What is spectroscopy?

Molecular structure and dynamics though absorption, emission, and scattering of light.

Molecular Window.

Heme iron complex (MW: 600-700) in the protein (MW > 10,000).

You can understand the molecular mechanism of the functions of heme proteins by spectroscopies.

Heme coordination structure and interactions between the surrounding amino acids and the heme iron complex in the proteins.
Iron Protoporphyrin IX, heme $b$
Prosthetic group
A flat and planar structure.
Itself Toxic $O_2^\cdot$, $H_2O_2$ are formed.
Insoluble

Heme $a$
Heme $a$ is a form of heme found
In cytochromes a and a3.
Cloning, expression and purification

*E. coli* K-12 genome

- Cloning
- pET21c
- Overexpression
- Purification

YddV contains a *b*-type heme with 1:1 stoichiometry.

Full-length YddV: 54 kDa
High Energies
Harmful, Dangerous
Cancer

Low Energies
Safe

radio waves
sub-mm
Infrared
visible light
Ultra-Violet
X-rays
gamma rays

LONG wavelengths
LOW energies

SHORT wavelengths
HIGH energies

red orange yellow green blue violet
energy

level 1

level 2

level 3

level 4

etc...

[Diagram of energy levels and transitions]
UV-Visible spectrometers
Absorbance spectra of 1.6 μM meso-tetra(N-methyl-4-pyridyl)porphyrin (TMPyP) and 1.6 μM zinc-tetrakis(4-sulfonatophenyl)porphyrin (Zn-TPPS) in 50 mM sodium phosphate (NaPi) buffer compared to the absorbance spectrum of the solid porphyrin crystals at room temperature (note the different scale).

Porphyrin is NOT planar, twisted, Insoluble, Aggregate, Broad spectra.

Porphyrin: Non-planar, Dimer or aggregation

Phenyl-porphyrin-Zn: Planar, Monomer, Diamagnetic, Aromaticity (Hückel's rule, 4n+2 rule, 18 delocalized $\pi$ electrons).

Benzene: six (4n+2, n=1) delocalized $\pi$ electrons.
UV-visible spectra of PfHRP2 reconstituted with various metallo-porphyrins. PfHRP2 was reconstituted with metallo-porphyrin in 200 mM sodium phosphate (pH 7.2) containing 1.1 μM PfHRP2 and a suspension of 100 μM FePPIX, SnPPIX, ZnPPIX, or PPIX for 5 min at 37 °C. Unbound metallo-porphyrin was then removed by gel filtration through a PD-10 column before the spectra of the reconstituted PfHRP2 (0.56 μM) were recorded in PBS.

Protoporphyrin IX: Dimer or aggregations, no binding to the protein.
Absorption spectra of Zn-cyt. C
Zn-porphyrin is planar, diamagnetic and has aromaticity.
Mb Fe(II) + CO

Soret band or $\gamma$–band

Visible region
Visible bands
Q bands
$\alpha$-band, $\beta$-band

High Energy: Ultraviolet (UV)
Low Energy: Infrared (IR)
Distal side
External axial ligand
$O_2$, CO, OH$, H_2O$

Internal axial ligand
Imidazolate

Proximal side

Fe(II)

Distal side pH 9: OH$^{-}$

pH 5: H$_2$O or nothing (vacant)
Heme Fe(II)-O₂  Oxy-hemoglobin,  Oxy-myoglobin
Heme Fe(II)      Deoxy-hemoglobin,  Deoxy-myoglobin
Heme Fe(II)-CO   Carbonmonoxy-hemoglobin,  Carbonmonoxy-myoglobin
Heme Fe(III)     Met-hemoglobin,  Met-myoglobin
Fe(II) weak ligand field, high spin state

Fe(II) strong ligand field, low spin state

Diagram shows the energy level splitting of d-orbitals under weak and strong ligand fields, with corresponding electronic configurations and spin states.
Fe(II):  O₂, CO, NO  
Fe(III):  CN⁻, OH⁻, F⁻, H₂O, NO

5-coordinated
External axial ligands: $O_2$, CO, NO, CN\(^{-}\), H\(_2\)O, OH\(^{-}\)

Distal side

6-coordinated

Ligand field
Strong: $O_2$, CO, NO – heme Fe(II), OH\(^{-}\), CN\(^{-}\) - heme Fe(III)
Low spin complex

Weak: vacant, H\(_2\)O – heme Fe(II), H\(_2\)O, F\(^{-}\), N\(^{3-}\) - heme Fe(III)
High spin complex

5-coordinated

Internal axial ligands: His, Cys
Proximal side
High spin complex: Iron is located out of the heme plane.

Low spin complex: Iron is located on the heme plane.
High spin complex: Iron is located out of the heme plane.

Low spin complex: Iron is located on the heme plane.
Electron Spin Resonance Spectroscopy: ESR Spectroscopy

Electron Paramagnetic Resonance Spectroscopy: EPR Spectroscopy

Heme Fe(III) complex, NO-heme Fe(II) complex: EPR OK
Fe(III) Spin EPR OK
Fe(II) No Spin NO EPR
NO Spin, NO-Fe(II) Spin EPR OK
ESR spectrum of Fe(II)NO HRI complex

**ESR spectral parameters of Fe(II)NO complexes**

<table>
<thead>
<tr>
<th>proteins</th>
<th>$g_3$</th>
<th>$A_3$ (mT)</th>
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<tbody>
<tr>
<td>HRI</td>
<td>2.010</td>
<td>1.70</td>
</tr>
<tr>
<td>CBS</td>
<td>2.008</td>
<td>1.66</td>
</tr>
<tr>
<td>CooA</td>
<td>2.015</td>
<td>1.60</td>
</tr>
<tr>
<td>sGC</td>
<td>2.005</td>
<td>1.60</td>
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<tr>
<td>cyt c'</td>
<td>2.010</td>
<td>1.60</td>
</tr>
<tr>
<td>Hb + IHP</td>
<td>2.008</td>
<td>1.65</td>
</tr>
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</table>

- The hyperfine triplet arises from coupling to the single $I = 1$ $^{14}$N nucleus of bound NO.
- 5-coordinated Fe(II)NO complex.
Fe(III)  Fe(II)  Fe(II)  Fe(II)

6-coordinated  6-coordinated

Cys  CO  NO

5-coordinated
Caloric restriction improves thermotolerance and reduces hyperthermia-induced cellular damage in old rats  FASEB J. 14, 79 (2000)

Figure 2. Caloric restriction lowers liver radical content both before and after heat stress. EPR spectra (77 K) of liver biopsies collected from (A) young euthermic control, (B) old CR sham-stressed control, (C) old CR heat-stressed, (D) old CON sham-stressed control, (E) old CON heat-stressed, and (F) E. coli MnSOD showing an immobilized manganese(II) EPR spectrum. The spectra show at least three species: Mn(II), a six-line feature centered at $g = 2.03$ hyperfine splitting $\approx 90$ G; features consistent with Fe(III) at $g = 2.25$ and 1.935; and a likely semiquinone radical at $g = 2.005$, $\Delta H_{pp} \approx 10$–15 G. Heat stress marginally elevated metal and semiquinone radical content in the CON group but lowered concentrations of these species in CR animals.
Characterization of a member of the NnrR regulon in *Rhodobacter sphaeroides* 2.4.3 encoding a haem–copper protein
Microbiology 148, 825 (2002)

Fig. 5. EPR spectrum of the as-isolated protein isolated from a strain expressing the NnrS-HT obtained with an IBM-Bruker ER-200 X-band EPR (9.51 Hz) and APD Cryogenics LTR-3 Helitran helium flow system operating at 13 K. Microwave power was 2 mW, field modulation was 0.0032 T and the EPR frequency was 9.513 GHz. T, tesla (=10⁴ gauss).
ESR spectrum of Fe(III)HRI

(A) ESR spectrum of the Fe(III)HRI complex and (B) Crystal field diagram for low-spin Fe(III) heme proteins

- Coordination structure of HRI
  - His-Fe(III)-Cys

<table>
<thead>
<tr>
<th>No.</th>
<th>proteins</th>
<th>axial ligand</th>
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<tr>
<td>1, 2, 3</td>
<td>CBS</td>
<td>His/Cys</td>
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<tr>
<td>4, 5, 6</td>
<td>CooA</td>
<td>Cys/Pro</td>
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<tr>
<td>7, 8, 9</td>
<td>P450</td>
<td>Cys/H₂O</td>
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<tr>
<td>10, 11, 12</td>
<td>P450 + N ligands</td>
<td>Cys/N</td>
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<tr>
<td>13, 14, 15, 16</td>
<td>Hb/Mb + imidazole</td>
<td>His/imidazole</td>
</tr>
</tbody>
</table>
Fe(III)  Fe(II)

Cys

6-coordinated
Raman Spectroscopy: The Molecular Detective

- Phonons (Rotational, Vibrational)
- Magnons
- Plasmons

Investigating isolated SWNTs.
Studying ultra thin films.
Studying Phase transitions under High Pressure and Temperature.

They are
1. Metal-Insulator and Charge-Ordering Transitions:
   \( \text{La}_{0.33}\text{Sr}_{0.66}\text{FeO}_3, \text{Pr}_{0.63}\text{Ca}_{0.33}\text{MnO}_3, \text{Fe}_3\text{O}_4, \text{CaFeO}_3 \)
2. Structural Transitions:
   Carbon Nanotubes, BN Nanotubes, LaB\(_6\).
Fig. 4. (a) Schematic illustration of a typical scattering spectrum containing the Rayleigh, Stokes and anti-Stokes bands. (b) A typical optical absorption spectrum of a heme protein. The Soret and Q bands originate from the $\pi$ to $\pi^*$ electronic transitions of the heme prosthetic group. Those in the 280 and 200 nm regions are assigned to the electronic transitions of the aromatic side-chain groups (Trp, Tyr and Phe) and the peptide backbone, respectively.
The Raman effect results from the interaction between electromagnetic radiation and a molecule. When an incident electromagnetic field, $E_i$, interacts with a molecule, an electric dipole is induced in the molecule, given by $P = \alpha \cdot E_i$, in which $\alpha$ is the polarizability of the molecule and $P$ is the induced dipole. The intensity of the resulting Raman signal is proportional to the square of the magnitude of this induced dipole moment $P$. Furthermore, when the incident photon energy ($h\nu_o$) coincides with the electronic transition energy of the molecule ($h\nu_{ex}$), the Raman intensity is strongly enhanced, typically by a factor of $10^2$–$10^6$. This is termed the resonance Raman (RR) effect. Metalloporphyrins present in heme-containing proteins have strong electronic transitions in the Soret (390–450 nm) and visible (500–600 nm) regions [34]. A typical example of the optical absorption spectrum, which reflects the electronic transitions, is shown in Fig. 4(b). When excited with a laser that has an output coincident these electronic transitions, the Raman spectrum of the heme moiety is resonance enhanced and that of the surrounding protein matrix is not. This allows the biophysicist to probe the heme binding site without spectral interference from the surrounding protein. Likewise, the vibrational modes of the aromatic residues, Try, Tyr and Phe, can be selectively enhanced with laser excitation at $\sim$280 nm [35], [36], [37], [38], [39], [40], [41], [42] and [43], and those of the polypeptide bonds can be resonance enhanced with $\sim$200 nm excitation [44] and [45].
Finger Print Regions

\( \nu_4 \): Heme redox marker band e.g. 1374 Fe(III), 1359 Fe(II)

\( \nu_3 \): Bond character marker band

\( \nu_2, \nu_{10} \): Heme spin/cooordination marker band

<table>
<thead>
<tr>
<th></th>
<th>( \nu_3 )</th>
<th>( \nu_2 )</th>
<th>( \nu_{10} )</th>
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<tr>
<td>Fe(III)</td>
<td>Fe(II)</td>
<td>Fe(II)</td>
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<tr>
<td>( 5cHs )</td>
<td>1487-1495</td>
<td>1465-1473</td>
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<tr>
<td>( 6cLs )</td>
<td>1501-1510</td>
<td>1493-1502</td>
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<tr>
<td>( \sim1573 )</td>
<td>( \sim1560 )</td>
<td>( \sim1580 )</td>
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<td>( \sim1580 )</td>
<td>( \sim1580 )</td>
<td>( \sim1580 )</td>
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<tr>
<td>( \sim1627 )</td>
<td>1601-1620</td>
<td>1615-1630</td>
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<tr>
<td>( \sim1638 )</td>
<td>1615-1630</td>
<td>1615-1630</td>
<td></td>
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</tbody>
</table>
$\nu_4$: Heme redox marker band

$\nu_3$: Bond character marker band

$\nu_2, \nu_{10}$: Heme spin/coordination marker band
High frequency region: Oxidation state, spin state, coordination number
Low frequency region: 
Fe-CO, Fe-C-O, Fe-O2
Convolution spectra

Difference spectra
Resonance Raman spectra

Fe(II)-O₂

λ_{ex} = 413.1 nm

^{16}O₂ - ^{18}O₂

<table>
<thead>
<tr>
<th>Raman Shift (cm⁻¹)</th>
<th>( v_{\text{Fe-O}} )</th>
<th>( v_{\text{O-O}} )</th>
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</thead>
<tbody>
<tr>
<td>WT</td>
<td>561</td>
<td>1141</td>
</tr>
<tr>
<td>Y45F</td>
<td>563</td>
<td>1150</td>
</tr>
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</table>

Tyr45 forms a hydrogen bond(s) with O₂.
Resonance Raman spectra

Fe(II)-^{16}O_{2}

\( \lambda_{ex} = 413.1 \) nm

D_{2}O

Tyr45 forms a hydrogen bond with proximal O.
Resonance Raman spectra

Fe(II)-$^{16}$O$_2$

$\lambda_{ex} = 413.1$ nm

D$_2$O

WT

Y45F

Tyr45 forms a hydrogen bond with proximal O?
Resonance Raman spectra

Fe(II)-CO

$\lambda_{ex} = 413.1$ nm

$^{12}\text{C}^{16}\text{O} - ^{13}\text{C}^{18}\text{O}$

<table>
<thead>
<tr>
<th></th>
<th>$\nu_{\text{Fe-CO}}$</th>
<th>$\nu_{\text{C-O}}$</th>
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<tr>
<td>WT</td>
<td>497</td>
<td>1958</td>
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<tr>
<td>Y45F</td>
<td>505</td>
<td>1954</td>
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</table>
Summary

Fe(II)-O$_2$  Half life > 3 days  
$K_d$ O$_2$  0.077, 0.67 µM

Crystal structures are important to characterize the heme surrounding.