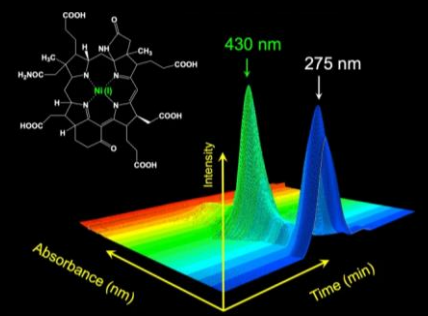
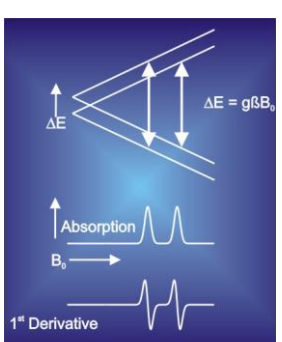


Workshop on Biomolecular Spectroscopy

EC Duin 2022

1



Part E

Practical Aspects of EPR Spectrometry

2

Practical Aspects of EPR Spectrometry

1) Metal-Ion Type Identification
2) Optimal Measuring Conditions (*T*,*P*)
3) The X-band EPR Spectrometer
4) Spectrometer Parameters
5) Spin Intensity
6) Redox Titrations
7) Freeze-Quench Experiments
8) Simulation of EPR Spectra
9) EPR on Whole Cells/Cell Extract
10) Site-Directed Spin Labeling (SDSL) EPR
11) Exercises

3

1) Metal-Ion Type Identification

- Which redox state is EPR active?

Metal Ion	Electron Configuration	Spin State
Fe^{2+}	d^6	S = 0 (ls) or S = 2 (hs)
Fe^{3+}	d^5	S = 5/2 (hs)
Ni^{1+}	d^9	S = ½
Ni^{2+}	d^8	S = 0 or S = 1
Ni^{3+}	d^7	S = ½
Cu^{1+}	d^{10}	S = 0
Cu^{2+}	d^9	S = ½

Prepare different samples: 1) as such
2) reduced (dithionite)
3) oxidized (ferricyanide)

- How many unpaired electrons? Different spin states!

4

Metal-Ion Type Identification

- Has the metal a nuclear spin?

Atom	Isotope	Spin (abundance)
V	50, 51	^{50}V, 6 (0.25); ^{51}V, $^{7}/_{2}$ (99.75)
Mn	55	$^{5}/_{2}$
Fe	54, 56, 57, 58	$^{1}/_{2}$ (2.119)
Co	59	$^{7}/_{2}$
Ni	58, 60, 61, 62	$^{3}/_{2}$ (1.14)
Cu	63, 65	^{63}Cu, $^{3}/_{2}$ (69.17); ^{65}Cu, $^{3}/_{2}$ (30.83)
Mo	92, 94, 95, 96, 97, 98, 100	^{95}Mo, $^{5}/_{2}$ (15.92); ^{97}Mo, $^{5}/_{2}$ (9.55)
W	180, 182, 183, 184, 186	$^{1}/_{2}$ (14.3)

Is the signal going to be split into 2 I + 1 lines?

- In general: The spin–orbit coupling parameter is positive for systems with less than half filled outer shells and negative for those with more than half filled shells, which means that the former have $g<g_e$ and the latter have $g>g_e$.

5

2) Optimal measuring Conditions (T, P)

- There is a need to measure at lower temperatures!
- EPR frequencies (1-100 GHz) are in the microwave range!
- Aqueous solutions will warm up in the EPR cavity at RT! This effect is absent in frozen samples.

Do-it-yourself microwave source

6

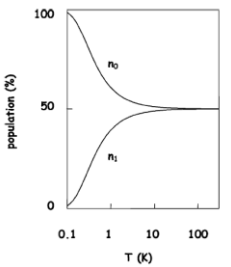
The Need for Lower Temperatures

The energy difference between the two energy level due to the Zeeman splitting is very small, ~0.3 cm^{-1} for X-band EPR.

Based on the Boltzmann distribution

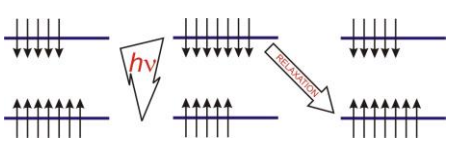
$$n_1 = n_0 e^{-\left(\frac{\Delta E}{kT}\right)}$$

it can be shown that only at low temperatures there will be enough difference in the population of the S = -½ level (n_0) and the S = ½ level (n_1) to create a signal.



7

Spin-Lattice Relaxation



EPR on metalloproteins:
- the relaxation rate *de*creases with *de*creasing temperature; and
- the relaxation rate is anisotropic (*i.e.* is different for different parts of the spectrum).

When too much power is applied the signal will saturate: Power saturation!

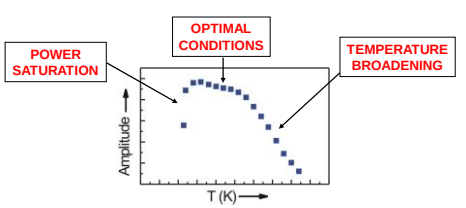
8

Heisenberg Uncertainty Principle

- Due to the uncertainty principle, EPR spectra will broaden beyond detection at higher temperatures. At lower temperatures the spectra will sharpen up.
- This sharpening up of the spectrum by cooling the sample is, however, limited by a temperature-*in*dependent process: *in*homogeneous broadening/*g*-strain.
- The protein or model molecules in dilute frozen solutions are subject to a statistical distribution in conformations, each with slightly different 3D structures and, therefore, slightly different *g*-values, which manifest themselves as a constant broadening of the EPR line independent of the temperature.

9

What to Do?



- Optimal measuring conditions (*T*,*P*) are determined by the interplay of the Boltzmann distribution, the Heisenberg uncertainty relation, the spin–lattice relaxation rate, and the conformational distribution of molecular structure.
- How do I find the correct measuring condition?
  1) ***Make a Curie Plot***
  2) ***Make Power Plots***

10

Power Plots

- The power in EPR is expressed in ***decibels (dB) attenuation***
- X-band microwave sources have a constant output that is usually leveled off at 200 mW (= 0 dB):

$$P(\mathrm{dB}) = -10 \times \log(0.2/P(\mathrm{W}))$$

- logarithmic scale: every -10 dB attenuation means an order-of-magnitude reduction in power.
- A good X-band bridge operates at power levels between 0 and -60 dB

11

Power Plots

Relationship between the amplitude, gain and the power in dB:

$$\left(\frac{amplitude}{gain}\right) \cdot 10^{-dB/20} = \text{constant}$$

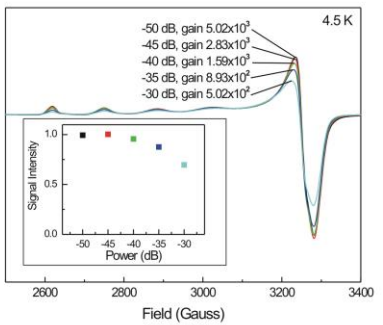
Both power and gain scales are logarithmic!

Need for low temperatures and high power, but this could lead to power saturation!

Practical rule: the amplitude of a *non*-saturated EPR signal does not change if a reduction in power by -1 dB is compensated by an increase in gain by one step.

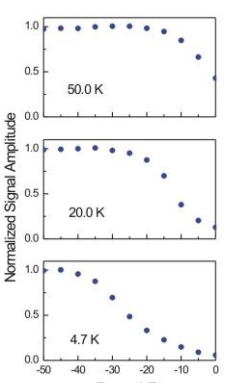
12

Power Plot (Copper Perchlorate)

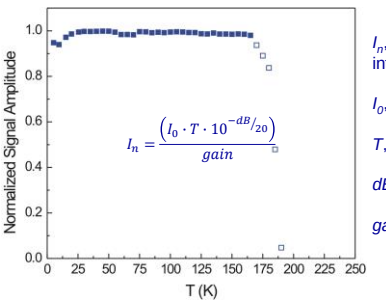


Power Plot (Copper Perchlorate)

- The relaxation rate ***in***creases with ***in***creasing temperature.
- Therefore if a signal does not saturate at a certain power at a certain temperature it will also not saturate at the same power at a higher temperature.
- The ***temperature behavior*** or ***Curie behavior*** will be different for different species.



Curie Plot (Copper Perchlorate)



I_n, normalized value for the intensity;

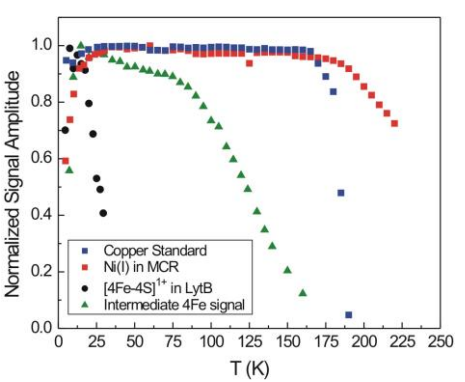
I_0, observed intensity;

T, absolute temperature in K;

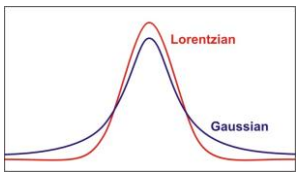
dB, reading of the attenuator;

gain, gain

Curie Plot



Line Shape

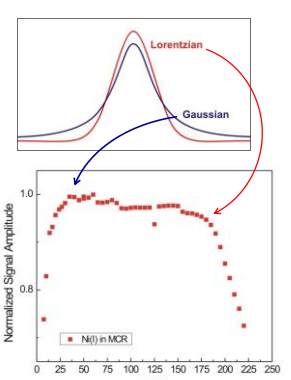


- The basic form of an EPR peak is described by the ***Lorentz distribution***. The Lorentzian line shape is also frequently called the ***homogeneous line shape***.
- In biological samples the paramagnet in each molecule has a slightly different structural surrounding and thus a slightly different *g*-value.
- This structural inhomogeneity is reflected in the form of an ***inhomogeneous line shape*** in addition to the Lorentzian shape.
- At low temperature the contribution from homogeneous broadening is small and the line shape can be described by the ***Gaussian distribution***.

17

Line Shape

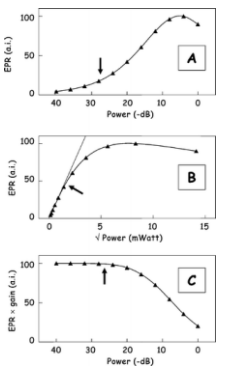
- At relatively high temperature a Lorentzian line shape will be observed, while a Gaussian line will be observed at relatively low temperatures
- The Gaussian shape will be broader.
- Preference to measure at the higher temperature end of Curie plot
- Practice better signal-to-noise at the lower end.



18

Power Plots

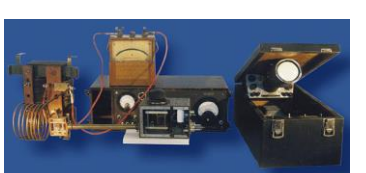
- Note that there are different ways to compose power plots.
- Plot A shows the signal intensity vs. power (-dB) with no correction.
- Plot B show the signal intensity versus $\sqrt{P}$ (in mW). In this case there is a linear relationship as long as the sample does not saturate (indicated by the straight line).
- Plot C uses the earlier described method on slide 165.



19

3) The X-band EPR Spectrometer

- In 1944, E.K. Zavoisky discovered magnetic resonance. Actually it was EPR on $CuCl_2$.

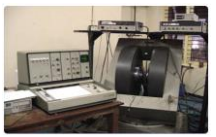


E.K. Zavoisky's first EPR system

20

EPR Spectrometer

Varian E3



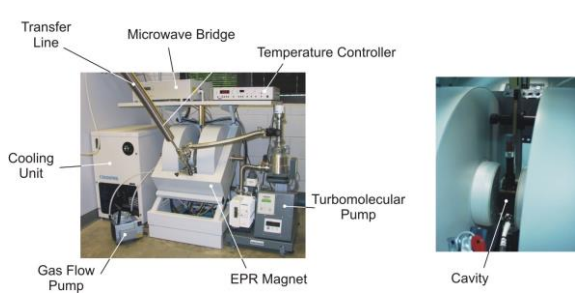
Varian E4

Bruker ElexSys X-band

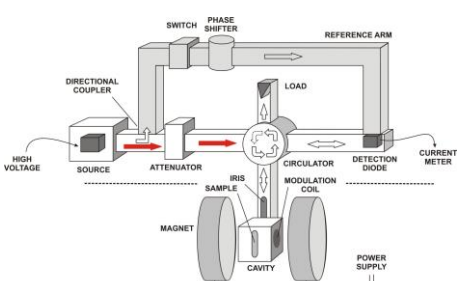
Bruker ElexSys W-band

X-Band EPR Spectrometer

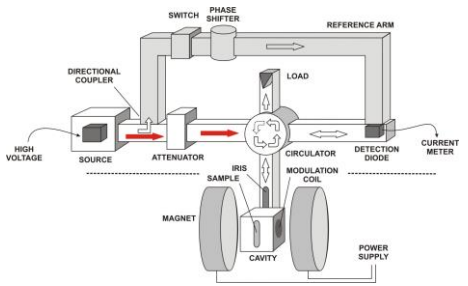
X-Band EPR Spectrometer



Cavity

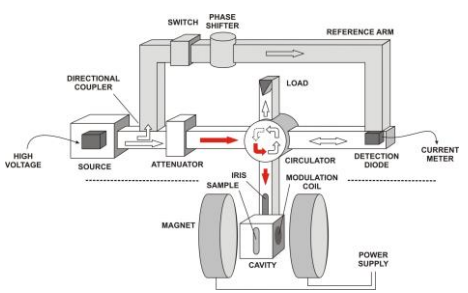


- On the left is a monochromatic source of microwaves of constant output (200 mW) and slightly (10%) tunable frequency.



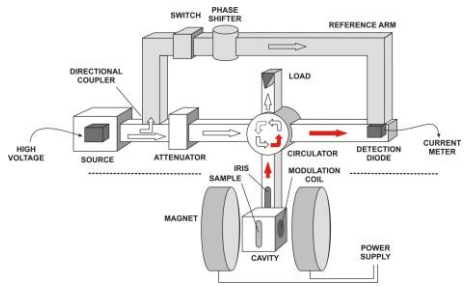
- The produced radiation is transferred by means of a rectangular, hollow wave guide to an attenuator where the 200 mW can be reduced by a factor between 1 and 10^6.

25



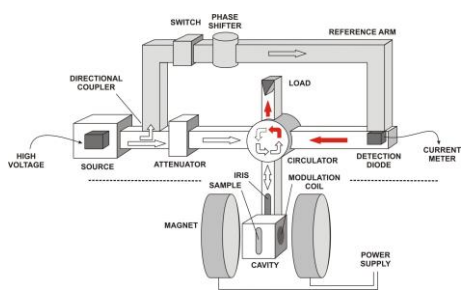
- The output of the attenuator is transferred with a waveguide to a circulator that forces the wave into the resonator/cavity.
- The entrance of the resonator is marked by the iris, a device to tune the amount of radiation reflected back out of the resonator.

26



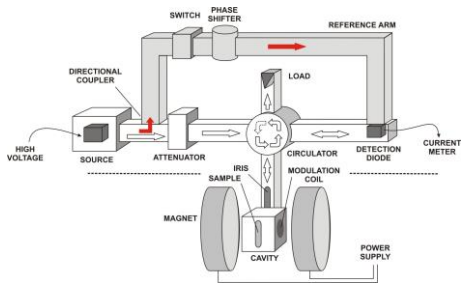
- The reflected radiation returns to the circulator and is directed to the diode for the detection of microwave intensity.

27



- Any remaining radiation that reflects back from the detector is forced by the circulator into the upward waveguide that ends in a wedge to convert the radiation into heat.

28



- A small amount of the 200 mW source output is directed through the reference arm directly to the detector to produce a constant working current.
- The reference arm contains a port that can be closed and a device to shift the phase of the wave.

29

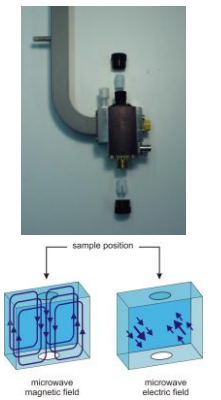
X-Band EPR Spectrometer

- Most EPR spectrometers are reflection spectrometers.
- They measure the changes (due to spectroscopic transitions) in the amount of radiation reflected back from the microwave cavity containing the sample.
- The detector should only detect the microwave radiation coming back from the cavity.

30

Cavity/EPR Resonator

- A microwave cavity is simply a metal box with a rectangular or cylindrical shape which resonates with microwaves much as an organ pipe resonates with sound waves.
- The resonator is designed to set up a pattern of standing microwaves in its interior.
- Standing electromagnetic waves have their electric and magnetic field components exactly out of phase - where the magnetic field is maximum, the electric field is minimum.



31

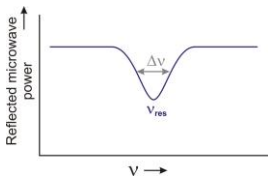
Cavity/EPR Resonator

- Resonance means that the cavity ***stores*** the microwave energy; therefore, at the resonance frequency of the cavity, no microwaves will be reflected back, but will remain inside the cavity.
- Energy can be lost to the side walls of the cavity because the microwaves generate electrical currents in the side walls of the cavity which in turn generates heat.

32

Cavity/EPR Resonator

- Cavities are characterized by their *Q* or quality factor, which indicates how efficiently the cavity stores microwave energy.



- We can measure *Q* factors easily:

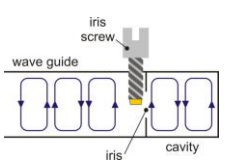
$$Q = (\nu_{res})/(\Delta\nu)$$

where ν_{res} is the resonant frequency of the cavity and $\Delta\nu$ is the width at half height of the resonance.

33

Cavity/EPR Resonator

- In order for the microwaves to enter the cavity one of its end walls must have an opening: ***iris***.
- The size of the iris controls the amount of microwaves which will be reflected back from the cavity and how much will enter the cavity.
- Just before the iris is a small metal plate (attached to the iris screw). Moving this plate up or down changes the amount of coupling.
- Only for one unique position is the cavity ***critically coupled***: all waves enter the cavity, and no radiation is reflected out.

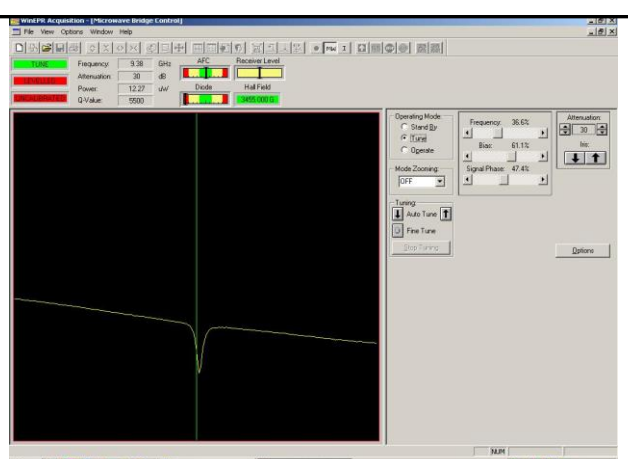


34

Cavity/EPR Resonator

- How do all of these properties of a cavity give rise to an EPR signal? When the sample absorbs the microwave energy, the *Q* is lowered because of the increased losses and the coupling changes.
- The cavity is therefore no longer critically coupled and microwaves will be reflected back to the bridge, resulting in an EPR signal.

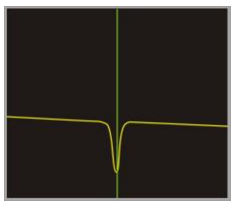
35



36

Tuning the Microwave Cavity and Bridge

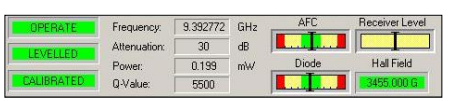
- **Locate and center the "dip" on the display**.
- The pattern is a display of the microwave power reflected from the cavity and the reference arm power as a function of the microwave frequency.



- The dip corresponds to the microwave power absorbed by the cavity and thus is not reflected back to the detector diode.
- By centering the dip on the display monitor, the microwave source is set to oscillate at the same frequency as the cavity resonant frequency

37

Tuning the Microwave Cavity and Bridge



- **Tune the signal (reference) phase.** Adjust the Signal Phase until the depth of the dip is maximized and looks somewhat symmetric.
- **Adjust the bias level**. Adjust the Bias until the Diode meter needle is centered.
- **Critical coupling of the cavity**. Power is increased and the iris screw is adjusted to keep the diode current in the center.

38

4) Spectrometer Parameters

- ***Center Field*** and ***Sweep Width***

- For initial broad scans, a Sweep Width around 5000 Gauss is recommended. Set the Center Field value to 2600 Gauss. This means that the scan will start at 100 Gauss and stops at 5100 Gauss (*2500 Gauss below and 2500 Gauss above the Center Field value*).
- This scan will cover the complete area available with our magnet where signals might be detectable.

39

Spectrum Settings

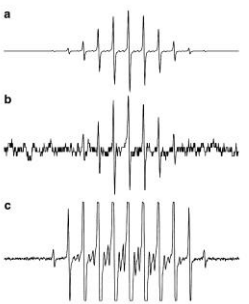
- ***Points***
- Standard 1024 points. Can be increased to 4096 for wide scans to keep the resolution.
- It is advisable, however, to rescan the interesting parts of a wide scan.
- Subtractions are not possible if the amounts of points between the two spectra are different.

40

Spectrum Settings

- ***Gain***

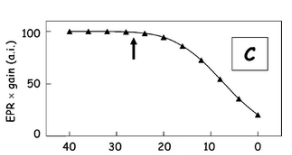
- Use the full range of the digitizer (a), coincides with the screen display.
- If the receiver gain is too low the effect of digitization will be evident in the spectrum (b)
- At too high gain the signals will be clipped due to an overload in the signal channel (c).



41

Microwave Bridge Parameters

- ***Microwave power level***. The EPR signal intensity grows as the square root of the microwave power in the absence of saturation effects. When saturation sets in, the signals broaden and become weaker. Several microwave power levels should be tried to find the optimal microwave power.



42

Phase Sensitive Detection

- Enhancement of the sensitivity of the spectrometer: less noise from the detection diode and the elimination of baseline instabilities due to the drift in DC electronics.
- The magnetic field at the site of the sample is modulated (varied) sinusoidally at the modulation frequency. If there is an EPR signal, the field modulation quickly sweeps through part of the signal and the microwaves reflected from the cavity are amplitude modulated at the same frequency.
- Only the amplitude modulated signals are detected. Any signals which do not fulfill these requirements (i.e, noise and electrical interference) are suppressed.

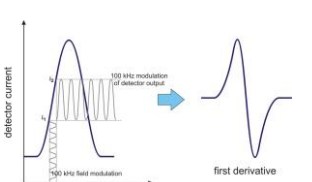
43

Phase Sensitive Detection

For an EPR signal which is approximately linear over an interval as wide as the modulation amplitude, the EPR signal is transformed into a sine wave with an amplitude proportional to the slope of the signal.

As a result the first derivative of the signal is measured.

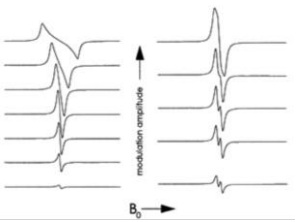
Two new parameters: ***modulation amplitude***, and modulation ***frequency***.



44

Field Modulation

- With more magnetic field modulation, the intensity of the detected EPR signals increases; however, if the modulation amplitude is too large (larger than the linewidths of the EPR signal), the detected EPR signal broadens and becomes distorted.



- A good compromise between signal intensity and signal distortion occurs when the amplitude of the magnetic field modulation is equal to the width of the EPR signal. Also, if we use a modulation amplitude greater than the splitting between two EPR signals, we can no longer resolve the two signals.

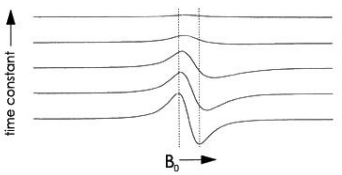
45

Signal Channel Parameters

- ***Modulation frequency***: normally set to 100 kHz
- ***Modulation amplitude***: You can start with 6 Gauss. The larger this value the lower the value needed for the Receiver Gain, which means less noise. Excessive field modulation, however, broadens the EPR lines and does not contribute to a bigger signal. As a rule-of-thumb this value has to be smaller than the line width of your signal.

46

Time Constant



- To further improve the sensitivity, a ***time constant*** is used to filter out more of the noise.
- Time constants filter out noise by slowing down the response time of the spectrometer. As the time constant is increased, the noise levels will drop. If we choose a time constant which is too long for the rate at which we scan the magnetic field, we can distort or even filter out the very signal which we are trying to extract from the noise. Also, the apparent field for resonance will shift.

47

Signal Channel Parameters

- ***Time Constant*** and ***Conversion Time***: If the Time Constant is too large in comparison with the Conversion Time (the rate at which the field is scanned) the signals we want to detect will get distorted or will even be filtered out.
- A longer Conversion Time, however, also improves the signal to noise ratio in a different way: The signal channel incorporates an integrating ADC (Analog to Digital Converter) to transfer the analog EPR spectra to the digital data acquisition system. An important side effect of using the integration method for the conversion is that it integrates the noise out of the signal.
- With a sweep width off about **1000** Gauss a Conversion Time of **163.84** msec and a Time Constant of **163.84** msec can be used.

48

Signal Averaging

- Very weak signals might get lost in the noise. You can increase your signal to noise ratio by signal averaging. The resultant signal to noise is proportional to √N, where N is the number of scans.
- With a perfectly stable laboratory environment and spectrometer, signal averaging and acquiring a spectrum with a long scan time and a long time constant are equivalent. Unfortunately perfect stability is impossible to attain. Slow variations result in baseline drifts. For a slow scan (>15 min) the variations can cause broad features in the spectrum dependent on the sample concentration and the gain used. If you were to signal average the EPR signal with a scan time short compared to the variation time, these baseline features could be averaged out.

49

Spectrometer Parameters

- ***Center Field***, ***Sweep Width, Gain, Microwave power level***: sample dependent
- ***Modulation frequency***: normally set to 100 kHz
- ***Modulation amplitude***: normally set to 6 Gauss.
- ***Time Constant*** and ***Conversion Time***: same value!
- sweep width off **1000** Gauss; both **163.84** msec
- ***Number of X-Scans***: normally set to 1

50

5) Spin Intensity

- Also known as ***spin counting***
- To calculate the amount of signal in a protein sample, the spin intensity can be compared with that of a standard with a known concentration (Copper perchlorate: 10 mM)
- Since an EPR spectrum is a first derivative, we have to integrate twice to obtain the intensity (I_0 = area under the absorption spectrum).
- In addition, corrections are needed for a number of parameters, to 'normalize' the spectra. Only then a direct comparison of double integral values of standard and unknown is possible:

51

Normalized Signal Intensity

$$I_n = \frac{\left(I_0 \cdot d^2 \cdot T \cdot 10^{-dB/20}\right)}{\left(g_p^{av} \cdot f \cdot a\right)}$$

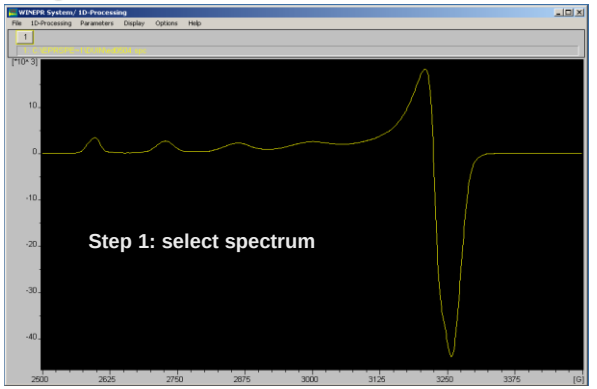
where

I_n	normalized double integral
I_0	observed intensity
d	distance between the starting and ending points (in Gauss)
T	absolute temperature in *K*
dB	reading of the attenuator
f	tube calibration factor
a	gain

and $g_p^{av} = \frac{2}{3}\sqrt{\frac{g_x^2+g_y^2+g_z^2}{3}} + \frac{(g_x+g_y+g_z)}{9}$

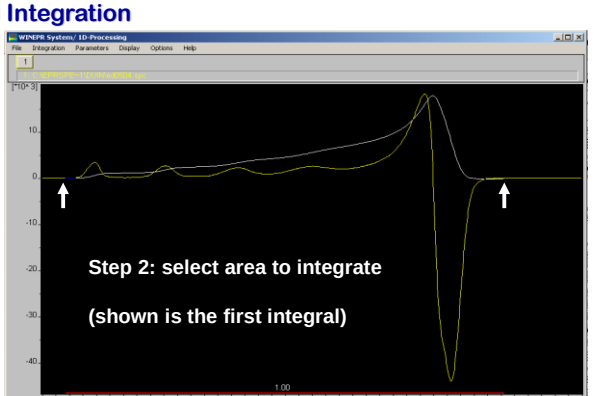
52

Signal Integration



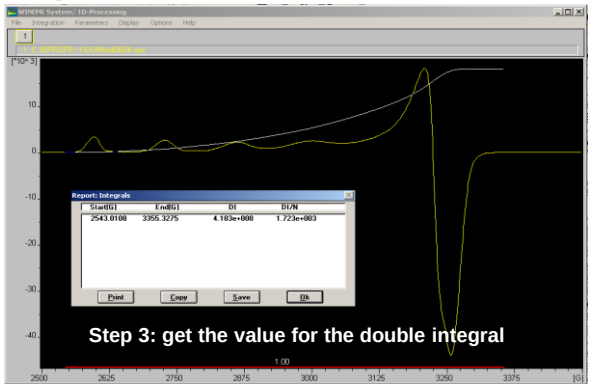
53

Signal Integration



54

Signal Integration



55

Comparison with 'Spin' Standard

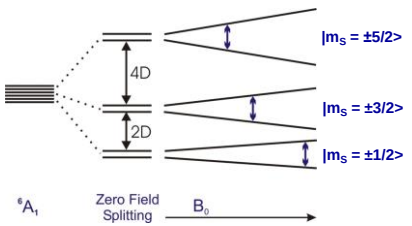
$$I_n = \frac{\left(I_0 \cdot d^2 \cdot T \cdot 10^{-dB/20}\right)}{\left(g_p^{av} \cdot f \cdot a\right)}$$

$$C_u = \frac{I_{n(u)} \cdot C_{st}}{I_{n(st)}}$$

- Keep measuring conditions the same: temperature, modulation amplitude, sweep time, amount of points, amount of scans (These are not averaged!)
- Measure samples on the same day!
- Correct for spin: $S(S+1)$

56

Signal Intensity of High Spin Systems



- For Kramers' systems each Kramer pair can give rise to its own resonance.
- Each of these can be described in terms of an effective $S = ½$ spectrum with three ***effective g-values***.

57

Signal Intensity of High Spin Systems

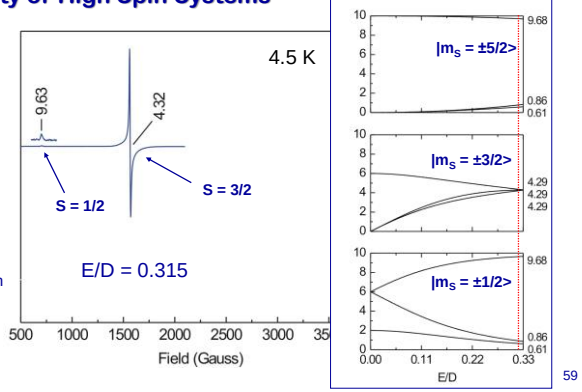
- The effective spin-Hamiltonian suggests an easy way for quantification of high-spin spectra: one simply applies the double-integration procedure to the effective $S_{eff} = 1/2$ spectrum *as if* it were a real $S = 1/2$ spectrum, however, *with a correction for the fractional population of the relevant doublet. Most of the time not possible!*

- Exception: For *high spin ferric hemoproteins* ($D \approx +10$ cm^{-1}) in X-band at $T =$ 4.2 K the fractional population of the $|m_S = \pm1/2>$ doublet is very close to unity (0.999) therefore, quantification of the spectrum does not require a depopulation correction.

58

Signal Intensity of High Spin Systems

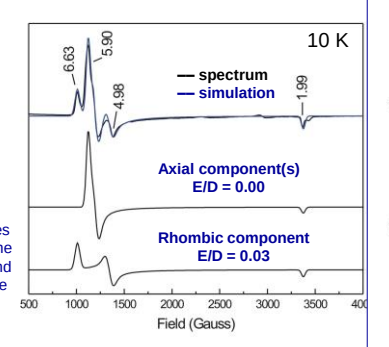
Intensity cannot be determined without correction for the Boltzmann distribution



59

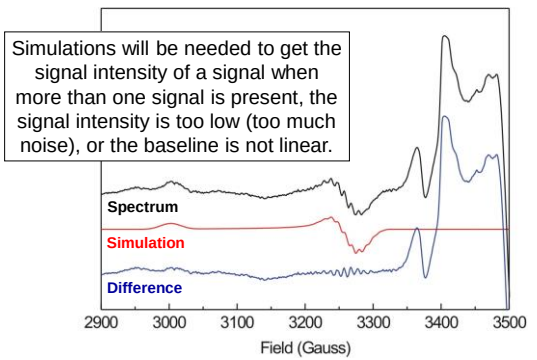
Signal Intensity of High Spin Systems

At low temperatures all electron are in the m_s=±1/2 doublet and the intensity can be determined.



60

Signal Intensity ???



61

6) Redox Titrations

- With species that are only paramagnetic at a certain redox potential it is possible to do a redox titration and obtain the ***midpoint potential*** (E_m) of the redox couple.
- This is particular useful if you are studying proteins that are involved in electron transfer pathways.
- In these experiments the protein is titrated in both the oxidative direction with ferricyanide and in the reductive direction with dithionite. The potential can be measured with a combination Ag/AgCl electrode,
- A mixture of redox dyes is added to stabilize the redox potential outside the E_m region

62

Redox Titrations

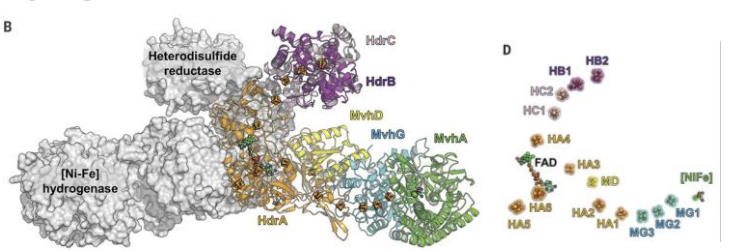
- When there is only a single paramagnetic species present the intensity of this signal can be determined directly.
- When more than one species are present you have to look for unique features, or the different components have to be simulated and their intensities determined.
- Plots of the intensity vs. the potential are generated.
- The points in the plot can be fitted with the ***Nernst equation:***

$$E = E_0 + \frac{RT}{nF} ln\left(\frac{[ox]}{[red]}\right)$$

R (gas constant) = 8.314 J K^{-1} mol^{-1}; F (Faraday constant) = 9.649×10^4 C mol^{-1}; n is the number of moles of electrons

63

Hydrogenase:heterodisulfide Reductase

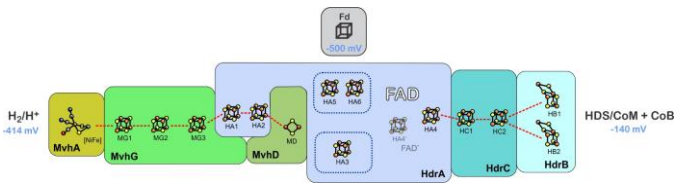


- Hydrogenase:heterodisulfide reductase complex from *Methanothermobacter marburgensis*

Wagner (2017) Science, 357, 699-703

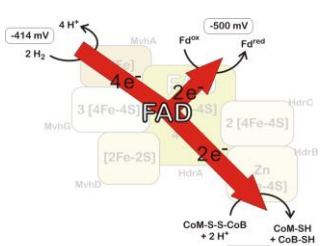
64

Hydrogenase:heterodisulfide Reductase



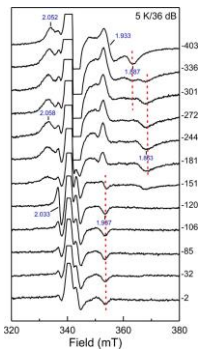
- FAD-based electron bifurcation: Electrons from hydrogen (-414 mV) are used to break down the heterodisulfide CoB-S-S-CoM (-200 mV) and are used to reduce Ferredoxin (-500 mV).
- Except for two (HB1 and HB2), all clusters are involved in electron transport.

Electron Bifurcation



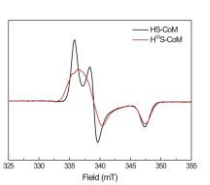
- For electron bifurcation to occur, the FAD accepts two electrons at intermediate potential.
- The first electron donated is the energetically more favorable high potential and goes towards the CoM-S-S-CoB site.
- This leaves the FAD in a hot red state and the second electron has a much lower potential and can reduce Ferredoxin.

Redox Titrations: H_2ase:HDR complex

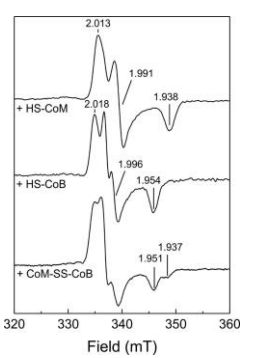


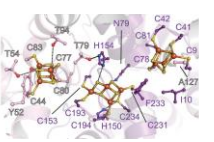
- Most of the FeS Clusters have an E_m value below -400 mV.
- *g* = 1.863 signal with E_m = -153 mV
- No other signals present in the absence of HS-CoM
- *g* = 1.987 signal detected in the presence of HS-CoM

Non-cuboidal Clusters

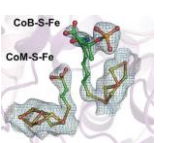


Labeling studies with $H^{33}S$-CoM (^{33}S: I = 3/2)

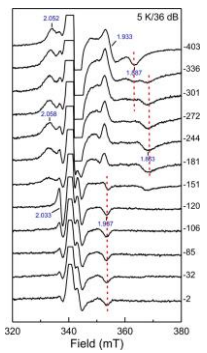


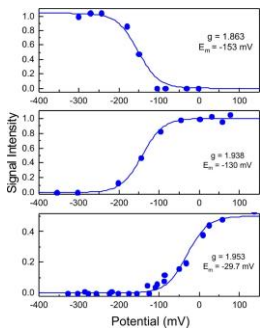


HC1 HB1 HB2



Redox Titrations: H_2ase:HDR complex



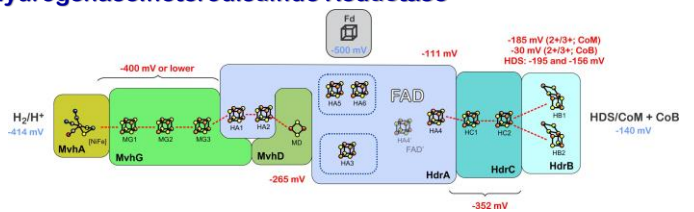


'regular' $[4Fe\text{-}4S]^{1+/2+}$

Non-cuboidal with CoM

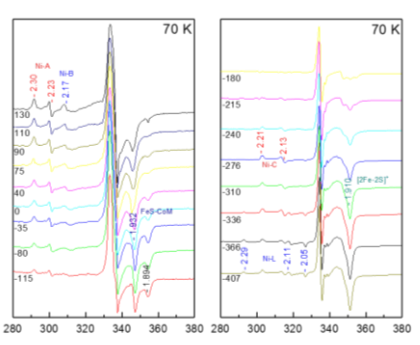
Non-cuboidal with CoB

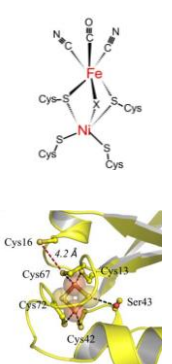
Hydrogenase:heterodisulfide Reductase



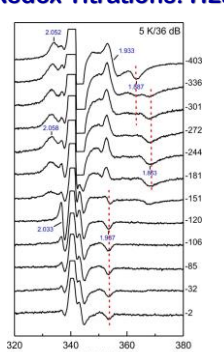
- For electron bifurcation to occur, the FAD accepts two electrons at intermediate potential.
- The first electron donated is the energetically more favorable high potential and goes towards the CoM-S-S-CoB site.
- This leaves the FAD in a hot red state and the second electron has a much lower potential and can reduce Ferredoxin.

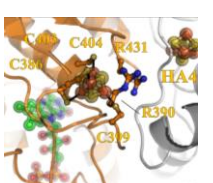
Redox Titrations: H_2ase:HDR complex





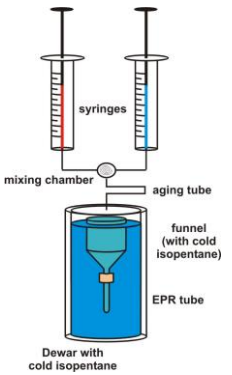
Redox Titrations: H2ase:HDR complex





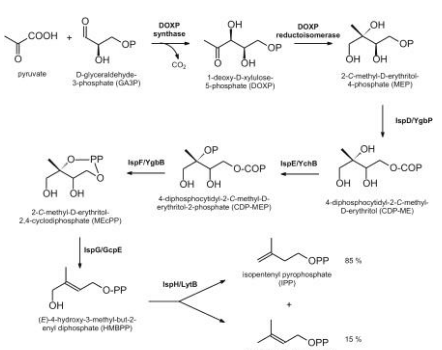
- Is the HA4 clusters the one that causes the g = 1.863 signal?
- The FAD needs a high potential cluster to accept the first electron.
- The [4Fe-4S] cluster is coordinated by a non-canonical sequence and has two neighbored basic residues.
- C-x_{12}-C-x_3-C-C

7) Freeze-Quench Experiments



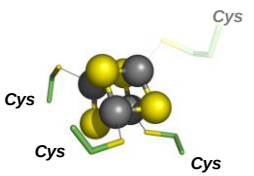
- To follow a reaction involving paramagnetic species ***freeze-quench experiments*** can be performed.
- In this experiment enzyme is mixed with substrate and other compounds and EPR samples are made by rapid mixing and freezing.
- Multiple samples have to be made to get insight into the formation/disappearance of an EPR signal.

Role of IspG in Isoprene Synthesis

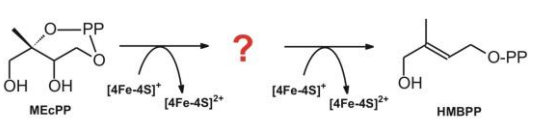


Role of IspG in Isoprene Synthesis

- IspG contains a single [4Fe-4S] cluster.
- The cluster is very unstable.
- Cluster falls apart when exposed to molecular oxygen.
- Instability probably caused by incomplete coordination.

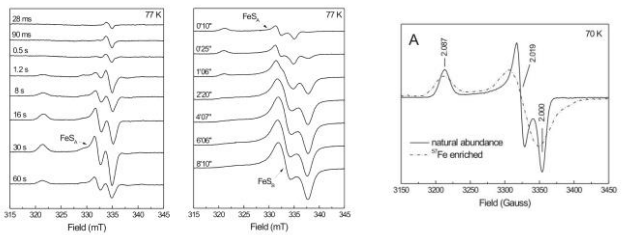


Role of IspG in Isoprene Synthesis



- The reaction is a reductive elimination of a hydroxyl group involving 2 electrons.
- A [4Fe-4S] cluster can only donate 1 electron at-a-time.
- Formation of radical species expected.

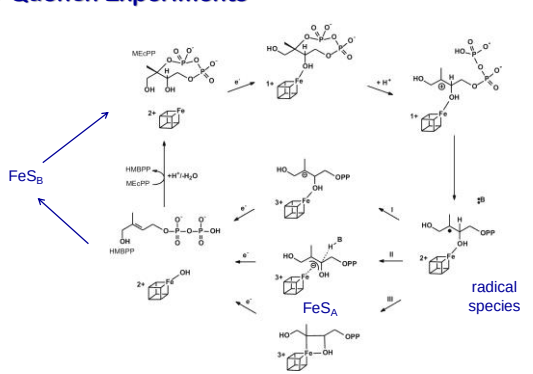
Freeze-Quench Experiments with IspG



- A transient isotropic signal is detected with maximal intensity at 90 ms.
- A transient rhombic signal, FeS_A, reaches maximal intensity at 30 s.
- A second rhombic signal, FeS_B, accumulates over time and reaches maximal intensity at 4 min.
- Signal not isotropic. ^{57}Fe labeling indicates Fe origin.

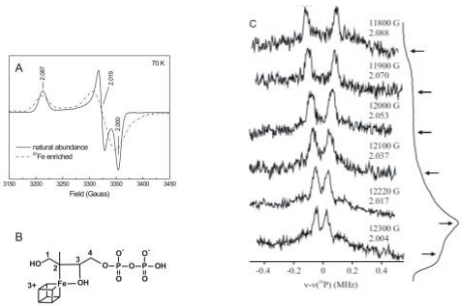
77

Freeze-Quench Experiments



78

ENDOR

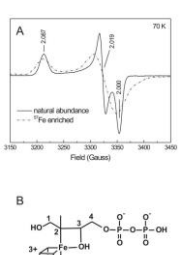


A) EPR spectrum obtained for IspG upon incubation with the substrate MEcPP and the reductant dithionite

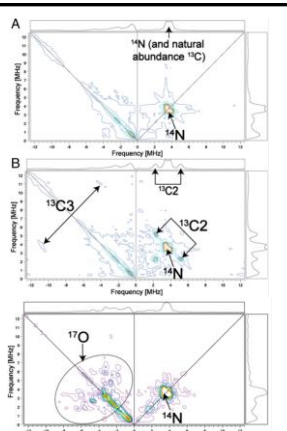
B) Proposed structure for the reaction intermediate

79

2D-HYSCORE



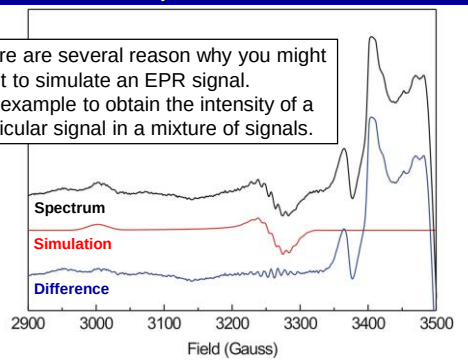
^{13}C and ^{17}O HYSCORE Spectra of the FeS_A species in IspG



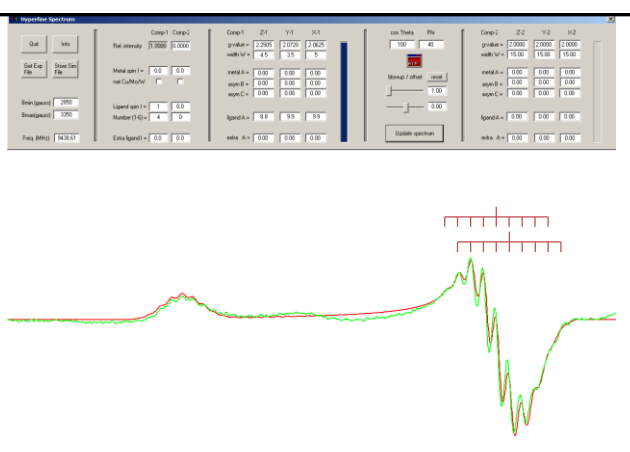
80

8) Simulation of EPR spectra

- There are several reason why you might want to simulate an EPR signal.
- For example to obtain the intensity of a particular signal in a mixture of signals.

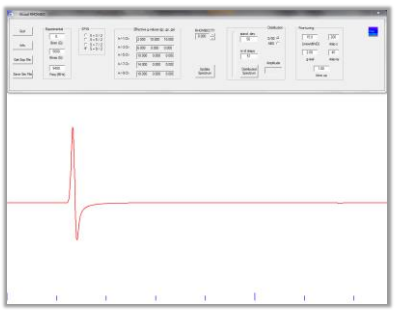


81



82

Visual Rhombo

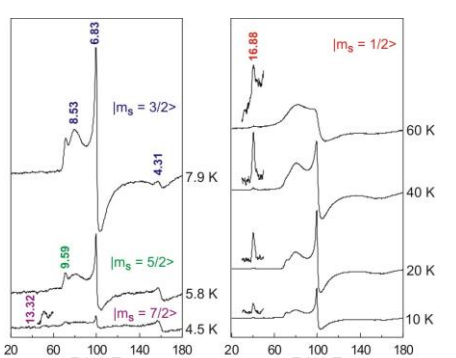


- Estimates of the effective *g*-values

83

Simulation

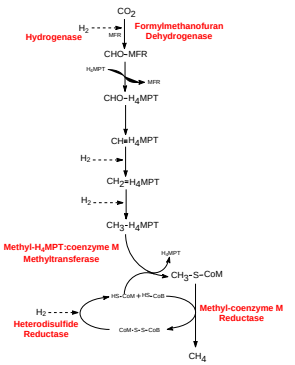
Clostridium pasteurianum
$[2Fe\text{-}2S]^{2+}$



84

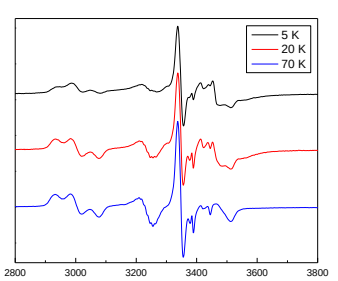
9) EPR on Whole Cells/Cell Extract

- CO_2-reducing pathway of methanogenesis, which uses H_2 and CO_2 as substrates.
- The reduction of CO_2 to CH_4 proceeds via coenzyme-bound C1-intermediates, methanofuran (MFR), tetrahydromethanopterin (H_4MPT), and coenzyme M (HS-CoM).



EPR on Whole Cells

- Overview of all paramagnetic species
- Behavior under different growth conditions
- Estimates of the amount of species present (simulations and integration)

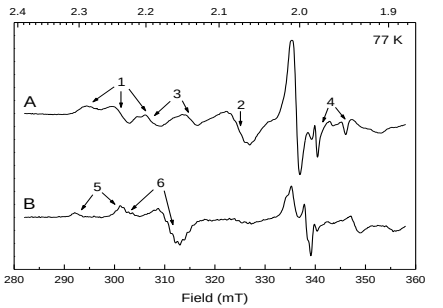


EPR on Whole Cells

A: 80% H_2/20% CO_2

B: 80% N_2/20% CO_2

1) MCR (red2 form)
2) MCR (red1 form)
3) Hydrogenase (Ni-C form)
4) Heterodisulfide reductase
5) Hydrogenase (Ni-A form)
6) MCR (ox1 form)



10) Site-Directed Spin Labeling (SDSL) EPR

Provides specific information on the location and environment of an individual residue within large and complex protein structures.

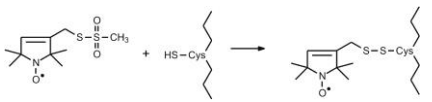
A. Motion: Determine rotational mobility of label at different protein sites.

B. Accessibility: An amino acid can be on the surface of a protein and accessible to water, or it can be placed inside the structure and is less accessible or not accessible at all. An amino acid can also be deep in the membrane space.

C. Distance: Measure the distance between 2 or more amino acids in one system or between systems.

The Technique

- Specific Cys residues are labeled with a spin label: 2,2,5,5-tetramethyl-1-oxyl-3-methyl methanethiosulfonate (MTSL).

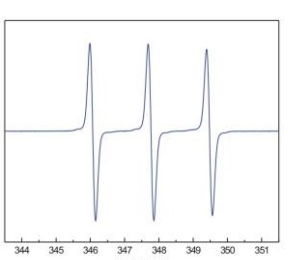


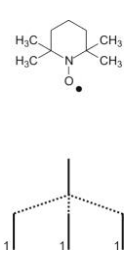
- Drawback: Cys residues have to be introduced in the structure at the positions of interest. All other accessible Cys residues need to be deleted.

89

Nitroxide Spin Labels

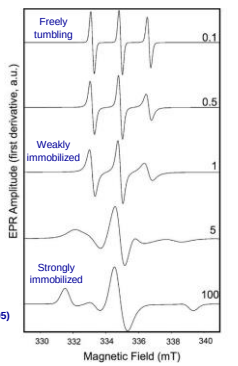
Hyperfine Interactions: TEMPO



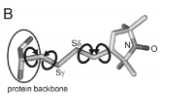


90

Nitroxyl Lineshapes



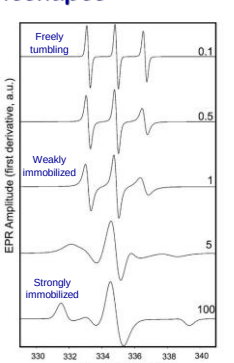
Thomas Stockner (2105) Biochm. Soc. Trans. 43:1023-1032

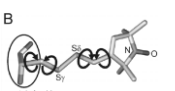


- Conventional X-band EPR spectra are sensitive to rotational motion in the range 0.1 to ~100 nsec.
- In the fast motional limit (~0.1 nsec) three lines of approximately equal height are observed.

91

Nitroxyl Lineshapes





- In the motional narrowing region the three lines become broader and since the total intensity does not change the line amplitude decreases.
- These changes, however, vary for each of the three lines.

92

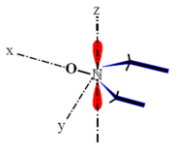
Nitroxyl Lineshapes

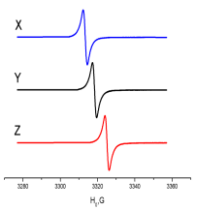
g_x=2.0091, g_y=2.0061, g_z=2.0023

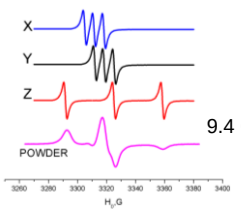
The field shift between the X- and Z- orientations is

$\Delta H = h\nu/g_x\beta - h\nu/g_z\beta \cong h\nu\Delta g/4\beta \sim 11G$

I=1: A_x= 6.2, A_y = 6.3, A_z=33.6







9.4 GHz

Nitroxyl Lineshapes

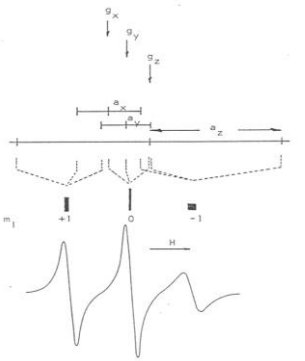
In the motional narrowing region, the dependence of the width of an individual hyperfine line on the nuclear spin state (m_I) can be expressed as

$$\Delta B(m_I) = A + B\,m_I + C\,m_I^2$$

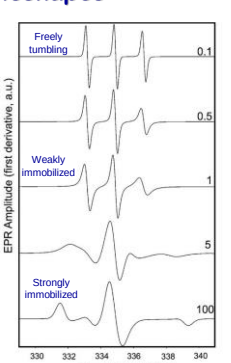
The dependence of the linewidth on the nuclear spin state (m_I) indicates that each line will have a different width. The '*A*' term broadens all lines equally; the '*C*' term broadens the low-and high-field lines but does not affect the center line (for which m_I = 0); the '*B*' term is negative and causes the high-field (m_I = -1) line to broaden and the low-field (m_I = 1) line to narrow.

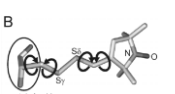
Nitroxyl Lineshapes

As the molecule tumbles, the smaller splitting for m_I = 0 is averaged more effectively than the larger splittings, which causes differences in the linewidths of the three hyperfine lines.



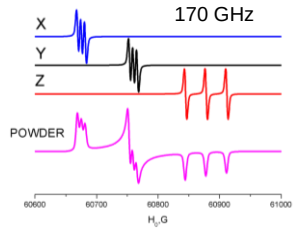
Nitroxyl Lineshapes





- Strongly immobilized corresponds to the slow motion limit (>100 nsec). The spectrum is very similar to the 'powder' or 'rigid limit' spectrum that is obtained for any nitroxide in the absence of rotational motion and for a dilute powder or frozen solution

Nitroxyl Lineshapes



g_x=2.0091, g_y=2.0061, g_z=2.0023

I=1, A_x= 6.2, A_y = 6.3, A_z=33.6

- High field EPR spectroscopy is the *g*-resolved spectroscopy, the regions corresponding to different orientations of the magnetic axis relative to the external magnetic field do not overlap.
- Note that using a different frequency will change the time scale of the experiment.

97

A. Motion

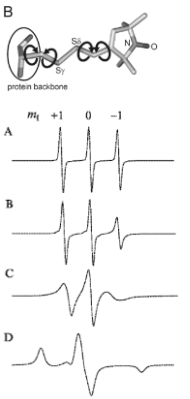
Attachment of the spin label to even a small unstructured peptide can result in some degree of motional restriction, and this restriction increases significantly in the presence of local secondary structure.

98

MTSL-15 AA peptide

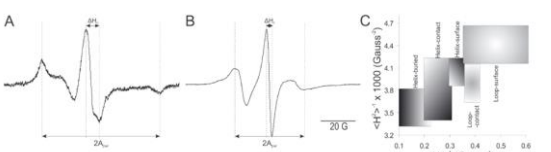
The motion of the spin label side chain is sensitive to tertiary contacts and protein structure in the local environment of the spin label.

A. dilute solution of MTSL fast motional limit (~0.1 nsec)
B. attached to 15 AA peptide with random coil structure
C. attached to same peptide with an α-helical structure
D. No motion (slow motion limit, >100 nsec) or in frozen solution



99

Spin Label Mobility Based on EPR Spectra Line Shape

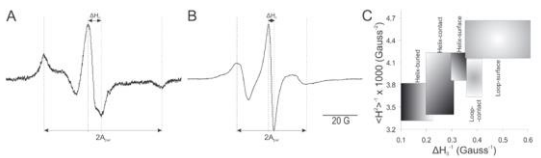


- (A) motionally restricted spin label of a buried side chain of a helix and (B) increased mobility of an exposed side chain of the same helix forming β spectrin lipid-binding domain
- The separation between the outer hyperfine extrema ($2A_{par}$) and the peak-to-peak separation of the central line width (ΔH_0) provide a measure of label mobility.

Czogalla, A. (200&) Acta Biochim Polonica 54: 235-244

100

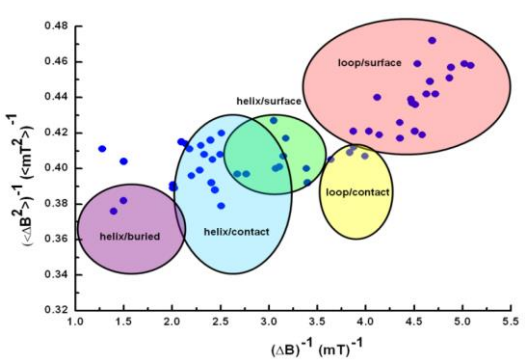
Spin Label Mobility Based on EPR Spectra Line Shape



- (C) Mobility map constructed as a plot of the inverse second moment of the EPR spectrum ($<H^2>^{-1}$) *versus* inverse central line width (ΔH_0^{-1}), which indicates the correlation between the measured parameters and regions of protein topology.

101

Mobility Map



102

B. Accessibility

An amino acid can be located on a solvent-exposed surface, buried within a protein, or within a membrane bilayer.

103

Power Saturation Studies

- Under nonsaturating conditions, the amplitude of the spectral lines are proportional to the incident microwave power, increasing linearly with the square root of the incident power, $\sqrt{P}$ or $P^{1/2}$.
- Under saturation conditions the increase becomes less than linear.
- When paramagnetic relaxation reagents interact with the spin label, they enhance the relaxation rate and allow the sample to absorb more power before becoming saturated.

104

Power Saturation Studies

Three common reagents:

Oxygen
- small and hydrophobic
- generally found in the center of lipid bilayers of membranes and in hydrophobic pockets of proteins
- Only present to a small extent in solution

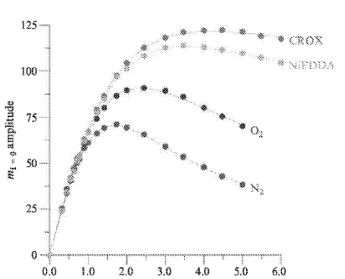
Ni(II) ethylendiaminediacetate (NiEDDA)
- Neutral/Water soluble

Chromium Oxalate (CROX)
- Negative/Water soluble

105

Power Saturation Studies

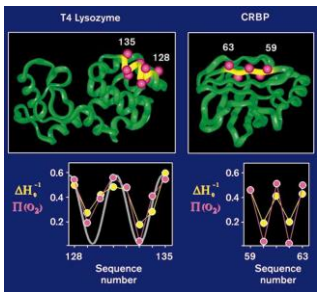
The spin label is in a water-soluble environment. CROX and NiEDDA prevent saturation (in comparison with the N_2 curve), while O_2 does have a much smaller effect.



106

T4 lysozyme

- Oxygen accessibility and probe mobility were measured as a function of sequence number for spin labels attached to T4 lysozyme (T4L) and cellular retinol binding protein (CRBP).

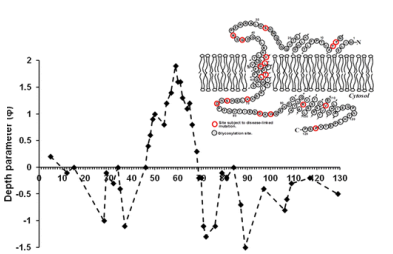


- The correlation between the two parameters indicates that the most mobile sites are also the most oxygen accessible.
- The repeat period of about 3.6 for T4L is consistent with the α-helical structure of this segment of the protein.

107

Depth Parameter

$$\Phi = \ln\left[\frac{\Delta P_{1/2}(O_2)}{\Delta P_{1/2}(\text{NiEDDA})}\right]$$



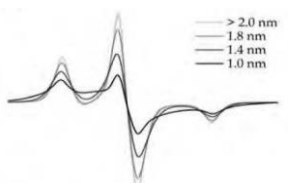
- $[O_2]$ is highest in the center of the membrane and lowest at the surface. The opposite is true for the [NiEDDA].
- The natural log of this ratio yields Φ, a parameter with a linear dependence on depth into the bilayer.

108

C. Distances

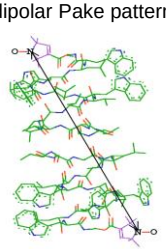
- Measure the distance between 2 spin labels.
- This can be intramolecular distances between two labels in the same monomer or intermolecular distances between sites on different proteins.

  - Binding processes, conformational changes
  - In the range ~8-20 Å, interactions between the two paramagnets give rise to distance-dependent line broadening in CW EPR.



Distances: Pulse EPR

DEER (Double Electron-Electron Resonance) is a pulse EPR technique that measures the dipolar frequency between two spins. Deer can cancel out all interactions resulting in an EPR spectrum except the dipole interaction in spin pairs. The dipolar Pake pattern shown is an FT of the DEER echo.

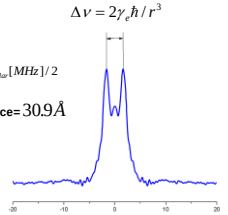


Example: spin labeled Gramicidin A

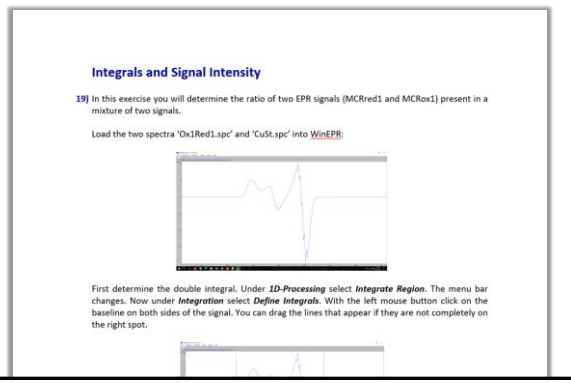
$$\Delta\nu = 2\gamma_e\hbar / r^3$$

$$\frac{5.2\cdot 10^4}{r^3[\text{Å}]} = \Delta\nu_{dipolar}[MHz]/2$$

Interspin distance = 30.9Å



11) Exercises



Integrals and Signal Intensity

19) In this exercise you will determine the ratio of two EPR signals (MCRred1 and MCRox1) present in a mixture of two signals.

Load the two spectra 'Ox1Red1.spc' and 'Cu5t.spc' into WinEPR:

First determine the double integral. Under **1D-Processing** select **Integrate Region**. The menu bar changes. Now under **Integration** select **Define Integrals**. With the left mouse button click on the baseline on both sides of the signal. You can drag the lines that appear if they are not completely on the right spot.

High Spin

Clostridium pasteurianum
$[2Fe\text{-}2S]^{2+}$

S = 9/2

What is the E/D value, what is the sign of D?