line width.

### 4.2 Saturation Transfer Electron Spin Resonance

#### 4.2.1 Qualitative Theory

STESR was developed to study slower molecular dynamics with rotational correlation times \((\tau_1)\) of >200 ns.\(^{34}\)

The timescale of conventional ESR is determined by the spin–spin relaxation time \(T_2\) (nanoseconds). The ESR timescale can be extended to a longer spin–lattice relaxation time \(T_1\) (microseconds) if a signal sensitive to spin saturation is observed. This can be done by saturating the signal with intense microwaves, creating a “hole” in the absorption spectrum, and subsequently observing signal recovery. When the saturating microwave is switched off (or decreased to nonsaturating levels) the signal recovers with the rate determined by the spin–lattice relaxation time, \(T_1\). The onset of motion provides an additional relaxation mechanism: spectral diffusion. The saturation is relieved as the resonating-saturated spins rotate away from the resonance field and the unsaturated spins come into resonance. The “hole” broadens out across the spectrum and the intensity of the signal increases. The second harmonic, out-of-phase ESR signal \((V_2^\prime\)\), collected at moderate saturation, is particularly sensitive to spectral diffusion. The line shape of \(V_2^\prime\), in the presence of saturation, bears a strong resemblance to the absorption spectrum with the intensity lowered in the spectral regions most sensitive to the spectral diffusion (see Figure 6c).

#### 4.2.2 Instrumental Parameters

The STESR signal is influenced by nitroxide relaxation times, spectral diffusion, spin saturation level and the modulation frequency with which the “hole” is observed. Hence the instrumental parameters which affect any of these must be precisely controlled. The saturating microwave field averaged over the sample volume is set to 0.25 G. The microwave power is adjusted to this level using the microwave field conversion factor \((c)\), corrected for a filling factor \((\eta)\) and dielectric losses, which lower the \(Q\) factor (Equation 13):

\[
\langle H_i^2 \rangle_{st} = c\eta P Q
\]

where \(P\) is incident microwave power. The power-to-field conversion factor is determined experimentally by the saturation of Fremy salt [peroxymamine disulfonate (PADS)] for which the half-saturation field is 0.1067 G.

The modulation frequency and amplitude, which determine the frequency of the stepping on- and off-resonance, i.e. the interval between burning and observing the “hole”, must also be calibrated. Modulation broadening of a narrow line-width sample, e.g. Fremy salt, is used for this purpose. The observed line width \((\Delta H_{pp})\) is dominated by modulation broadening when the modulation amplitude is ~10 times the intrinsic line width \(\Delta H_{pp}(0)\), i.e. \(\Delta H_{pp} = H_m - \Delta H_{pp}(0)\). Commonly used values for the modulation field are 5 G and 50 kHz.

Finally, since the \(V_2^\prime\) STESR signal is 90° out of phase (phase quadrature) with modulation, the precise phase nil must be found. An error of 1° in setting the phase quadrature can result in significant line-shape changes due to leakage of a more intense in-phase signal. The most popular phase nulling method is by interpolation of the unsaturated in-phase signal: two or three readings are taken within 15° on each side of the putative nil and the phase at which the signal is zero is found by linear interpolation. A general description of the experimental procedures and calibration can be found in Fajer and Marsh\(^{35}\) and Squier and Thomas.\(^{36}\) Digital post-acquisition methods have also been proposed but are not widely used.

As a footnote, protein mobilities measured by conventional ESR and STESR were independently verified by optical methods – fluorescence and phosphorescence anisotropy. Bovine serum albumin labeled with a dual probe bearing a spin label moiety and the optical probe eosin was measured using optical methods and ESR/STESR.\(^{37}\) The agreement between the fluorescence/phosphorescence and ESR was excellent.

#### 4.3 Time Domain Methods

Time domain ESR relies on the perturbation of the equilibrium magnetization by an intense microwave pulse which is then followed by one of the following: (a) conventional ESR to observe the return of magnetization to equilibrium – saturation recovery ESR; (b) refocusing of the magnetization in the \(xy\)-plane – spin-echo ESR; or (c) free induction decay (FID) of the magnetization in the \(xy\)-plane which is then Fourier transformed (FTESR).

The development of time domain ESR posed a formidable technical challenge. The microwave pulse must be short (a few nanoseconds) and strong enough to
cover a 70-G wide spectrum of nitroxides. The resonators must dissipate the pulse energy within tens of nanoseconds before the loss of magnetization coherence and the signal must be digitized with a subnanosecond dwell time owing to the short nitroxide relaxation times. Fortunately, technological advances in microwave sources, resonator design and data acquisition electronics in the last decade have facilitated development of commercial Fourier transform spectrometers and the time domain method has become increasingly popular. The various time domain ESR techniques are illustrated in Figure 7(a–c).

Saturation recovery electron spin resonance (SRESR) is a hybrid of continuous wave and pulse methods in which the pulse saturates a spin population at a desired field thereby creating a “hole” in the absorption spectrum (Figure 7a). The kinetics of recovery are determined by various relaxation pathways: spin–lattice relaxation, nuclear relaxation, Heisenberg spin exchange (HSE) or spectral diffusion. These competing pathways can be resolved by varying the pulse duration. SRESR has been used successfully in the determination of spin–lattice correlation times and spin exchange.

Spin-echo electron spin resonance (SEESR) uses a sequence of pulses; in Hahn echo a 90° pulse is followed by a 180° pulse $t_1$ seconds later (Figure 7b). The first pulse tips the magnetization into the $xy$-plane where individual spins rotate with their respective Larmor frequency, $\omega$. The difference in Larmor frequencies, which arises from different resonant fields, leads to dephasing of the magnetization in the $xy$-plane which is then refocused by the 180° pulse. Spins lagging by $\Delta \omega t_1$ before the refocusing pulse are now $\Delta \omega t_1$ ahead. At time $2t_1$, spins are brought into coherence and an echo is formed. In this way static differences in Larmor frequency due to different resonant fields or different local fields (inhomogeneous broadening) are annihilated. The dependence of the echo amplitude on time $t_1$ reveals Larmor frequency fluctuations that cannot be refocused by the 180° pulse. These fluctuations contain information about molecular dynamics, spin exchange and dipolar interactions. The decrease of the spin echo as a function of $t_1$ is a measure of the $T_2$ relaxation time. The decay of the echo amplitude is often recorded as a function of spectral position by stepping the magnetic field, resulting in a 2-D spectrum: an inhomogeneously broadened spectrum along the field axis and a homogeneous line shape on the $T_1$ axis. Since the inhomogeneous broadening often obscures a multitude of phenomena affecting ESR line width, then the ability to obtain a pure, homogeneously broadened spectrum is of considerable value.

2-D FTESR is the most versatile technique of time domain ESR (Figure 7c). All the spins are excited simultaneously with a strong, short microwave pulse which tips the magnetization into the $xy$-plane. The length and strength of the signal determine the spectral range covered, e.g. for nitroxides with a 200-MHz spectral range, 2-kW pulses 5 ns in duration are needed.

Coherently excited spins precess about a magnetic field at their Larmor frequency. This precession can be detected as an oscillating signal in the $xy$-plane which decays in time as the spins lose coherence. This FID signal is Fourier transformed into the frequency domain to yield an absorption spectrum. Application of two or more pulses spaced by varying intervals allows sampling of spin coherences in multiple dimensions. In the simplest of these experiments, two-pulse spin-echo correlation spectroscopy (SECSY), the first pulse tips the magnetization into the $xy$-plane where the spins become frequency labeled during the evolution time $t_1$. A second pulse reverses the magnetization during the collection time $t_2$, canceling inhomogeneous broadening. Fourier transformation with respect to $t_1$ and $t_2$ yields an absorption spectrum along the $f_2$ axis and a homogeneous line width along the $f_1$ axis. Thus SECSY is an FTESR equivalent of field-stepped SEESR. The isolation of the homogeneous line shapes out of an inhomogeneously broadened spectrum is used to study molecular dynamics.

Three pulse sequences are used in 2-D ELDOR experiments (e.g. Figure 7c). The first pulse creates a transverse magnetization in the $xy$-plane which evolves for time $t_1$. The second pulse stores this frequency encoded magnetization along the $z$-axis allowing for

---

**Figure 7** Time domain ESR methods: (a) saturation recovery; (b) spin echo; (c) 2-D FTESR (ELDOR).
Figure 8 2-D ELDOR of PD-TEMPONE. In addition to auto-peaks occurring at \( f_1 = f_2 \), there are off-diagonal cross-peaks due to HSE. (Reproduced with permission from J. Gorcester, J.H. Freed, J. Phys. Chem., 88, 4678–4693 (1986).)

magnetization transfer to take place during mixing time \( T_M \). A third pulse transforms the magnetization back into the \( xy \)-plane where it is observed during time \( t_2 \). Magnetization transfer changes the resonant frequency of a spin from \( f_a \) to \( f_b \), creating an off-diagonal cross peak at \( (f_a, f_b) \) (Figure 8). The time evolution of the amplitude of the cross peak, measured by varying \( T_M \), is used to determine the time course of magnetization transfer. The biophysically relevant phenomena causing magnetization transfer include HSE, modulation of dipolar interactions, nuclear flips and, most importantly, spectral diffusion due to rotational motion.

5 APPLICATIONS OF SPIN LABELING

The orientational difference of magnetic interactions (referred to anisotropy) forms the basis of spin labeling techniques in biological research. In the absence of motion, each field position corresponds to a defined orientation of the label with respect to the field. The intensity of the signal at a particular field position is directly proportional to the population of molecules with that given orientation. Hence an ESR spectrum can be used to determine the range of orientations present in a sample. Partially or fully averaging the \( g \)- and hyperfine tensor anisotropy results in spectral line shapes determined by the frequency and amplitude of molecular motion. ESR can also be used to measure intra- and intermolecular distances. The presence of paramagnetic centers in the vicinity of spin labels modulates spin relaxation pathways in a distance-dependent manner. In this section we shall discuss how ESR is used in the investigation of molecular orientation, molecular dynamics, ligand binding, intra- and intermolecular distance measurements and the determination of various levels of proteins structure.

For each of these applications a qualitative description of the physical principles allowing for these measurements will be given, followed by examples. More extensive reviews of these topics can be found in a monograph by Lichtenstein, a series edited by Berliner and Reuben and separate reviews by Hubbell et al., Marsh and Horvath, Millhauser et al. and, most recently, Hustedt and Beth.

5.1 Protein Orientation

5.1.1 Orientation of a Single Molecule

The anisotropy of the Zeeman and hyperfine interactions confers orientational sensitivity to ESR spectra. Nitroxide spin labels with a \( z \)-axis parallel to the magnetic field generate a spectrum with a splitting of 70 G. Spins oriented perpendicular to the field display a splitting of 14 G (Figure 9).

The center position of the spectrum, determined by the \( g \)-tensor, is sensitive not only to the position of the \( z \)-axis but also to the orientation of the \( x \)- and \( y \)-axes. At 9 GHz the center is shifted 5 G downfield (left) for a spin with its \( y \)-axis aligned with the magnetic field and another 4 G for spins with \( x \)-axis parallel to \( H_0 \) (Figure 9).

The effective \( g \)- and hyperfine splitting tensors for a spin placed at an arbitrary polar angle \( (\theta, \phi) \) with respect to the field are given by Equations (14) and (15), respectively:

\[
g(\theta, \phi) = g_{xx} \sin^2 \theta \cos^2 \phi + g_{yy} \sin^2 \theta \sin^2 \phi + g_{zz} \cos^2 \theta
\]

(14)

Figure 9 Orientational sensitivity of ESR spectra. Splitting of the spectrum changes when the \( z \)-axis of nitroxide rotates with respect to the magnetic field. The center of the spectrum changes when nitroxide rotates about any axis.
ESR LABELING IN PEPTIDE AND PROTEIN ANALYSIS

\[ A^2(\theta, \phi) = A_{\max}^2 \sin^2 \theta \cos^2 \phi + A_{\min}^2 \sin^2 \theta \sin^2 \phi + A_z^2 \cos^2 \theta \]  

It follows, then, that the resonance field for a given orientation \((\theta, \phi)\) is given by Equation (16):

\[ H_{\text{res}}(\theta, \phi, m) = \frac{h\nu}{I_g(\theta, \phi)} + m_1 A(\theta, \phi) \]  

Equation (16) describes the orientational resolution of ESR line shapes: a spin with a specific orientation can be found at a defined position along the field axis. The ESR intensity at any field position is directly proportional to the number of spins at the orientation defining \(H_{\text{res}}\). An ESR spectrum can also be considered as an orientational distribution function, \(N(\theta)\). \(N(\theta)\) is approximated by an orthonormal set of spherical harmonics and has been developed and applied to samples with cylindrical and planar symmetry.\(^{48,49}\)

Alternatively, the orientation can be modeled in terms of a Gaussian distribution with a width \(\Delta \theta\) and center \(\theta_0\) (Equation 17):

\[ \rho(\theta) = \exp \left[ -\ln 2 \frac{(\theta - \theta_0)^2}{\Delta \theta^2} \right] \]  

The ESR spectrum, \(Y(H)\), is created by calculating a resonance field \(H_{\text{res}}\) for every \(\theta\) within the Gaussian distribution of orientations and placing a Lorentzian first derivative line width at \(H_{\text{res}}\) with the intensity weighted by \(\rho(\theta)\) (Equation 18):

\[ Y(H) = \rho(\theta) \frac{(H - H_{\text{res}}) \Delta H_{pp}}{[(H - H_{\text{res}})^2 + \Delta H_{pp}^2]^2} \]  

where the peak-to-peak width of the Lorentzian \((\Delta H_{pp})\) is defined by the spin–spin relaxation time, \(T_2\) (Equation 19):

\[ \Delta H_{pp} = \frac{2}{\sqrt{3} \gamma T_2} \]  

ESR is one of the very few biophysical techniques directly sensitive to orientational disorder. The spectra in Figure 10(a–c) illustrate this sensitivity. As the width of the Gaussian distribution increases, the ESR resonances broaden to a powder pattern limit which is characteristic of isotropically disordered spins.

In summary, the parameters describing orientational distribution (axial, azimuthal angles and their disorder) can be obtained from spectral parameters: spectral splitting, center of the spectrum and the line width respectively. This can be achieved either by graphical methods or from the automated fitting of full spectral line-shape parameters. Graphical methods compare the effective splitting and width of the resonance to graphs obtained from computer simulations. The automated method allows modeling of more complex bimodal distributions, in both \(\theta\) and \(\phi\), and can be linked to standard fitting routines such as Levenberg–Marquardt\(^{50}\) or Simplex.\(^{51}\)

5.1.2 Macromolecular Assemblies

Of considerable interest is the orientation of molecules within macromolecular assemblies, e.g. proteins within lipid membranes or contractile proteins in the muscle
fibers. If the assembly of proteins is ordered and oriented at a specific angle with respect to the magnetic field, the orientational distribution of the labeled components of the assembly can be easily determined. The spectra of such samples oriented with the symmetry axis parallel to the field are the same as for a single spin (Equation 18). When the orientation of the spin label with respect to the protein is known, the ESR spectra are interpreted in terms of the orientation of the labeled domain with respect to the assembly which is of biological interest. This is achieved using Eulerian transformations between three frames; molecular frame (defining orientation of the label within the protein), sample frame (orientation of proteins within the assembly) and laboratory frame (orientation of the sample in the magnetic field) (Figure 11a and b).

The ESR spectra are simulated taking into account the orientational distribution in each of the frames. The magnetic tensors are rotated from molecular to laboratory axes using directional cosine matrices (L) according to Equation (20):

\[ \mathbf{A}_{\text{lab}} = L_{\text{mol}}^T L_{\text{sam}}^T L_{\text{lab}}^T \mathbf{A}_{\text{NO}} L_{\text{lab}} L_{\text{sam}} L_{\text{mol}} \]  

where \( L_{\text{mol}} \), \( L_{\text{sam}} \) and \( L_{\text{lab}} \) are cosine matrices defined in Equation (21) for each of the Eulerian transformations and \( L^T \) is their transpose:

\[ L = \begin{bmatrix}
\cos \beta \cos \alpha \cos \gamma & \sin \beta \sin \alpha \cos \gamma & -\sin \beta \cos \gamma \\
-\sin \alpha \sin \gamma & \cos \alpha \sin \gamma & \cos \alpha \cos \gamma \\
-\sin \alpha \cos \gamma & \cos \beta \sin \alpha \sin \gamma & \sin \beta \sin \gamma \\
\sin \beta \cos \alpha & \sin \beta \sin \alpha & \cos \beta
\end{bmatrix} \]  

(21)

The resonant field for each spin packet is calculated as shown in Equation (22):

\[ H_{\text{res}} = \frac{\hbar}{\beta g_{\text{eff}}} + m_1 \sqrt{A_{zz}^2 + A_{yz}^2 + A_{xz}^2} \]  

(22)

Note that the subscripts of the \( g \)- and hyperfine tensors in Equation (22) denote elements in the laboratory frame.

5.1.2.1 Protein Orientation in Membranes  
The Eulerian transformation approach was introduced by Griffith et al. to determine lipid orientation in membrane bilayers. The orientation of the spin label nitroxide with respect to the lipid molecule is well defined. A stack of lipid membranes is tilted with respect to the magnetic field at a known angle, and the spectra can be defined solely by the orientational distribution in the sample frame of reference (\( \Omega_{\text{sample}} \)).

Membrane-bound proteins are investigated in a similar manner. The orientation of a spin-labeled ligand of the
erythrocyte anion transporter, Band 3, was determined by flowing red blood cells into thin, flat, sample cells. The flow shear oriented the red blood cells parallel to the flow and the sample cell was rotated both parallel and perpendicular to the field in order to vary $\Omega_{lab}$.

A global analysis of the tilt series resulted in a full description of the label orientation with respect to the normal membrane axis. The derived spin label orientation was found to be consistent with orientation results determined independently by analyzing the anisotropy of motion.

5.1.2.2 Muscle Proteins

Similar approaches have been used to describe the orientation of various muscle proteins. Force generation in muscle is believed to be brought about by the reorientation of the myosin cross bridges. Since ESR is sensitive to the orientation of the proteins, muscle field proved to be a fertile ground for ESR applications. Muscle fibers form a naturally ordered assembly with the fiber axis defining the cylindrical symmetry required for ESR. Proteins can be labeled directly in muscle cells or labeled as isolated components and exchanged for corresponding unlabeled proteins in the muscle sample. These include most of the thin filament proteins—actin, troponin C (TnC), troponin I (TnI) and tropomyosin—and also the thick filament components—myosin heavy chain, regulatory light chain and essential light chain. The orientation of many of these components has been extensively studied as a function of the intermediate states of the acto-myosin cycle and also during muscle activation. Thomas and Cooke have established that in the absence of ATP, myosin heads attach themselves strongly and stereospecifically to actin. Muscle relaxation, in the presence of ATP, produced disorder consistent with head detachment and Brownian motion.$^{(56)}$ Subsequent studies using various nucleotide analogs to trap the intermediate adenosine triphosphatase (ATPase) states have revealed a sequence of orientational changes of the catalytic domain: a nonstereospecific attachment of transient, weakly bound heads followed by an equally large orientational disorder of the strongly attached heads in the prepower stroke state.$^{(75,76)}$ Force generation was associated with the disorder-to-order transition. The postpower stroke state, with the hydrolysis product adenosine diphosphate (ADP) in the active site, showed a local domain heterogeneity, but overall the catalytic domain was well oriented, $\Delta \theta \pm 8^\circ$ (Figure 12). Release of ADP (rigor state) resulted in a slight change in the twist and the tilt angle of the heads.$^{(77)}$ During isometric contraction, when most of the myosin heads should be in the prepower stroke state (immediately prior to the rate-limiting step of the cycle), no species were observed at a different angle to that of the postpower stroke heads.$^{(102)}$ These findings excluded a simple model in which the catalytic domain (accounting for most of the myosin head mass) generates a force while rotating by 45° from one well-defined angle to another.

A different story emerged when the labels were placed in the regulatory domain of the myosin head. Two distinct populations, centered 36° apart, were observed in contraction (predominantly prepower stroke heads), whereas in rigor, only one population was observed.$^{(57)}$ Clearly, the rotation of the head is limited to the regulatory domain with the catalytic domain shifting from a disordered to ordered structure.

The disorder-to-order transition was also found for other muscle proteins. For example, spin-labeled TnC is well ordered prior to Ca$^{2+}$ activation but becomes disordered in the presence of Ca$^{2+}$ or activating myosin heads.$^{(58)}$ The loss of stereospecific, protein–protein interactions is reflected by changes to the conformational homogeneity and is the basis of many molecular mechanisms. ESR, with its capacity to see directly both the homogeneity and is the basis of many molecular mechanisms. ESR, with its capacity to see directly both the order and the disordered populations, complements more popular methods such as X-ray crystallography or electron microscopy image reconstructions which ignore disorder.

5.2 Protein Dynamics

5.2.1 Sensitivity of Conventional Electron Spin Resonance

Conventional ESR is used to study molecular dynamics on the nanosecond timescale. This timescale corresponds to motions of peptides and small proteins, or the mobility of labels on the surface of large proteins. Sensitivity of the ESR signal to motion arises from rotational modulation of the magnetic tensor anisotropy. The anisotropy...
of Zeeman or hyperfine interactions results in different resonant fields for different spin orientations. Therefore, rotational motions which change the spin label orientation will modulate the ESR line shape. This can be explained by using a two-site exchange example: two spins, A and B, resonate with frequencies \( w_A \) and \( w_B \) and exchange their positions at a rate \( 1/\tau_{ex} \). When the exchange rate is significantly slower than the difference in resonant frequencies (\( 1/\tau_{ex} \ll \Delta w_{AB} \), slow exchange), the spectrum consists of resonances A and B centered at \( w_A \) and \( w_B \), respectively, and the line widths are determined by \( T_2 \) (Figure 13). The effective relaxation rate \( 1/T_{2\text{eff}} \) is the sum of the intrinsic \( 1/T_2 \) rate and the exchange rate \( 1/\tau_{ex} \). When the exchange frequency increases, the line widths start to broaden. Further increase of the exchange rate causes partial averaging of the resonance positions. The observed difference in resonant frequencies is reduced according to Equation (23):

\[
\Delta w_{AB} = \Delta w_{AB}^0 \sqrt{1 - \frac{8}{\tau_{ex}^2 \Delta w_{AB}^0}} \tag{23}
\]

For exchange rates faster than the difference in resonant frequencies (\( 1/\tau_{ex} \gg \Delta w_{AB} \), fast exchange), the two resonant peaks coalesce into one peak at the average frequency (assuming equal populations of A and B) (Figure 13). The line width is determined by the effective relaxation time, \( T_{2\text{eff}} \), with contributions from \( T_2 \) of species A and B and the exchange broadening (Equation 24):

\[
\frac{1}{T_{2\text{eff}}} = \frac{1}{T_{2A}} + \frac{1}{T_{2B}} + \Delta w_{AB}^2 \frac{\tau_{ex}}{8} \tag{24}
\]

In other words, at the fast exchange limit (\( 1/\tau_{ex} \gg \Delta w_{AB} \)), the contribution of the exchange rate to line width disappears and the spectrum consists of a single peak at \( 1/2(w_A + w_B) \) with an average line width of \( 1/T_{2\text{A}} + 1/T_{2\text{B}} \) (Figure 13).

The above considerations generally hold true for any spins exchanging between different environments such as different local magnetic fields, dipolar interactions or association with different macromolecular assemblies. Spin label reorientation with respect to the magnetic field is also a form of exchange where the rotational correlation time \( \tau_c \) is the exchange rate and the anisotropy of the g- and hyperfine interactions defines the frequency difference. At X-band, \( \Delta \nu = (A_{zz} - A_{ss})/\hbar = 500 \text{ MHz} \) or \( (g_{ex} - g_{zz})\hbar \gamma / \hbar = 185 \text{ MHz} \).

As for the two-site exchange discussed above, various motional regimes of ESR can be defined: fast (\( \tau_c \approx 10^{-11} - 10^{-9} \text{ s} \)), slow (\( \tau_c \approx 10^{-3} - 2 \times 10^{-7} \text{ s} \)) and very slow (\( \tau_c > 2 \times 10^{-7} \text{ s} \)). Fast and slow motion are of the order of \( T_2 \) (15–30 ns) for electron spin and have visible effects on conventional ESR spectra which measure the transverse component of magnetization. The very slow motions do not affect transverse magnetization, but they do affect longitudinal magnetization which decays with a correlation time \( T_1 \) (1–15\( \mu \text{s} \)). These slow motions can be detected (indirectly) using saturation transfer, pulsed ELDOR or saturation recovery ESR.

5.2.1.1 Fast Motion (\( \tau_c \approx 10^{-11} - 10^{-9} \text{ s} \)) In the fast motional regime, the motion completely averages the anisotropy of the g- and hyperfine tensors. The rotational rate is obtained from the line width broadening using Redfield’s perturbation theory. The broadening itself is a function of the nuclear quantum spin number as different nuclear manifolds have varying anisotropy values (Equation 25):

\[ \Delta H(m_l) = A + B m_l + C m_l^2 \tag{25} \]

The coefficient \( A \) is equal to homogeneous broadening and coefficients \( B \) and \( C \) assure differential broadening of lines belonging to different nuclear manifolds. These coefficients are obtained from the line widths of the Lorentzian lines according to Equations (26) and (27):

\[
B = \frac{\sqrt{3}}{4} \Delta H(0) \left\{ \frac{V(0)}{V(+1)} - \frac{V(0)}{V(-1)} \right\} \tag{26}
\]

\[
C = \frac{\sqrt{3}}{4} \Delta H(0) \left\{ \frac{V(0)}{V(+1)} + \frac{V(0)}{V(-1)} - 2 \right\} \tag{27}
\]

where \( V(m_l) \) is the peak-to-peak height of a given nuclear manifold resonance and \( \Delta H(0) \) is the peak-to-peak line width of the central line.
Factors $B$ and $C$ are equal for isotropic motion and $\tau_{ij}^{iso}$ is calculated directly from Equations (28) and (29):

$$
\tau_{B}^{iso} = -1.22 \times 10^{-9} B
$$

(28)

$$
\tau_{C}^{iso} = 1.19 \times 10^{-9} C
$$

(29)

where $B$ and $C$ are expressed in gauss and $\tau$ in seconds.\(^{(61)}\)

In the case of anisotropic motion, $B \neq C$, and the rates of rotation about the nitroxide $z$-, $x$- and $y$-axes are different. The ratio of $C$ and $B$ can be used to define the anisotropy as the coefficients are independent of the rate of motion. Various models of anisotropic motion are considered in excellent reviews by Marsh\(^{(61)}\) and Beth and Robinson.\(^{(67)}\) If the molecule is diffusing in an isotropic medium, then the rotational correlation times about the nitroxide $z$-axis ($\tau_z$) and about an axis perpendicular to $\tau(\tau_{ij})$ are given by Equations (30) and (31):

$$
\tau_{ij} = \frac{2\tau_0 \tau_{ij}}{3\tau_0 - \tau_{ij}}
$$

(30)

$$
\tau_{ij} \text{ is calculated directly from Equations (28) and (29):}
$$

$$
\tau_{ij} = \frac{2\tau_0 \tau_{ij}}{3\tau_0 - \tau_{ij}}
$$

(31)

where $\tau_0$ and $\tau_{ij}$ describe spin relaxation and are related to the anisotropy of the magnetic interactions (Equations 32 and 33):

$$
\tau_{20} = \frac{1.11 \times 10^{-7} 5(\delta A)B - 8(\delta g)HC}{H\Delta A - \Delta g\delta A - \delta g\Delta A}
$$

(32)

$$
\tau_{22} = \frac{3.69 \times 10^{-8} 8\Delta gHC - 5\Delta AB}{H\delta A - \Delta g\delta A - \delta g\Delta A}
$$

(33)

where $\Delta A$ and $\delta A$ are given by hyperfine anisotropy (Equations 34 and 35):

$$
\Delta A = A_{zz} - \frac{1}{2}(A_{xx} + A_{yy})
$$

(34)

$$
\delta A = \frac{1}{2}(A_{xx} - A_{yy})
$$

(35)

with equivalent expressions for $g$-anisotropy. The indices in Equations (34) and (35) are permuted to calculate the values of $\tau_{ij}$ and $\tau_{ij}$ for the rotation about the $x$- and $y$-axes of the nitroxide.

The above equations hold for anisotropic motion about a specific nitroxide axis in an isotropic medium. An additional complication arises when diffusion takes place within a strongly orientating potential such as in a lipid membrane, or within the steric confines of a protein. The field position of resonances now depends on the amplitude of motion, which defines the time average of available angular space; e.g. if the nitroxide can move only within an angular cone, then only the resonances corresponding to the orientations within the cone are averaged. Motionally averaged spectra are described in terms of order parameters ($S$) – time averages of the direction cosines of the diffusion axis with respect to the local director axis. For an isotropic diffusion within the cone angle $\theta_c$, components of an ordering tensor are given by Equations (36) and (37):

$$
S_{zz} = \frac{1}{2}(\cos^2 \theta_c + \cos \theta_c)
$$

(36)

$$
S_{xx} = S_{yy} = -\frac{1}{2}S_{zz}
$$

(37)

$S_{zz}$ and $S_{xx}$ are used to define the motionally averaged magnetic $g$-tensors $g_\parallel$ and $g_\perp$ in terms of its average value $g_0$ and the anisotropy $\Delta g$ and $\delta g$ (Equations 38 and 39):

$$
g_\parallel = g_0 + \frac{2}{3}\Delta gS_{zz} + \frac{2}{3}\delta g(S_{xx} - S_{yy})
$$

(38)

$$
g_\perp = g_0 - \frac{1}{3}\Delta gS_{zz} - \frac{1}{3}\delta g(S_{xx} - S_{yy})
$$

(39)

with equivalent expressions for the effective hyperfine splitting $A_\parallel$ and $A_\perp$. The latter two are resolved in the experimental line shapes; see Figure 14. Thus $S_{zz}$ of the nitroxide $z$-axis can be easily obtained from Equations (38) and (39) and the corresponding cone angle from Equation (36).

5.2.1.2 Slow Motion ($\tau_r \approx 10^{-9} \sim 2 \times 10^{-7}$ s) For $\tau_r > 2$ ns, Redfield's theory does not hold. The equation of motion for an electron spin is solved using a stochastic Liouville equation (SLE), developed by Schneider and Freed.\(^{(60)}\) Although the description of the SLE approach is beyond the scope of this article, one can rationalize the effect of slow motion on ESR spectra in terms of the two-site exchange. The low- and high-field extremes of the powder spectra correspond to nitroxides lying with the $z$-axis parallel to the magnetic field. Rotation (i.e. exchange with any other orientation) results first in an exchange broadening of the line width and then partial averaging of the anisotropy. Line width and effective splitting are

![Figure 14](image-url) Definition of the parallel and perpendicular hyperfine splitting for calculation of order parameters.
used to determine $\tau_r$ (Equations 40 and 41):

$$\tau_r = d_m \left( \frac{\Delta H_m}{\Delta H_m^{\infty} - 1} \right)^{b_m}$$  \hspace{1cm} (40)

$$\tau_r = a \left( 1 - \frac{A'_{zz}}{A_{zz}^{\infty}} \right)^{b}$$  \hspace{1cm} (41)

where $\Delta H_m$ and $A_{zz}^{\infty}$ are the line widths at half-height and hiperfine splitting, respectively, and the superscripts denote their rigid limit values. Coefficients $d_m$, $b_m$, $a$ and $b$ are calculated from SLE simulations. Their precise values depend on the motional model used for the simulations. For a Lorentzian line width $b = 3.0 \text{G}$ and isotropic Brownian diffusion, $d_m = 11.5 \text{ns}$, $b_m = -0.943$, $a_m = 21.2 \text{ns}$, $b_m = -0.778$, $a = 0.54 \text{ns}$ and $b = -1.36$. Values for different line widths or motional models can be found in Marsh.\textsuperscript{(61)}

It should be noted that the calculated $\tau_r$ values depend strongly on the chosen rigid limit values. A user-friendly simulation and optimization program based on the SLE was developed by Budil et al.\textsuperscript{(50)} Sensitivities of the conventional ESR and STESR spectra are illustrated in Figure 15(a) and (b).

**Examples.** Side-chain and polypeptide backbone dynamics are determined using the above formalism. Spin labels attached to the surface of small $\alpha$-helical peptides exhibit subnanosecond motions observed by ESR which compare well with motions predicted by molecular dynamics simulation programs.\textsuperscript{(45,62)} Scanning of the label position along a peptide length reveals a V-shaped gradient of the label mobility. The cone angle for random motion in the middle of the peptide was half the value found at either terminus. Interestingly, the C-terminus was found to be more flexible than the N-terminus, which explains the decreased stability of the C-terminus as compared with the N-terminus in $\alpha$-helices.\textsuperscript{(45,46)} Backbone dynamics observed in isolated peptides are further modulated by tertiary interactions. A survey of 30 cysteine mutants of T4 lysozyme with spin labels at various structural sites (on the surface of helices, within the helix termini, interhelical loops, buried sites and sites involved in tertiary contacts) revealed a characteristic pattern of spin label mobility in relation to the secondary structure of the protein.\textsuperscript{(31)} When the second moment of the spectrum (defined as the reciprocal

![Figure 15](image.png)

**Figure 15** Sensitivities of (a) the conventional ESR spectra and (b) STESR spectra.

![Figure 16](image.png)

**Figure 16** Reciprocal of the square of the splitting versus reciprocal of the central resonance line width. The spectral parameters cluster according to the labeled protein structural elements.\textsuperscript{(31)} [Reprinted with permission from H.S. McHaourab, M.A. Lietzow, K. Hideg, W.L. Hubbell, *Biochemistry*, **35**, 7692–7704 (1996). Copyright 1996 American Chemical Society.]
of maximum splitting squared) is plotted against the reciprocal of the central field line width ($\Delta H_{max}^{-1}$), sites in similar environments are clustered together (Figure 16). The clustering reflects the degree of motional restrictions, with the second moment related to the averaging of the hyperfine anisotropy and the central line width to the averaging of the $g$-tensor. When motional restrictions increase, the averaging decreases and both the second moment and the line width of the resonances increase. Hence the second moment and line width can be used as semiempirical diagnostic tools to evaluate the secondary and tertiary structure of a labeled site.

5.2.1.3 Very Slow Motion ($\tau_r > 10^{-9} \text{s}$) When $\tau_r > 100 \text{ ns}$, conventional ESR line shapes are no longer sensitive to motion. The rate of angular exchange is too small to affect the hyperfine or $g$-anisotropy and the line shapes become insensitive to very slow motions. To study these biologically important motions, a related ESR technique was developed, STESR.

Isotropic Motion. In the presence of power saturation, the second harmonic out-of-phase ($V_2'$) line shape resembles an absorption spectrum (Figure 6c), with the intensity reflecting the effective relaxation at that point. Since effective relaxation is related to spectral diffusion and spectral diffusion is a function of the rotational correlation time, the $V_2'$ line shape reflects rotational mobility. The rate of spectral diffusion ($\tau_{sd}$) is a function of the resonant field $H_{res}$. Some field positions are more sensitive to angular rotation than others and $\partial H_{res}/\partial \theta$ varies across the spectral line shape. For instance, the rate of spectral diffusion is zero at the turning point $H^{*} = H_{res}(\theta = 0^{\circ})$, but increases in the intermediate fields (Equation 42):

$$\tau_{sd}(H_{res})^{-1} = \left(\frac{8}{3\pi^2}\right) \left(\frac{\partial H_{res}}{\partial \theta}\right)^2 T_2^2 \tau_r^{-1}$$

(Equation 42)

To the first approximation, the change of the signal intensity ($I$) at any field position is proportional to the change of the spin–lattice relaxation time due to spectral diffusion (Equation 43):

$$I(H_{res}) = I_0(H_{res}) \frac{T_1^{\text{eff}}(H_{res})}{T_1}$$

(Equation 43)

where $I_0$ is the rigid limit intensity in absence of motion and $T_1^{\text{eff}}$ is the intrinsic $T_1$ modified by spectral diffusion according to Equation (44):

$$T_1^{\text{eff}}(H_{res}) = T_1^{\text{eff}} \frac{1}{1 + \left(I_0(H_{res})/T_2\right) T_0^r \tau_{sd}(H_{res})^{-1}} + T_1^{\text{eff}} \tau_{sd}(H_{res})^{-1}$$

(Equation 44)

Since $T_1^{\text{eff}}$ is a function of the field position (spin angle with respect to field), it is customary to define the line height at precise positions in the spectrum: $L''$, $C'$ or $H''$ at $\theta = 35^\circ$ (two-thirds of the way between resonant field corresponding to $\theta = 90^\circ$ and $\theta = 0^\circ$) and normalize it to the intensity at $H^*$ ($L$, $C$ and $H$ positions) for which spectral diffusion is zero.

By substituting Equation (44) for the effective relaxation rate in Equation (43) a semiempirical expression for the $P'/P$ ratio dependence on $\tau_r$ is obtained (Equation 45):

$$\frac{P'}{P(H_{res})} = \frac{I_0(H_{res})}{T_0(H^*)} \frac{1 + a/\tau_r}{1 + b/\tau_r}$$

(Equation 45)

The parameters $a$, $b$ and $I_0(H_{res})/I(H^*)$ can be estimated from Equations (42) and (44) by numerically evaluating sensitivity $\partial H_{res}/\partial \theta$ at each spectral position. In practice, these values are obtained from fits to the experimental curves of line-height ratios from spectra of molecules undergoing Brownian diffusion with a known $\tau_r$. Spin-labeled hemoglobin or bovine serum albumin tumbling in media of a known viscosity (water–glycerol mixtures) is used for this purpose. The rotational rate of hemoglobin (the abscissa in Figure 17) is calculated from the Stokes–Einstein equation for a sphere of radius $r$, tumbling in a medium with viscosity $\eta$ (Equation 46):

$$\tau_r = \frac{4\pi\eta r^3}{3kT}$$

(Equation 46)

![Figure 17](image-url) Dependence of $V_2'$ diagnostic ratios on the rotational correlation time. The curves are simulated with Equation (63) using the parameter values from Table 1.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>$I_0(H_{res})/I(H^*)$</th>
<th>$a$ (µs)</th>
<th>$b$ (µs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$L''/L$</td>
<td>1.88</td>
<td>6.18</td>
<td>67.9</td>
</tr>
<tr>
<td>$C'/C$</td>
<td>1.01</td>
<td>0</td>
<td>21.1</td>
</tr>
<tr>
<td>$H''/H$</td>
<td>2.17</td>
<td>21.7</td>
<td>210</td>
</tr>
</tbody>
</table>

Table 1 STESR parameters from maleimide spin label–hemoglobin calibration curves

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**Note:** The image appears to be a graph and a table, which are not transcribed here as they do not contribute to the natural text representation. The table includes parameter values for the simulation of $V_2'$ diagnostic ratios.
The parameter values listed in Table 1 were obtained for maleimide spin-labeled hemoglobin tumbling in different water–glycerol mixtures. In principle, these values should be transferable from one laboratory to another. In practice, each cavity is sufficiently different that separate calibrations are often constructed. New calibrations are also necessary for different spin labels. Changes to the magnetic tensors and relaxation times alter STESR line shapes. In rare cases, full numerical simulation of the $V g'$ line shape is used to determine the correlation time, but the computational time required is still prohibitive.$^{(69)}$

**Anisotropic Motion.** The effective rotational correlation times ($\tau_{\text{eff}}$) obtained from such calculation curves reflect rates for isotropic rotation. However, isotropic motion is not very common in biological systems. For example, the nonspherical shape of the diffusing molecules or the restoring potential of the media results in anisotropic motion. Intuitively, rotation about the long axis of a cylinder is faster than the tumbling motion around its short axis. Assigning an isotropic $\tau_i$ to an anisotropic motion is obviously in error. For elongated molecules correlation times for rotation about the major and minor axes are given by Equations (47) and (48):

$$\tau_i = \frac{f_i}{4kT(1 + f_i/2f_\perp)} \quad (47)$$

$$\tau_\perp = \frac{f_\perp}{6kT} \quad (48)$$

where $T$ is absolute temperature and the frictional coefficients $f_i$ and $f_\perp$ are a function of the shape of the molecule.$^{(64,65)}$ For a cylinder of length $2a$ and radius $b$ the frictional coefficients are given by Equations (49) and (50):$^{(66)}$

$$f_i = 8\pi\eta ab^2[0.96(1 + \delta_i)] \quad (49)$$

$$f_\perp = \frac{8\pi\eta a^3}{3[\ln(a/b) + \delta_\perp]} \quad (50)$$

where $\delta_\perp$ and $\delta_i$ are as given by Beth and Robinson (Equations 51 and 52):$^{(67)}$

$$\delta_i = 0.688 \left(\frac{b}{a}\right) - 0.202 \left(\frac{b}{a}\right)^2 \quad (51)$$

$$\delta_\perp = -0.661 + 0.891 \left(\frac{b}{a}\right) \quad (52)$$

The anisotropic diffusion tensor ($D$) creates an additional complication. The effect of the molecular rotation on the spectral line shape is a function of the label orientation with respect to the diffusion axis. If the principal axis of diffusion is parallel to the $z$-axis of the spin label, the motion interconverts the $x$- and $y$-components only. If it is parallel to the $x$-axis, then the $y$- and $z$-components will be mixed. To describe fully anisotropic diffusion of the anisotropic tensor, six parameters are needed: three diffusion coefficients about the $x$-, $y$- and $z$-axes and three Eulerian angles describing the orientation of the diffusion tensor with respect to the magnetic tensor.

The problem is simplified if either the diffusion tensor ($D$) and/or the magnetic tensors ($g$ or $A$) are axially symmetric: the elements of the diffusion tensor are related to the correlation times by $\tau_{\parallel} = 1/(6D_{\perp})$ with a corresponding expression for $\tau_i$. It has been shown that the effective correlation time obtained from the $L''/L$ and $H''/H$ line-height ratios of STESR spectra ($m_1 = \pm 1$) can be described in terms of $D_{\perp}$ and the angle $\theta$ between the diffusion and magnetic tensor axis (Equation 53):$^{(68)}$

$$\tau_{\text{eff}}(\pm 1) = \frac{1}{3[D_{\parallel}\sin^2\theta + D_{\perp}(1 + \cos^2\theta)]]} \quad (53)$$

When $\theta = 0^\circ$ the outer manifolds reflect $D_{\perp}$ which defines the $z$- and $x$-($y$-) element conversion ($D_{\parallel}$ leaves the nitroxide $z$-direction unchanged). If $\theta = 90^\circ$ the intensity of $L''$ and $H''$ is determined by $D_{\parallel}$, which now interconverts the $z$- and $x$-, $y$-axes.

In some cases, anisotropic rotation is about a single axis and the motion can be described by a uniaxial model. The mobility of transmembrane peptides or proteins in lipid membranes is a good example. A uniaxial model, with a single diffusion tensor element $D_{\parallel}$ and an angle $\theta$ defining the relative orientation of the magnetic and diffusion axes, is sufficient to simulate STESR spectra of membrane-bound proteins.$^{(69)}$ If $\theta$ is not known, then $\tau_{\text{eff}}(\pm 1)$ gives an upper estimate of $0.5\tau_{\parallel} (\theta = 90^\circ)$. It is important to realize that the changes of the $\theta$ angle, brought about by conformational changes, might result in STESR line-shape changes which can be mistakenly interpreted as changes in protein dynamics. A quick diagnostic for the presence of anisotropic motion is the comparison of the effective correlation times estimated from the $C'/C$ ratio and from $L''/L$ ($H''/H$). If they agree,
then the motion is likely to be isotropic. If they are different, then either the overlap of the nuclear manifolds is different in hemoglobin calibration spectra (unlikely) or the motion is anisotropic. If the change in the STESR spectra is to be interpreted in terms of changed motional rate and not changed anisotropy of motion, then at least the ratio of the correlation times \( \tau_{\text{eff}}(\pm 1)/\tau_{\text{eff}}(0) \) should stay constant.

Another motional model commonly encountered in biology is restricted diffusion. In such a model, the motion is isotropic but constrained in amplitude. The smaller the amplitude of motion, the slower is the apparent mobility derived from isotropic calibration. When the amplitude is \(<30^\circ\), the effective correlation time can be a factor of 10 larger than the actual \( \tau_r \) and for amplitudes >60°, \( \tau_{\text{eff}} \) approaches \( \tau_r \). For small amplitudes, there is no isotropic line shape which will match the STESR spectrum of restricted motion. The amplitude effect is not just reflected in one or two places in the spectrum, but rather it is distributed across the whole line width.

An extensive review by Beth and Robinson deals with the effects of anisotropic motion on STESR spectra and the theoretical simulations of line shapes. Numerical simulations are based on the transition rate matrix which couples neighboring angular zones with the rate of angular reorientation. The SLE approach and spin density matrix method have also been used. Both approaches have been applied successfully to isotropic and anisotropic motional models. The continuous increase in computational speed bodes well for the routine application of STESR simulations to analyze experimental data.

5.2.1.4 Examples in Muscle Proteins Microsecond motions are common for large macromolecular complexes (1 MDa) such as are present in muscle. The timescales of force generation, the actomyosin ATPase cycle and muscle activation coincide with the micro- to millisecond timescale of STESR, thereby making it the method of choice. The first application of the method established the dynamics of myosin, its subfragments and actin. Thomas et al. showed that the myosin head is capable of moving independently of the large myosin filament. Such motion was a prerequisite for force production. When bound to actin, in the rigor state (no ATP) the myosin heads were immobilized but when ATP was added the heads detached and were free to move.

Subsequent studies in muscle fibers at various intermediate states of the acto-myosin ATPase cycle have established a progressive decrease of catalytic domain mobility during the contractile cycle: the 10-µs motion of relaxed and weakly attached heads became 80 µs just before force was generated and was completely “frozen out” in the postpower stroke states of ADP and rigor. In the ADP state the head, although globally rigid, retained “breathing motions”, which were suppressed on the release of nucleotide. It is believed that this gradient in protein mobility reflects tighter and more stereospecific binding as myosin progresses through the contractile cycle (Figure 12).

The dynamics of the myosin head are complicated by the fact that this elongated protein does not behave like a rigid body. A comparison of the dynamics of the catalytic and regulatory domains revealed a three-fold difference in the rate of motion for the two domains. Moreover, the two domains were found to have dramatically different orientational distributions. These results highlight the complexity of the conformational changes in the actomyosin system: force generation is not synonymous with force transmission and both events involve changes of dynamics and orientation.

This complex behavior of myosin is in contrast to that of actin. Neither the orientation nor the dynamics of actin monomers, as probed by labels attached near the myosin binding site, were affected by head attachment. The absence of any orientational changes in contracting muscle fibers was also observed using spin-labeled toxin phalloidin bound rigidly to the interface between the actin monomers. This agrees with the current model of actin’s passive role in force production in providing “tracks” for myosin motor protein to “walk” on.

Force activation involves a complex pathway with subtle changes in protein–protein interactions. It is mediated by the conformations and dynamics of the participating molecules. Smooth muscle is activated via phosphorylation of myosin light chain 2, whereas skeletal muscle is regulated by a thin filament based system involving \( \text{Ca}^{2+} \) binding to TnC. STESR spectra of phosphorylated myosin with a probe bound to myosin light chain 1 have implied increased motional freedom of the head. This finding supports a model in which unphosphorylated heads are tied to the surface of myosin filaments and inhibited from binding to actin. Phosphorylation abolishes the electrostatic attraction to the filament surface allowing the heads to interact with actin.

In skeletal muscle, binding of \( \text{Ca}^{2+} \) to TnC initiates a signaling pathway from the thin to thick filament which ultimately activates muscle contraction. Biochemical changes in the affinity of myosin for actin and of TnC for \( \text{Ca}^{2+} \) have a structural basis that is readily observed by both STESR and conventional ESR. The mobility and orientation of TnC (and TnI) has been found to be similarly affected by the binding of myosin heads to actin or by \( \text{Ca}^{2+} \) binding to TnC. Interestingly, TnC was capable of sensing not only the binding of the myosin heads to actin but also the intermediate ATPase states.

5.2.1.5 Examples in Membranes Rotational diffusion of membrane-bound proteins is often the best way of
The biological significance of dynamic structural changes is best illustrated by the Ca-ATPase \textsuperscript{(92)} whose molecular dynamics correlate with transport activity.\textsuperscript{(93,94)} As shown by ST-EPR, allosteric interactions between Ca-ATPase polypeptide chains and catalytically important domain interactions involved in the transport cycle are regulated by both alterations in membrane lipid composition, anesthetics, and the regulatory protein phospholamban.\textsuperscript{(95–97)} Thus, physiological regulators of calcium transport modulate catalytically important motions and provide a structural basis for \(\beta\)-adrenergic stimulation in the heart.

Protein dynamics measured by STESR and conventional ESR have differentiated between two models of steroid biosynthesis in mitochondria: the shuttle mechanism and the ternary complex of adrenodoxin, P450 and adrenodoxin reductase. Adrenodoxin was found to form binary complexes (but not ternary complexes) with either P450 or adrenodoxin reductase, supporting a shuttle mechanism.\textsuperscript{(98)}

An excellent example of the potential of STESR in describing complex anisotropic motions is in the study of the transmembrane anion transporter Band 3 by Hustedt and Beth.\textsuperscript{(69)} The STESR spectra were simulated using a uniaxial model for protein rotation. The diffusion rate and the angle between the magnetic and diffusion tensor were freely floated in the least-squares fits to experimental spectra. The uniqueness of the solution was corroborated by the orientational study of Band 3 in oriented erythrocytes.\textsuperscript{(55)}

5.2.2 Mobility and Time Domain Methods

The measurement of the molecular dynamics by time-resolved ESR methods is still in its infancy. Specialized hardware is necessary to perform such experiments. Spectral diffusion due to the reorientation of spins can be observed either by recovery from saturation at the resonant field (saturation recovery ESR) or by arrival of saturation originally induced at some other nonresonant field (pulsed ELDOR). The initial promise of these methods was not fulfilled when it was shown that the nuclear relaxation, which couples different nuclear manifolds, contributed significantly to spectral diffusion. Combining pulsed ELDOR and saturation recovery differentiates between nuclear relaxation and rotational spectral diffusion and can be used to measure the true rotational correlation time.\textsuperscript{(99)}

2-D FTESR methods appear to be more promising. Nuclear relaxation is seen as cross peaks between the manifolds and can easily be distinguished from homogeneous broadening and spectral diffusion broadening.\textsuperscript{(100)} In the limits of fast motion, \(\tau_r\) is obtained directly from the homogeneous line width and is defined by the pure \(T_2\) (similar information is obtained from the spin-echo experiments). For slower motions, mixing time between the pulses is varied (2-D ELDOR) and the dependence of spectral broadening on mixing time is used to determine \(\tau_r\). Correlation times in the range 1–30\(\mu\)s have been measured for small peptides tumbling in viscous media.\textsuperscript{(101)}

5.3 Kinetic Experiments

Elucidation of molecular mechanisms involves primarily two approaches: (a) entrapment of reaction intermediates with a subsequent reconstruction of the sequence of events and (b) transient kinetics in which the reactions are synchronized with the observed spectral changes. Each of these approaches have potential problems. In the “trapping” approach, the states have to be related to the kinetic intermediates. There are cases in which states trapped with substrate or product analogs are not lying on the kinetic pathway. On the other hand, transient experiments are easier to interpret, but technically more challenging owing to lower signal levels, fast acquisition times and difficulties in spectral assignment. The two approaches should be considered complementary. In an ideal world, “trapping” approaches should be used to identify and assign signals collected during transient experiments.

Historically, optical spectroscopy was used for transient kinetics owing to inherently higher sensitivity, but ESR is making substantial inroads.\textsuperscript{(102)} Recent advances in resonator design allow for millisecond resolution on microliter samples in the submillimolar concentration range.\textsuperscript{(103)} The DR developed for this purpose is capable of measuring millisecond kinetics in a single shot on 100\(\mu\)L of a 40\(\mu\)M sample with an 8-ms deadtime.\textsuperscript{(104)} The further development of this DR/stop-flow configuration allows the recording of a full spectrum within 100 ms.\textsuperscript{(105)}

Transient ESR was used to resolve the stages of channel formation in lipid membranes. Phospholipid vesicles and membrane channel colicin were mixed rapidly and the time course of the protein absorption to the membrane surface was clearly resolved from the insertion of the channel into the membrane.\textsuperscript{(106)} For colicin the process was fairly slow, with a timescale of seconds, but the formation of another channel annexin was followed on the millisecond timescale.\textsuperscript{(107)} The millisecond time resolution makes ESR a viable
alternative to optical methods in investigations of kinetic processes.

The photolysis of caged compounds, cATP and cCa$^{2+}$, to study conformational transients has been used primarily in the muscle field and in the study of Ca$^{2+}$-ATPase. A single ultraviolet (UV) pulse (10 ns in duration with an energy flux of 150 mJ cm$^{-2}$ at 351 nm) from an excimer laser is capable of liberating 0.5 mM ATP (Figure 18a and b). The magnetic field is locked into a position where the initial and final states display a large spectral difference and the intensity at that position is followed in time. In myosin, the pre- and post-ATP hydrolysis states have different mobilities at a labeled residue near the catalytic site, with correlation times of $\tau_i > 100$ ns and $\tau_i \approx 80$ ns. This mobility difference and the associated line-shape difference was utilized in measuring the rate of transition between the two states (43 s$^{-1}$) and was found to correspond to the hydrolysis rate of ATP$^{108}$ (Figure 18a and b). Similar experiments in muscle fibers, measuring both the orientation and the mobility, established that the rapid disorder of myosin heads follows nucleotide binding but precedes hydrolysis. These experiments also determined that the rate of hydrolysis is the same in fibers as in solution.$^{102}$

Transient ESR of the Ca$^{2+}$-ATPase following cATP photolysis revealed local domain changes around the labeled site which correlate well with the formation of the phosphoenzyme intermediate.$^{109}$ A larger and more motionally restricted label was also used to observe global changes, e.g. shape or oligomerization state. No such changes were observed by transient STESR during the ATPase cycle.$^{110}$

Biological photocycles encountered in rhodopsin and bacteriorhodopsin are special cases of cycles that are easily synchronized. The photoisomerization of retinal in bacteriorhodopsin initiates a series of proton transfer reactions via short-lived intermediates culminating in the loss of $H^+$ at the extracellular surface. Some 50 µs after photoactivation, an intermediate M decays to N when Asp96 transfers a proton to the Schiff base. During the decay of the N state, Asp96 regains a proton from the cytoplasmic site and bacteriorhodopsin reverts to the ground state, thereby completing the cycle. Labels have been attached to a number of cytoplasmic, interhelical and extracellular loops in the vicinity of Asp96 and their mobility was followed after irradiation with light.$^{111–113}$ Cytoplasmic sites and those near Asp96 all showed significant changes which coincided with the decay of the M state and recovered with the decay of the N state.$^{112}$ The efforts of Hubbell’s and Steinhoff’s groups to describe the molecular mechanism of rhodopsin and bacteriorhodopsin are an excellent example of the power of ESR methods to evaluate both static and transient molecular structures. In summary, the combination of high sensitivity, short mixing deadtimes, and temporal resolution makes ESR an increasingly popular method to study transient kinetics.

5.4 Protein Folding

In the last few years, site-specific spin labeling has been applied to protein folding problems.$^{114,115}$ The advantage of the ESR approach to protein folding lies in site specificity as the denaturation of local domains can be followed independently of global denaturation. This approach relies on differences in the mobility of spin labels in folded and denatured proteins. The folded protein provides steric restrictions due to secondary structure and tertiary contacts whereas the denatured one does not. The ESR spectra for the denatured fraction are a composite of sharp, motionally averaged line shapes in contrast to broader, immobilized spectra observed for the folded protein. Fractions of protein in each form are easily calculated by spectral subtractions and by line shape integrations.$^{115}$ Cooperativity and stability of the given region are determined from spectral titration with a denaturing agent, e.g. GdnCl, urea or heat. Differences in the melting of hydrophobic and aqueous surfaces of the β-strand pore of FepA receptor were observed by cysteine scanning of the polypeptide chain lining the channel. The hydrophilic surface was more stable and cooperative in the transmembrane portion of the strand than the
extramembraneous strand ends. The residues exposed to the lipid exhibited noncooperative melting and did not denature completely even at the highest concentrations of denaturants.\(^\text{(116)}\)

ESR is also capable of sensing multiphase folding intermediates. Carbonic anhydrase was found to denature via an intermediate characterized by a compact and stable molecular core with a more dynamic periphery.\(^\text{(117)}\) In the presence of the chaperonin GroEL, the intermediate core was destabilized and partially melted, explaining how GroEL allows for the refolding of misfolded proteins.\(^\text{(118)}\) The development of new resonators, as mentioned previously, has facilitated detailed analysis of folding kinetics. The initial phases (<20 ms), ascribed to helix formation, were recently resolved by stop-flow ESR.\(^\text{(104)}\)

5.5 Ligand Binding

Two less well known applications of spin labeling are the determination of the binding of small ligands and the aggregation of large (the latter reviewed in section 5.7.4). The binding of small ligands is followed by changes in their mobility. Spin label analogs of ligands have sharp, motionally narrowed spectra when free in solution. Binding to a larger target slows the motion of the label and the spectra become broadened. Spectral resolution between the broad/bound species and the narrow/free ligands allows quantification of bound species and hence calculation of binding isotherms. The binding of mellitin to \(\alpha\)-crystallin\(^\text{(119)}\) and the binding of nucleotide analogs to ATPases were determined by this method. Binding studies are not limited to ligands carrying a spin label. Competition of unlabeled and labeled analogs is used to determine the \(K_d\) of unlabeled ligands. The binding of ATP, ADP, adenosine triphosphate (ATP), adenosine imidotriphosphate (AMPNNP) and adenosine methylenetriphosphate (AMPPCP) to myosin was determined by the displacement of spin-labeled ATP.\(^\text{(120)}\)

A good example of ESR applications in ligand binding is the association of lipid spin labels with intrinsic membrane proteins. The differences in mobility are small and the spectra of bound and free labels are not resolved. Small broadening due to exchange between free and restricted environment was simulated to extract the equilibrium constant and the number of binding sites.\(^\text{(61)}\)

Finally, the binding of metals to proteins can be established by ESR. Mn(II) has a characteristic six-line spectrum when free in solution, but no signal when bound to protein. The decrease of free Mn(II) signal upon addition of a protein identifies the fraction of bound metal.\(^\text{(121)}\) As shown in the example with ATP analogs, displacement of bound Mn(II) by other metals can be used to determine their binding affinity.

5.6 Distance Measurements

ESR is capable of measuring short distances (2–25 Å) between selected sites. The method relies on the distance-dependent interactions between two spin labels (spin label–spin label method), or an interaction between a spin label and a paramagnetic metal (spin label–spin probe method). The physical basis for the coupling between the two spins is the exchange interaction \(J\) arising from the overlap of the orbitals of unpaired electrons and the dipolar interaction between magnetic moments of the two spin labels. The spin label–spin probe method relies on the enhancement of the relaxation of the spin labels by paramagnetic metals which have considerably faster relaxation rates and provide an efficient relaxation pathway for nitroxides.

5.6.1 Spin Label–Spin Label Method

5.6.1.1 Exchange Interaction, Distances <8 Å

At distances shorter than 8 Å, the \(\sigma\)- or \(\pi\)-orbitals of the neighboring unpaired electrons can overlap, creating a single spin state: singlet (spins are antiparallel) or triplet (spins are parallel) state. Unlike nuclear spin, electron spin coupling propagates both through space and through covalent bonds. Through-bond coupling drops off very quickly and is insignificant at a distance greater than a few bonds. The through-space coupling strength \(J\) is a function of the interspin distance \(r_{\text{dd}}\) (Equation 54). \(J\) diminishes exponentially, with a 1-Å interaction distance from the initial value of \(J_0 = 300\) G.

In the strong exchange regime, when \(J\) is much larger than hyperfine interactions, the combined nuclear spin is 2 and the spectrum displays a five-line pattern. For weak coupling \((J < A_0)\), the interaction asymmetrically broadens the low- and high-field resonances. The shift of the downfield edge of the low-field resonance in the presence of the second label \(\Delta H_{\text{dd}}\) is proportional to \(J\) and hence to the interspin distance.

\[
\Delta H_{\text{dd}} \approx J = J_0 \exp(-\beta r_{\text{dd}})
\]  

For example, a 0.1-Gauss broadening implies an interspin distance of ~8 Å, defining the upper limit of the sensitivity.

Spin exchange in peptides labeled with two probes has been used to determine helical folds.\(^\text{(122)}\) Some proteins contain \(\alpha\)-helices between \(\beta\)-strands or at the end of \(\alpha\)-helices and their detection in solution is difficult. Fiori and Millhauser utilized a difference of distance between the \(i\) and \(i + 4\) residues along the \(\alpha\)- and \(3_{10}\)-helices to distinguish between the two helical forms. The \(i\) to \(i + 3\) distances are similar in both helices (6–8 Å), but the \(i\) to \(i + 4\) distance for the \(3_{10}\)-helix is outside the range of the exchange interaction (10–16 Å) and within the
range for an α-helix (7–11 Å). Placing pairs of labels in the $i$, $i+3$ and $i+4$ positions along the synthetic helices identified the predominantly α-helical regions in the N-terminus (equal broadening of $i$, $i+3$ and $i$, $i+4$ pairs) with the $3_10$-helix at the C-end (no broadening of the $i$, $i+4$ pair). A comparison of 16- and 21-residue peptides revealed a length-dependent equilibrium between the α- and $3_10$-helices. Shorter peptides favored the $3_10$-helix whereas the longer peptides favored the α-helix.

Transition between the two helical forms might provide a mechanical pathway for allosteric mechanisms. The α-helix has more residues per turn and is significantly shorter, hence the $3_10$-helix to α-helix transition will mechanically pull on the neighboring domains.

5.6.1.2 Dipolar Coupling Distances: 8–25 Å In addition to the exchange interaction, neighboring spins experience local fields induced by their respective magnetic dipoles. The local fields can add to or subtract from the external field splitting each of the resonances. This dipolar interaction has an $r^{-3}_{dd}$ dependence which results in discernible line-shape changes for distances up to 25 Å. The contribution of the exchange interaction for distances over 10 Å is negligible.

The strength of the dipolar interaction is a function of eight parameters: the interspin distance $r_{dd}$, the angle between the static magnetic field and the interspin vector and the six angles describing the orientation of each of the spin labels with respect to the interspin vector. Protein dynamics also affects these parameters on the timescale of the experiment: the global motion of the molecule modulates the angle with respect to the field, intradomain motions modulate the interspin distance and the interspin angles. The full solution of dipolar ESR line shapes in the presence of motion poses a formidable challenge.

The geometry of the NAD coenzyme bound to glyceraldehyde dehydrogenase (GAPDH) is the best example of the approaches taken to tackle this problem. Since GAPDH is a tetramer it can bind four molecules of coenzyme NAD. The relative orientation of the coenzyme was determined from the dipolar splitting of a spin label analog of NAD. Hustedt et al. used different microwave frequencies (9, 35 and 94 GHz) coupled with sophisticated fitting procedures to solve independently for most of the parameters listed above. The ESR structure was consistent with the geometry derived from molecular modeling using a crystal structure of the apo-enzyme.

Another example is the open form of α-helices in protic solvents. Alanine-rich peptides, incorporating the unnatural spin-labeled amino acid TOAC (2,2,6,6-Tetramethylpiperidine-1-oxyl-4-amino-4-carboxylic acid), showed a strong dipolar interaction for the $i$, $i+4$ labeled peptides with less interaction between the $i$, $i+3$ sites. Molecular modeling revealed a shorter interspin distance and larger backbone torsional angles, consistent with 3.8–3.9 residues per turn compared with 3.6 residues for a standard α-helix. The open configuration allows for the formation of additional hydrogen bonds with amide carbonyls while preserving helical hydrogen bonds. ESR proves to be an important tool in identifying subtle changes in helical folds induced by the local environment.

Short of solving the general case as above, two simpler cases are often encountered: (1) the static dipolar case in which all motions are frozen out, the labels are disordered on the surface of the protein and the protein itself is isotropically disordered in the magnetic field, and (2) the motionally averaged case in which the anisotropy of the dipolar interactions is averaged ($\tau_r < 6$ ns) and the spectrum is homogeneously broadened by spin–spin interactions.

In the first case, the nitroxides and the sample are rigid and each of the resonance peaks are broadened by the Pake function given in Equation (55):

$$\Delta H_{dd} = \pm \frac{3g\beta(3\cos^2\theta - 1)}{4r^3}$$

where $\theta$ is the angle of the interspin vector in the magnetic field.

Convolution of the resonances with the Pake pattern yields a characteristic line shape with dipolar wings in the low- and high-field regions of the spectra. The Pake function is obtained from the spectrum by a Fourier transform or by empirical calibrations. Empirical calibrations relate the line heights of the low- and high-field resonances to the line height of the central peak (Equation 56):

$$r_{dd} = 9.3 + \frac{0.77}{(d_1/d) + 0.36A_{zz} - 1.76}$$

where $r_{dd}$ is in ångstroms and $A_{zz}$ is in gauss, $d$ is the line height of central resonance, and $d_1$ is the difference between low-field peak and high-field trough.

Alternatively, the Van Vleck relation between the second moment of the central resonance $M_2$ and $r_{dd}$ is used in obtaining the distance (Equation 57):

$$M_2 = \frac{3\gamma^2\beta^2}{20r_{dd}^6}$$

where $r_{dd}$ is in ångstroms. The second moment is given by the Gaussian part of the peak-to-peak line width (Equation 58):

$$M_2 = \left(\frac{\Delta H_{pp}^G}{2}\right)^2$$
The Pake broadening approach was verified for interspin distances of doubly labeled rigid synthetic polypeptides in which distances in the range 8–24 Å were predicted from the structure. The method was also verified for larger molecules by comparison with an X-ray determined structure of spin-labeled insulin. The interspin distances in a crystal were in excellent agreement with those found in solution by ESR. Distribution of distances due to protein flexibility in solution was found to be twice as large in solution as in the crystal. It is important to note that ESR is one of the very few techniques which can estimate conformational heterogeneity of a protein in solution.

Static dipolar broadening is rapidly gaining popularity. It was used in the analysis of helix packing of lactose permease in the membrane. Spin labels placed on surfaces which faced each other displayed dipolar broadening. Analysis of the extent of broadening as the label is moved around the helix identified the relative rotation of neighboring helices. Dipolar broadening was also used in the elucidation of the opening/closure of K-channels as described in detail in section 5.7.4.

The static dipolar approach (case 1) fails when the proteins are not rigid on the nanosecond timescale of an ESR experiment. If the spin mobility is high enough to modulate the anisotropy of dipolar interactions, the rotational modulation of the interspin vector results in the broadening related to both the distance and the correlation time (Equation 59):

\[
\tau_r \leq \left( \frac{g_b \Delta H_{dd}}{h} \right)^{-1} = \left( \frac{3\pi g^2 h^2}{r_{dd}^3 h} \right)^{-1}
\]  

(59)

the rotational modulation of the interspin vector results in the broadening related to both the distance and the correlation time (Equation 60):

\[
\Delta H_{dd} = \frac{3}{10} \frac{\nu^4}{g_b^2 h^4} \frac{\tau_r}{r_{dd}^3} \left( 3 + \frac{5}{1 + \nu^2 \tau_r^2} + \frac{2}{1 + 4\nu^2 \tau_r^2} \right)
\]  

(60)

Line-shape comparisons of singly and doubly labeled samples reveal the extent of dipolar broadening from which the \(r_{dd}\) distance is calculated assuming (or measuring) an appropriate correlation time for the molecule. McHaourab et al. tested this approach on a series of labeled sites in T4 lysozyme (8 Å < \(r_{dd}\) < 23 Å) and found them to be in excellent agreement with the structure of the protein (Figure 19a and b). Note that the motional modulation regime is a function of both the interspin distance and the rotational correlation time, e.g. dipolar interactions of labels 15 Å apart are averaged for motions with correlation times of \(\leq 6\) ns.

The above examples illustrate the static and motionally averaged cases of spin–spin interactions. However, often the precise mechanism of spin–spin interactions is not known as there are contributions from static dipolar interactions, from the rotational modulation of the interspin vector and from the modulation of the interspin distance. Although there is currently no theory fully describing the changes in the ESR line shape, the presence of line-width broadening is always indicative of two labels being in the range 10–20 Å from each other. Even the simplest, qualitative statement of spin–spin distance can yield important structural information. The presence of spin–spin interactions has helped to elucidate changes in the topology of the cytoplasmic portion of rhodopsin following light activation. It has explained how rhodopsin initiates the phosphorylation cascade by rhodopsin kinase.

Interested readers are directed to an excellent review by Husted and Beth.

5.6.1.3 Very Long Distances: >25 Å The upper limit of 25 Å for the detection of dipolar interactions is defined by an observable broadening: ~3 G for inhomogenously
broadened samples of rigid samples and 0.2 G for the sharp, motionally narrowed line shapes. This limit can be extended by time-resolved techniques, which unlike the continuous-wave methods do not rely on line broadening. A variety of pulsed ESR methods have been developed for this purpose. Double electron–electron resonance (DEER) is one method in which the spin echo is observed as a function of time of an intervening pulse applied at a second frequency, \(^{131,132}\) or at the same frequency as in “2 + 1” resonance. \(^{133}\) In both cases, an amplitude of the spin echo is modulated by Pake dipolar interactions which are extracted by Fourier transformation of the echo modulation. In model compounds, the distances recovered by DEER ranged between 20 and 33 Å, \(^{132}\) whereas the “2 + 1” scheme elucidated an interspin distance of 35 Å between nitroxides attached to β-93 cysteine in tetrameric hemoglobin. \(^{133}\) Double quantum 2-D FTESR is the most recent technique that looks directly at spin–spin interactions i.e. the filtering out of a “normal” ESR spectrum originating from isolated spins. This method has been tested on solid polyproline peptides with an interspin distance of 18 Å. \(^{101}\)

5.6.2 Spin Label–Paramagnetic Metal Method

An entirely different way of determining distances is by coupling an unpaired spin of a nitroxide radical to a fast-relaxing spin of a paramagnetic metal, e.g. Cu(II) or Fe(III). The spin–lattice relaxation times of many metals are orders of magnitude faster than those of nitroxide spin labels and thus, when coupled to nitroxides, they provide an efficient relaxation path. The relaxation enhancement can be detected by either increased Lorentzian broadening or directly by the shortening of \(T_1\) or \(T_2\) of the spin label in the presence of the metal (Equation 61):

\[
\Delta \left( \frac{1}{T_{1s}} \right) = \frac{\mu^2 r^2}{6\pi T_{1f}} \left[ \frac{4}{5(w_1 - w_2)^2} + \frac{24}{5(w_1 + w_2)^2} + \frac{12}{5w_1^2} \right]
\]

(61)

All three methods have been tested in spin labeled hemoglobin in which a nitroxide at residue 93 of the β-chain was coupled to Fe(III) bound to the heme. The distance of 15 Å between the label and iron was in excellent agreement with molecular modeling. \(^{134}\)

The metal relaxation is not limited to naturally occurring metal binding sites. Site-specific spin labeling has recently been extended to the engineering of metal binding sites.

Voss et al. engineered Cu(II) binding sites to T4 lysozyme and lactose permease by introducing histidine residues in consecutive turns of the α-helix. \(^{135,136}\) Spin labels were placed between 8 and 18 Å away from the metal site by cysteine mutagenesis. The ESR line shapes were broadened by paramagnetic Cu(II) chelated by the histidine residues. Binding of diamagnetic metals did not affect the line shape. The spectra were analyzed in terms of the dipolar model of Leigh, \(^{137}\) which relates line-shape broadening to the interspin distances \(r_{dd}\) and metal relaxation time \(T_{1m}\) (Equation 62):

\[
\Delta H_{dd} = \frac{9\mu^2 T_{1m}}{hr^6}(1 - 3\cos^2 \theta)^2
\]

(62)

where \(\mu\) is the magnetic moment of the metal.

A convenient experimental parameter is the amplitude of the central line. The amplitude decreases with decreasing spin–spin separation. Calibration curves derived from computer simulations were used to estimate the interspin distances in two model systems, lactose permease and T4 lysozyme. Excellent agreement was found, not only for the rigid samples for which Leigh’s model was originally developed, but also for motionally narrowed spectra due to the mobility of the nitroxides with respect to the protein. For Leigh’s model to hold, the interspin vector must not move on the same timescale as dipolar interactions, which is \(T_1\) of the metal. This is satisfied for molecules larger than 15 kDa (\(\tau_r > 6\) ns) and for metals such as Cu(II) where \(T_1\) is 1–3 ns. The distances obtained for T4 lysozyme in solution at room temperature were approximately 1 Å shorter than those obtained from frozen proteins. \(^{136}\) This small underestimation of the distance is compensated by the biological advantage of performing experiments at room temperature and by the increased fidelity in measurements of small-amplitude changes in sharper, motionally narrowed spectra. Another elegant application of this method involved the determination of helix packing in lactose permease. Interspin distances between three helices labeled with nitroxide labels and a metal site containing Cu(II) determined the relative orientation of the helices and their relative tilt. \(^{138}\)

Currently this method is limited to distances between 10 and 20 Å for Cu(II) with the X-band. Inspection of Leigh’s Equation (61) suggests that the distance range might be extended to ~50 Å by using lower ESR frequencies (S-band), metals with a larger magnetic moment (Gd\(^{3+}\)) or shorter \(T_1\) (Ni\(^2+\)) and also by direct measurement of relaxation times. \(^{139}\)

5.6.3 Collision Exchange

A variation of spin–spin interactions is the relaxation of spin labels by collisions with soluble paramagnetic agents such as metals or O\(_2\). Collisions lead to the HSE, enhancing spin–lattice relaxation according to Equation (63):

\[
\Delta T_1^{-1} = k W_r
\]

(63)
where \( k \) is a factor accounting for the efficiency of collisions and statistics of diffusion in two or three dimensions and \( W_\varepsilon \) is the bimolecular collision frequency. The collision frequency is of interest because it reflects the accessibility of the labeled site to the relaxant. Comparison of \( W_\varepsilon \) for various sites reveals which residues are exposed or hidden and their secondary structure content and identifies tertiary interactions.

Relaxation enhancement is measured directly by pulse methods (saturation recovery or spin echo ESR) or by continuous-wave power saturation. The amplitude of the ESR signal increases linearly with the microwave magnetic field \( (H_1 \propto P^{1/2}) \) until the Boltzmann equilibrium population difference is perturbed and the signal between excited and ground states decreases. Samples with a long \( T_1 \) saturate easily and addition of relaxing agents relieves this saturation (Figure 20).

The peak-to-peak amplitude \( (A) \) of the first-derivative spectrum is given by Equation (64): \(^{(140)}\)

\[
A = \frac{A_0 \sqrt{P}}{1 + (\sqrt{2} - 1)(P/P_{1/2})^2} \tag{64}
\]

where \( \varepsilon \) depends on the resonance line shape and varies between 0.5 for purely Lorentzian and 1.5 for Gaussian line shapes; \( A_0 \) is an instrument scaling factor and \( P_{1/2} \) is the half-saturation power (the power at which the signal is half of what it would be in the absence of saturation). \( P_{1/2} \) is determined either graphically or by the fitting of experimental curves to Equation (64). The \( P_{1/2} \) value is then used in calculating \( T_1 \) according to Equation (65):

\[
T_1 = \frac{2^{2/3} - 1}{\sqrt{2} \Lambda^2 P_{1/2} / T_2} \tag{65}
\]

where \( \Lambda \) is an instrumental factor which depends on the power-to-magnetic field conversion of the resonator. Since \( T_2 \) for nitroxides is \( 2-3 \) orders of magnitude smaller than \( T_1 \) and because \( T_2 \) is proportional to the peak-to-peak line width of the central line \( (T_2 \propto 1/\Delta H_0) \), the collision frequency is determined from Equations (63) and (65) (Equation 66):

\[
W_\varepsilon \propto \frac{\Delta P_{1/2} \Delta H_0^{DPPH}}{\Delta H_0 P_{1/2}^{DPPH}} \tag{66}
\]

In order to account for differences in resonators and spectrometers between various laboratories, a dimensionless accessibility parameter \( \pi \) was defined; \( \pi \) normalizes \( W_\varepsilon \) to the half-saturation power and line width of a diphenylpicrylhydrazyl (DPPH) standard. \(^{(141)}\) (Equation 67):

\[
\pi = \frac{\Delta P_{1/2} \Delta H_0^{DPPH}}{P_{1/2}^{DPPH}} \tag{67}
\]

Trends in accessibility to various relaxing agents are used to determine the local environment of spin labels. The relaxants can be nonpolar such as \( O_2 \) partitioning into lipid bilayers, or polar with preference for the aqueous phase. The latter includes neutral relaxants such as \( \text{NiAA} [\text{nickel(II) acetylacetone}] \) and \( \text{NiEDDA} [\text{nickel(II) ethylenediaminediacetate}] \) and charged relaxants such as \( \text{CROX} [\text{potassium tris(oxalatochromate)}] \). \(^{(142)}\)

An important application of collisional relaxation is the determination of the secondary structure of peptides and proteins. Patterns of collisional accessibility along the polypeptide chain can reveal \( \alpha \)-helical folds, \( \beta \)-sheet strands, immersion in membranes and chain tilt within the membranes. These applications are described at length in section 5.7.2.

### 5.7 Structural Biology

The advent of site-specific spin labeling established ESR as a structural technique. \(^{(41-43,46,145)}\) In the first study, \(^{(30)}\) comparison of the relaxations enhancement of four labeled cysteine mutants of bacteriorhodopsin identified membrane embedded and surface exposed residues. Since then, these approaches have been refined and extended to establish (a) the topology of membrane bound proteins, (b) the secondary structure of proteins by cysteine scanning and following trends in accessibility and mobility of residues and (c) the tertiary folding of proteins by distance measurements between engineered sites. Most of the examples discussed below are from work of Hubbell et al.
5.7.1 Side-chain Environment – Immersion in Membranes

Differential effects of nonpolar (O₂) and polar (NiAA, CROX) relaxants are used to measure the immersion depth of membrane proteins. This technique relies on opposite concentration gradients for polar and nonpolar relaxants within lipid membranes. The concentration of nonpolar reagents increases with the immersion depth and the concentration of polar reagents decreases. The further the nitroxide is from the aqueous interface, the stronger is the relaxation enhancement by nonpolar reagents and the weaker is the effect of polar relaxants. The difference (Φ) between the polar and nonpolar reagents as defined in Equation (68) is thus a function of the immersion depth:

\[ \Phi = \ln \frac{\Delta P_{\text{nonpolar}}^{1/2}}{\Delta P_{\text{polar}}^{1/2}} \]  

(68)

Calibration curves of Φ are constructed using lipid spin labels with nitroxides at defined positions along the acyl chains and these curves are used to determine the immersion depth of labels attached to membrane-bound proteins (Figure 21a and b).

This relatively simple method, when used in conjunction with cysteine scanning, differentiates between membrane-bound and solvent-accessible surfaces of membrane-associated proteins or peptides. As the nitroxide is moved along the length of a polypeptide chain, ΔP₁/₂ for the polar agent shows minima and maxima for the residues interacting with the lipid bilayer and the surface-exposed residues, respectively. The nonpolar agent has a similar pattern of minima and maxima, but it is offset by 180° with respect to the polar relaxant, i.e. maximum relaxation will be observed for the residues interacting with the membrane and minimum relaxation for water-exposed residues. A similar phase shift is observed for the residues of helices lining the aqueous pores of channels. Residues facing the lumen of the pore show maximum relaxation enhancement for polar agents (minima for the nonpolar oxygen), while the residues facing the membrane environment have maxima for oxygen and minima for CROX, NiAA and NiEDDA.

Such is the case for the ferric enterobactin receptor FepA, the transmembrane β-strand of which was found to line an aqueous channel. The maxima of accessibility to NiEDDA was alternating with maxima to O₂, identifying the β-strand face as lining the channel and the side of the β-strand facing the lipid bilayer. Similar results have identified residues lining the aqueous channels in collicin, diphtheria toxin and annexin.

5.7.2 Secondary Structure Determination

Cysteine scanning also allows for secondary structure determination. One method, based on the exchange interactions between nitroxides attached to the i, i + 3 and i, i + 4 residues, identifies α- and 3₁₀-helices and was discussed in section 5.5.1.1. Other methods rely on changes in nitroxide mobility and accessibility to relaxing agents. The periodicity of steric interactions varies along the polypeptide chain, which in turn determines the nitroxide mobility and/or periodicity of relaxation effects. For example, an α-helix in an unevenly...
solvated environment (owing to the interaction with a membrane surface or another polypeptide chain) shows a pattern of flexibility and solvent accessibility with a 3.6-residue periodicity. β-Strands, on the other hand, will display a two-residue periodicity (Figure 22a and b).

This characteristic periodicity of 3.6 residues was observed for a number of helices of transmembrane proteins: rhodopsin, collcin, K-channel and the soluble protein T4 lysozyme. Periodicity of β-strands was observed for the transmembrane protein FepA receptor and water-soluble α-crystallin.

In some cases ESR has extended the structural information obtained by other methods, for example interhelical loops in rhodopsin. However, in other cases, the secondary structure determined by ESR was the only available source for example FepA receptor and α-crystallin.

An interesting use of SDSL ESR is to extend monomeric (subunit) structures determined by NMR and X-ray crystallography to the structures of functioning macromolecular complexes. The monomeric structure of the soluble (nonfunctional) form of the membrane pore annexin has been solved by X-ray crystallography. A mobility and accessibility profile of 26 single cysteine mutants in the helix–loop–helix motif has revealed a dramatic structural transition when annexin is inserted into the membrane to form a continuous, transmembrane α-helix. As was expected for a membrane pore made of the annexin trimer, one side of the helix was found to be highly solvated.

5.7.3 Tertiary Structure: Conformational Changes

The greatest potential for the above methodologies is the determination of tertiary structure. The current rate of structure determination by X-ray crystallography or NMR (~1000 per year) is too slow to solve for all 120,000 gene products. Fortunately, most proteins are built from well defined, common structural motifs, but are packed in different ways to give proteins their unique three-dimensional structure. It seems that instead of solving ab initio the atomic structure of each protein it will be simpler to determine the relative arrangement of common structural motifs. For instance,
a few chosen mutations can quickly establish whether given helices or β-strands are in a parallel or antiparallel arrangement.\textsuperscript{143,153}

Qualitative information about tertiary structure is obtained from mobility and solvent accessibility values, both of which are limited to sites of tertiary contacts, e.g. $\pi$ ($O_2$) values are 0–0.05 for buried sites and 0.3 for solvent-accessible surface sites.\textsuperscript{119} Additionally, in-phase tracking of accessibility to polar and nonpolar reagents and tracking of mobility patterns identifies surface residues and residues buried within a protein core. The surfaces involved in helix packing in rhodopsin\textsuperscript{147,148} and collicin\textsuperscript{144} and β-strand packing in α-crystallin\textsuperscript{151} have been identified by this in-phase behavior.

The tilt angle of polypeptide chains within lipid membranes is easily determined from the immersion depth of selected residues (see section 5.7.1). The immersion depth is calculated from the $\Phi$ parameter (ratio of nonpolar to polar accessibility), which is calibrated in terms of the distance from the membrane surface of lipid spin labels at defined positions. The average depth ($d$) of consecutive residues is compared with the distance along the chain ($d_0$). The tilt of the chain with respect to the bilayer normal is given by Equation (69):

$$\alpha = \cos^{-1}\left(\frac{d}{d_0}\right) \quad (69)$$

For β-barrels, the tilt information, combined with number of β-strands, can be used to estimate the diameter of the barrel.\textsuperscript{144}

Most of the examples identifying conformational changes are from membrane-bound proteins. In rhodopsin, helices flanking the ionone ring of retinal have been labeled with nitroxides and the interspin distance tracked upon photoisomerization of the retinal.\textsuperscript{130,154} The observed rigid body rotation, with an associated change of the tilt angle in one of the flanking helices, resulted in increased accessibility of the cytoplasmic loop. Increased exposure of the loop facilitates binding of transducin to rhodopsin, which is the first step in the phosphorylation cascade of signal transduction pathway.

Conformational changes accompanying insertion into a membrane and pore formation were observed by ESR for the small cytosolic protein annexin. A water-soluble monomer with a helix–loop–helix motif was rebuilt to form a continuous transmembrane helix in the presence of $Ca^{2+}$. The formation of long helix induced membrane insertion of annexin.\textsuperscript{107} In another example, smaller conformational changes were observed by varying the lipid environment of transmembrane proteins. Reconstitution of lactose permease into proteoliposomes induced a small 2-Å movement of neighboring helices.\textsuperscript{127}

A particularly rewarding example is that of conformational changes in T4 lysozyme: two structures solved by X-ray crystallography implied a hinge movement which opened the active site by 8 Å. Using strategically placed cysteines near the active site, this predicted opening of the active site was verified in solution.\textsuperscript{129} In addition to corroborating the presence of the two conformers, ESR was able to measure an equilibrium of closed and open structures, yielding a unique estimate of activation energy associated with catalysis.

The most spectacular application of ESR to the tertiary/quaternary structure of proteins was that of the bacterial potassium channel by Perozo.\textsuperscript{32,128} Nearly a third of the entire protein including two transmembrane helices and the interhelical region flanking a selectivity filter have been scanned with spin labels. This is a total of 62 mutants for the 160 amino acid polypeptide chain. The channel is formed by the tetrameric assembly of the two helices, with one helix (TM2) forming an aqueous pore and the other helix (TM1) located on the periphery. The structure has been solved independently by X-ray crystallography\textsuperscript{156} and by ESR from accessibility and mobility profiles.\textsuperscript{32} The ESR protein structure determination was further extended to the structural description of the channel opening. The channel is activated by lowering the pH. Sequence profiles of mobility and interspin distances were compared for the open and closed forms, revealing a physical opening of the central pore. The open form was brought upon by a rigid body rotation and tilting of the TM2 helices with an accompanying movement of the peripheral TM1 helices.\textsuperscript{128}

### 5.7.4 Assembly of Polypeptide Chains: Quaternary Structure

Differences in mobility between monomers and oligomers can be used to identify the oligomerization state of proteins. For small proteins, ESR line shapes are motionally narrowed, whereas the spectra of aggregates are considerably broader. Spectral resolution of monomeric and oligomeric forms in a composite spectrum allows for the determination of their respective concentrations in solution and hence thermodynamic parameters of oligomer formation. Oligomerization in a variety of solvents of cecropin AD, a small ion channel, was studied in this way.\textsuperscript{157}

The kinetics of the formation of amyloid plaques were monitored by the disappearance of the sharp central line of the spin-labeled amyloid protein monomer.\textsuperscript{158} Monomers were found to aggregate initially into an amorphous plaque precursor in which the protein was in equilibrium between soluble monomers and the aggregated protein. The precursor was an initiation site for fibril formation of which the amyloid
plaques are subsequently formed. Characterization of various assemblies and the equilibria between monomers and aggregates are of direct interest in understanding the molecular basis for diseases such as Creutzfeldt–Jacob (‘‘mad cow disease’’) and Alzheimer’s disease.

An alternative way of following the formation of aggregates is to utilize spin–spin interactions. Interacting monomers lead to a broadening of the ESR spectra, provided that the labels are within the range of spin exchange or dipolar interactions. Spin-labeled insulin B chain was found to aggregate on reduction of the interchain disulfide bonds, but the presence of α-crystallin was found to prevent aggregation. In the absence of crystallin, the spectra of B insulin displayed a broad Lorentzian pattern, characteristic of closely placed spins. This turned into a normal powder pattern upon binding to crystallin.¹¹⁹

Titration of spin–spin interactions with unlabeled proteins can be used to estimate the number of monomers forming an oligomer. As the concentration of unlabeled monomers increases, the probability of spin–spin interactions decreases and dipolar broadening is relieved leading to an increase in signal amplitude. For small oligomers, dilution with small amounts of unlabeled protein results in a greater increase of signal amplitude than for larger oligomers. The titration follows a binomial expansion and has been used to establish that the membrane-bound form of annexin is a trimer.¹⁰⁷

This qualitative approach has been used for annexin pores. While the crystal structure of the soluble form of annexin suggested a hexameric assembly, nothing was known about annexin in membranes. Among the possibilities were a trimeric ring and a hexamer consisting of stacked trimers. To distinguish between these alternatives, cysteines were introduced at the interface between the monomers forming a trimer and on the interface between the trimers forming a putative hexamer. In the soluble form, no dipolar interactions between any of the sites were observed, consistent with the monomeric form in solution. Addition of Ca²⁺, which triggers membrane binding, resulted in the broadening of the spectra within the trimer but not between the trimers, proving that a trimer and not a hexamer was forming the pore.¹¹⁷

6 CONCLUSION

ESR of protein is currently enjoying a renaissance of sorts. In addition to its contributions in studies of protein dynamics and orientation, ESR is being increasingly used as a structural technique. The advances of molecular biology facilitate targeting of chosen domains or scanning of the whole structure with spin labels. Comparison of dynamics, accessibility and distances at consecutive positions along the polypeptide chain is used in the determination of the secondary, tertiary and quaternary structure of proteins, which is of enormous importance in the post-genomic era. It is likely that the ease of determination of relative orientation of known domain motifs will make ESR a method of choice in high-throughput structural biology.

Technical advances in ESR, which include new probes, FTESR, higher magnetic fields, increase in absolute sensitivity, spectral dispersion and diversity of applications bode well for the continued development of ESR spectroscopy. Lastly, the development of powerful computational simulations makes ESR user-friendly and increases the number of ESR practitioners outside the die-hard community of spectroscopists.

ACKNOWLEDGMENTS

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LIST OF SYMBOLS

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>hyperfine interactions</td>
</tr>
<tr>
<td>A</td>
<td>peak-to-peak amplitude</td>
</tr>
<tr>
<td>A</td>
<td>hyperfine interaction tensor</td>
</tr>
<tr>
<td>A_{max}, A_{zz}</td>
<td>maximum hyperfine splitting</td>
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<tr>
<td>a₀, A₀</td>
<td>isotropic hyperfine splitting</td>
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<td>c</td>
<td>microwave field conversion factor</td>
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<tr>
<td>C/C</td>
<td>central manifold line-height ratio of the V spectrum</td>
</tr>
<tr>
<td>D</td>
<td>diffusion tensor</td>
</tr>
<tr>
<td>D_{∥}, D_{⊥}</td>
<td>elements of diffusion tensor for motion parallel and perpendicular to the z-axis of a nitroxide</td>
</tr>
<tr>
<td>E</td>
<td>energy</td>
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<tr>
<td>f</td>
<td>resonant frequency of a spin</td>
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<tr>
<td>f_{∥}, f_{⊥}</td>
<td>frictional coefficients</td>
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<td>g</td>
<td>Zeeman interaction tensor</td>
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<td>microwave field</td>
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<td>center field of a spectrum</td>
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<td>modulation amplitude</td>
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<td>resonant field</td>
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<td>J</td>
<td>coupling strength</td>
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<td>directional cosine matrices</td>
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<tr>
<td>L, C, H</td>
<td>turning points of the spectra, θ = 0°</td>
</tr>
</tbody>
</table>
$L''/L'; H''/H$  
line-height ratios of STESR line shape  

$M_2$  
second moment of the central resonance  

$m_I$  
nuclear spin quantum number, nuclear manifold  

$P$  
microwave power  

$P'/P$  
line-height ratios of $V_2'$ STESR spectra  

$P_{1/2}$  
half-saturation power  

$Q$  
resonator quality factor  

$r_{dd}$  
terms-derivative distance  

$S$  
electron spin  

$	ilde{S}$  
order parameter  

$T_1$  
spin–lattice relaxation time  

$T_{1\text{eff}}$  
effective spin–lattice relaxation time  

$T_2$  
spin–spin relaxation time  

$T_{2\text{eff}}$  
effective spin–spin relaxation time  

$V(m_1)$  
peak-to-peak height of a given nuclear manifold resonance  

$V_0$  
absorption spectrum  

$V_1'$  
first-derivative spectrum  

$V_2''$  
second-derivative, $90^\circ$ out-of-phase display  

$W_x$  
rate of bimolecular collisions  

$Y(H)$  
electron spin resonance spectrum  

$\beta$  
Bohr magneton  

$\Delta H_m$  
line width at half-height of a given nuclear manifold resonance line  

$\Delta H_{pp}$  
peak-to-peak resonance line width  

$\Delta \theta$  
width of Gaussian angular distribution  

$\Delta \nu_0$  
difference in resonant frequencies between spins A and B  

$\eta$  
resonator filling factor  

$\eta$  
viscosity  

$\gamma$  
magnetogyratic ratio  

$\Gamma$  
width (at half-height) of the resonance  

$\mu$  
magnetic moment of an electron  

$\nu$  
Larmor frequency  

$\Omega$  
orientational distribution  

$\pi$  
dimensionless accessibility parameter to relaxants  

$\pi'$  
normalized solvent accessibility to various quenchers  

$\Phi$  
differential accessibility to polar and nonpolar relaxants  

$\rho(\theta)$  
probability of the spins being orientated at angle $\theta$ with respect to the magnetic field  

$\tau_{\text{eff}}$  
effective rotational correlation time  

$\tau_{\text{eff}(m_1)}$  
effective correlation time obtained from the STESR calibration curves of $P'/P$ ratios  

$\tau_{\text{ex}}$  
exchange time  

$\tau_\perp$  
correlation time for rotation about axis perpendicular to nitroxide $z$-axis  

$\tau_\parallel$  
correlation time for rotation about the nitroxide $z$-axis  

$\tau_i$  
isotropic rotational correlation time  

$\theta_c$  
cone angle  

$\theta_0$  
center of Gaussian angular distribution  

$\theta, \phi$  
axial and azimuthal polar angles  

ABBREVIATIONS AND ACRONYMS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADP</td>
<td>Adenosine Diphosphate</td>
</tr>
<tr>
<td>AFC</td>
<td>Automatic Frequency Control</td>
</tr>
<tr>
<td>AMPPCP</td>
<td>Adenosine Methylene triphosphate</td>
</tr>
<tr>
<td>AMPPNP</td>
<td>Adenosine Imidotriphosphate</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine Triphosphate</td>
</tr>
<tr>
<td>ATPase</td>
<td>Adenosine Triphosphatase</td>
</tr>
<tr>
<td>ATP/S</td>
<td>Adenosine Thiotriphosphate</td>
</tr>
<tr>
<td>CROX</td>
<td>Potassium Tris(oxalatocromate)</td>
</tr>
<tr>
<td>DEER</td>
<td>Double Electron–Electron Resonance</td>
</tr>
<tr>
<td>DPPH</td>
<td>Diphenylpicrylhydrazyl</td>
</tr>
<tr>
<td>DR</td>
<td>Dielectric Resonator</td>
</tr>
<tr>
<td>ENDOR</td>
<td>Electron–Electron Double Resonance</td>
</tr>
<tr>
<td>ELDOR</td>
<td>Electron–Electron Double Resonance</td>
</tr>
<tr>
<td>ESR</td>
<td>Electron Spin Resonance</td>
</tr>
<tr>
<td>FID</td>
<td>Free Induction Decay</td>
</tr>
<tr>
<td>FTESR</td>
<td>Fourier Transform Electron Spin Resonance</td>
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<tr>
<td>GAPDH</td>
<td>Glyceraldehyde Dehydrogenase</td>
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<tr>
<td>HSE</td>
<td>Heisenberg Spin Exchange</td>
</tr>
<tr>
<td>LGR</td>
<td>Loop Gap Resonator</td>
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<tr>
<td>NAD</td>
<td>Nicotinamide Adenine Dinucleotide</td>
</tr>
<tr>
<td>NiAA</td>
<td>Nickel(II) Acetylatedonate</td>
</tr>
<tr>
<td>NiEDDA</td>
<td>Nickel(II) Ethylenediaminodiacetate</td>
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<tr>
<td>NMR</td>
<td>Nuclear Magnetic Resonance</td>
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<tr>
<td>PADS</td>
<td>Peroxylamine Disulfonate</td>
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<tr>
<td>SDSL</td>
<td>Site-directed Spin Labeling</td>
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<tr>
<td>SECSY</td>
<td>Spin-echo Correlation Spectroscopy</td>
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<tr>
<td>SEESR</td>
<td>Spin-echo Electron Spin Resonance</td>
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<tr>
<td>SLE</td>
<td>Stochastic Liouville Equation</td>
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<tr>
<td>STESR</td>
<td>Saturation Transfer Electron Spin Resonance</td>
</tr>
<tr>
<td>TnC</td>
<td>Troponin C</td>
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</tbody>
</table>
REFERENCES


