X-ray Absorption Spectroscopy: Application to Biological Systems

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IUCr, Aug. 21. 2017
Synchrotron Science

MACROMOLECULAR CRYSTALLOGRAPHY

STRUCTURAL BIOLOGY

SMALL ANGLE X-RAY SCATTERING

X-RAY SPECTROSCOPY
Synchrotron Science

SMALL ANGLE X-RAY SCATTERING

MACROMOLECULAR CRYSTALLOGRAPHY

X-RAY SPECTROSCOPY
Conformation

~50 – 7 Å

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Atomic Position

~1 – 5 Å

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Small Angle X-ray Scattering

Atomic Position

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Macromolecular Crystallography

Electronic Structure

>>1 Å

X-ray Spectroscopy
• Introduction of X-ray Absorption Spectroscopy (XAS)
• Importance of XAS
• Factors affecting the XAS Spectra : Information Content
• Some XAS Examples
• EXAFS and the Biological Complication
• Examples from Homogeneous Biological (and otherwise) Catalysis
• Single Crystal XAS : Combination of XAS & EXAFS
An edge results when a core electron absorbs energy equal to or greater than its binding energy.

Edges are labeled according to the shell the core electron originates from.

XAS is an element specific technique.

Cu K-edge ~9000 eV  Fe K-edge ~7000 eV
Cu L-edges ~930 eV   Fe L-edges ~720 eV
Cu M-edges ~70-120 eV Fe M-edges ~50-100 eV

S K-edge ~2472 eV  S L-edges ~200 eV
Pre-edge and Edge (XANES) - Electronic and Geometric Information

EXAFS (extended x-ray absorption fine structure) - Geometric Information

XAS or XAFS
Metal K-edge XAS

M 4p
M 3d
L 3p
M 1s
Metal K-edge XAS

\[ \text{M 4p} \rightarrow \text{M 3d} \rightarrow \text{L 3p} \]

energy

pre-edge

pre-edge
Metal K-edge XAS

M 4p
M 3d
L 3p
M 1s

energy

pre-edge

edge

pre-edge
Metal K-edge XAS

Energy diagram showing transitions from M 4p, M 3d, and L 3p levels to continuum. Pre-edge and edge transitions are highlighted.
Metal K-pre-edge absorptions arise due to a quadrupole-allowed dipole-forbidden 1s → 3d excitation ($\Delta l = \pm 2$) - weak

Metal K-rising edge absorptions are electric dipole allowed ($\Delta l = \pm 1$) - Intense
Importance of XAS Edges

Fast data acquisition time and high signal-to-noise ratio. Can be measured at room temperature without depreciation of data quality. Used to estimate:

The pre-edge region can be used to estimate:
- Ligand-field
- Spin-State
- Centrosymmetry

The rising-edge region can be used to estimate:
- Geometric Structure
- Metal-Ligand overlap via Shakedown transitions
- Ligand arrangement in certain cases
- Charge on the metal center
Interpretation of XAS Edges

Qualitatively

Uses edges as a “fingerprint” of the electronic structure.
Compare to known model complexes.
Use in PCA analysis.

Molecular Orbital-Based Approach

Obtain a more quantitative description.
Energy and intensity distributions LF theory.
Works well for bound state transitions.
Fails for rising-edge and beyond.

Multiple Scattering-Based Approach

Required to simulate rising edge.
FEFF, MXAN.
Difficult to relate back to an MO-based picture.

Band Structure Approach

Density of States.
Oxidation State

The rising-edge and the edge maxima shift to higher energy as the oxidation state increases.

Important consideration – similar ligand system.
Factors that Affect Metal K-edge Shape and Energy

Oxidation State
Both Fe samples. What oxidation states do they represent?
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Spin states are different! High-Spin ($S=2$) and Low-Spin ($S=0$)
Oxidation State

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High-Spin (S=2) and Low-Spin (S=0)

Both Ni samples. What oxidation states do they represent?
Oxidation State

Factors that Affect Metal K-edge Shape and Energy

Both Fe samples. What oxidation states do they represent?

Spin states are different!
High-Spin ($S=2$) and Low-Spin ($S=0$)

Both Ni samples. What oxidation states do they represent?

Ni is special case with little change upon oxidation.
Factors that Affect Metal K-edge Shape and Energy

Coordination Number and Geometry
Factors that Affect Metal K-edge Shape and Energy

Coordination Number and Geometry

Coordination no: 2 3 4

Normalized Absorption

Energy (eV)

4p_{x,y,z}
Factors that Affect Metal K-edge Shape and Energy

Coordination Number and Geometry

Coordination no: 2 3 4

Normalized Absorption

Energy (eV)

Energy levels:
- $p_z$
- $p_{x,y}$
- $4p_{x,y,z}$

2-coord Cu(I)
Factors that Affect Metal K-edge Shape and Energy

Coordination Number and Geometry

- 2-coord Cu(I)
- 3-coord Cu(I)

Energy (eV)

Normalized Absorption

Energy (eV)

Coordination no: 2 3 4

Cu

p_z  p_y,z

p_{x,y}  p_x

4p_{x,y,z}
Factors that Affect Metal K-edge Shape and Energy

Coordination Number and Geometry

Coordination no: 2  3  4

2-coord Cu(I)  
3-coord Cu(I)  
4-coord Cu(I)

Normalized Absorption

Energy (eV)

8980  8990  9000  9010

Factors that Affect Metal K-edge Shape and Energy
Focusing on Metal K-edge Shape and Energy

**Factors that Affect Metal K-edge Shape and Energy**

---

**Coordination Number and Geometry**

- **2-coord Cu(I)**
- **3-coord Cu(I)**
- **4-coord Cu(I)**

Rising edge has strong contribution from the $1s \rightarrow 4p$ transition.

In special cases where the $4p$ orbital is low-lying, the energy and intensity of the edge transition can be used to estimate coordination number/geometry.
Factors that Affect Metal K-edge Shape and Energy
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Covalency

Factors that Affect Metal K-edge Shape and Energy

Normalized Absorption

Energy (eV)

Covalency

Factors that Affect Metal K-edge Shape and Energy

Normalized Absorption

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Normalized Absorption

Energy (eV)
Factors that Affect Metal K-edge Shape and Energy

Covalency

Normalized Absorption vs. Energy (eV)

- M 4p
- M 3d
- M 1s
- L 3p

Energy (eV):
- 8980
- 9000
- 9020
Factors that Affect Metal K-edge Shape and Energy

Covalency

![Graph showing energy levels and transitions](image)

- Shakedown
- M 4p
- M 3d
- L 3p
- M 1s
Energy and intensity can be correlated with metal-ligand overlap using the VBCI model. Typically transition present in square-planar complexes.

In comparable systems: \( \text{Intensity} \propto \text{Covalency} \quad \text{Energy} \propto \frac{1}{\text{Covalency}} \)
Factors that Affect Metal K-edge Shape and Energy

Pre-edge Shape and Energy

![Graph showing pre-edge shape and energy](image-url)
Factors that Affect Metal K-edge Shape and Energy

Pre-edge Shape and Energy

Normalized Absorption vs. Energy (eV)

Energy (eV)

Normalized Absorption

Factors that Affect Metal K-edge Shape and Energy
Pre-edge intensity $\propto$ Deviation from Centrosymmetry $\propto$ Metal 3d-4p mixing
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Pre-edge intensity pattern is dependent on:
- Spin-State
- Oxidation-State
- Ligand-Field splitting
- Multiplet-Effects
Factors that Affect Metal K-edge Shape and Energy

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Pre-edge intensity pattern is dependent on:
- Spin-State
- Oxidation-State
- Ligand-Field splitting
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Pre-edge intensity-weighted average energy is modulated by LF strength
Factors that Affect Metal K-edge Shape and Energy

Pre-edge Intensity Pattern

Pre-edge intensity $\propto$ Deviation from Centrosymmetry $\propto$ Metal 3d-4p mixing
Factors that Affect Metal K-edge Shape and Energy
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Pre-edge Intensity Pattern

Normalized Absorption

Energy (eV)

0.0 0.4 0.8 1.2

Td Oh

Fe Fe Fe Fe

Sq-py Td Sq-Py* Oh

Pre-edge intensity $\propto$ Deviation from Centrosymmetry $\propto$ Metal 3d-4p mixing

4p orbitals: $t_{1u}$ symmetry
3d orbitals: $t_{2g}$ and $e_g$ symmetry
No Mixing = Weak pre-edge
Factors that Affect Metal K-edge Shape and Energy

Pre-edge Intensity Pattern

Oh
4p orbitals: $t_{1u}$ symmetry
3d orbitals: $t_{2g}$ and $e_g$ symmetry
No Mixing = Weak pre-edge

Td
4p orbitals: $t_2$ symmetry
3d orbitals: $t_2$ and $e$ symmetry
Mixing = Intense pre-edge

$\preceq Fe \preceq$ > $\preceq Fe \preceq$ > $\preceq Fe \preceq$ > $\preceq Fe \preceq$

Sq-py  Td  Sq-Py*  Oh

Pre-edge intensity $\propto$ Deviation from Centrosymmetry $\propto$ Metal 3d-4p mixing
Factors that Affect Metal K-edge Shape and Energy

Pre-edge Energy

Energy (eV)

Normalized Absorption

M 3d

L 3p

Ligand Field

Z_{eff}

M 1s

Z_{eff}
Factors that Affect Metal K-edge Shape and Energy

Pre-edge intensity-weighted average energy is modulated by Ligand-Field strength
Pre-edge intensity-weighted average energy is modulated by Ligand-Field strength.

$Z_{\text{eff}}$ or charge on the metal affects the energy of all energy levels equally, therefore has minimal effect on pre-edge energy position.

Factors that Affect Metal K-edge Shape and Energy

Pre-edge Energy

Normalized Absorption

Energy (eV)

8980 9000 9020
Pre-edge Example 1: Cobalamin

Vitamin $B_{12}$ derivative: Cobalamin

Problem: Determination of Co-C bond distance in Me-Cobalamin
Pre-edge Example 1: Cobalamin

Crystallography consistently gave a long Co-C distance than reasonable. Question – Could the diffraction data have error from beam-damage/decomposition?
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Me-Cbl and H₂O-Cbl have similar EXAFS
Pre-edge Example 1: Cobalamin

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Me-Cbl and H₂O-Cbl have similar EXAFS

Pre- and rising-edge data distinct
Pre-edge Example 1: Cobalamin

Crystallography consistently gave a long Co-C distance than reasonable. Question – Could the diffraction data have error from beam-damage/decomposition?

Near-edge data were used to show a) crystal structure was erroneous b) determine the Me-Co distance to atomic resolution.
Pre-edge Quiz

Match the Spectra and Assign Oxidation State

Energy (eV)

Normalized Absorption
Pre-edge Quiz

Match the Spectra and Assign Oxidation State

![Graph showing normalized absorption vs energy (eV) for two spectra with peaks at different energies.]

A

B

Energy (eV)
Pre-edge Quiz

Match the Spectra and Assign Oxidation State

A

6-coordinate

B

5-coordinate
Pre-edge Quiz

Match the Spectra and Assign Oxidation State

Which deviates more from centrosymmetry?

Which system has least 4p mixing?

Can you guess the oxidation state of A and B?
Pre-edge Quiz

Match the Spectra and Assign Oxidation State

Which deviates more from centrosymmetry? B

Which system has least 4p mixing?

Can you guess the oxidation state of A and B?
Pre-edge Quiz

Match the Spectra and Assign Oxidation State

Which deviates more from centrosymmetry?  B

Which system has least 4p mixing?  6-coordinate, octahedral, centrosymmetric

Can you guess the oxidation state of A and B?
Pre-edge Quiz

Match the Spectra and Assign Oxidation State

Which deviates more from centrosymmetry? B

Which system has least 4p mixing? 6-coordinate, octahedral, centrosymmetric

Can you guess the oxidation state of A and B? Look at edge & pre-edge energies
Pre-edge Quiz

Match the Spectra and Assign Oxidation State

Which deviates more from centrosymmetry? B

Which system has least 4p mixing? 6-coordinate, octahedral, centrosymmetric

Can you guess the oxidation state of A and B? Look at edge & pre-edge energies

A, green spectrum, Co$^{3+}$

B, black spectrum, Co$^{2+}$
What About EXAFS ??
EXAFS Data
EXAFS Data

experimental raw data/$I_0$
EXAFS Data

experimental raw data/I₀

2nd-order background
EXAFS Data

**Raw Absorption**

- **Energy (eV)**
  - 8200
  - 8400
  - 8600
  - 8800
  - 9000

- **Experimental Raw Data/I₀**
  - 0.0
  - 5.0
  - 10.0
  - 15.0

- **Normaled Absorption**
  - 0.0
  - 0.4
  - 0.8
  - 1.2

**2nd-order background**

- Well normalized data:
  - pre-edge = 0
  - post-edge = 1

---

- **Experimental raw data/I₀**
  - 0.0
  - 2
  - nd-order background
EXAFS Data

- **Experimental raw data**: Raw absorption vs. energy.
- **Normalized data**: Well normalized data with pre-edge = 0 and post-edge = 1.
- **Expanded post-edge region**: Expanded view showing EXAFS structure observed.

2\textsuperscript{nd}-order background
The final EXAFS is multiplied by \( k^3 \) to compensate for the rapid attenuation with energy.

Less intuitive about local structure.
EXAFS Data & Fourier Transforms

The final EXAFS is multiplied by $k^3$ to compensate for the rapid attenuation with energy.

Less intuitive about local structure.

Fourier Transform (FT) is performed over a chosen $k$ range to yield the radial distribution.

Can be directly correlated to M-L distances.
EXAFS: Information Content

<table>
<thead>
<tr>
<th>How Many of What Type of atoms are at What Distance from the Absorbing Element</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distances</td>
</tr>
<tr>
<td>Coordination Nos.</td>
</tr>
<tr>
<td>Angle Information</td>
</tr>
<tr>
<td>Distance Range</td>
</tr>
<tr>
<td>Scattering Atom</td>
</tr>
<tr>
<td>Contamination Allowed</td>
</tr>
<tr>
<td>Typical sample concentration</td>
</tr>
</tbody>
</table>
Ordered Systems
Ordered Systems

- EXAFS more ordered (data to high $k$)

- Shell by Shell analysis is popular and FT based fitting is helpful.

- Rigid structure - similarity between related systems.
Biological Systems
Biological Systems

- EXAFS are more disordered (data to k=11-15 Å).
- Complete EXAFS analysis necessary for meaningful interpretation.
- Confidence mostly in first shell & second shell metal coordination.
Experimental Considerations

• Sample Requirement
  • ~1 mM in metal, 100 uL in volume, 20-30% glycerol/glassing agent.
  • 0.1-1 mM for heavy metals Z > Cu, ~2 mM for Z < Fe.
  • Duplicates for photoreducing systems.
Experimental Considerations

• **Sample Requirement**
  - ~1 mM in metal, 100 uL in volume, 20-30% glycerol/glassing agent.
  - 0.1-1 mM for heavy metals $Z > Cu$, ~2 mM for $Z < Fe$.
  - Duplicates for photoreducing systems.

• **Beamline Specification**
  - Liq He cryostat (10-15K) : **must**
  - 30+ element Ge Detector: **critical**
  - BL equipped with fast shutters, beam filters, ease of detuning: **critical**
  - Automated data measurement: **required**
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  • 30+ element Ge Detector: critical
  • BL equipped with fast shutters, beam filters, ease of detuning: critical
  • Automated data measurement: required

• Measurement Time
  • Time : 5-15 hours (per-sample, excluding duplicates)
  • Reproducibility : At least once
Combining Experiment and Theory
Combining Experiment and Theory

XAS pre- & near-edge

Electronic information: covalency, bond strength, type of ligands
Combining Experiment and Theory

**XAS pre- & near-edge**

**EXAFS**

Electronic information: covalency, bond strength, type of ligands

Structure: Information on Ligands

*How many, What type, How far.*
Combining Experiment and Theory

XAS pre- & near-edge

EXAFS

Electronic information: covalency, bond strength, type of ligands

Structure: Information on Ligands
How many, What type, How far.

Theoretical Correlation

Detailed Electronic Information
Combining Experiment and Theory

XAS pre- & near-edge

EXAFS

Structure: Information on Ligands
How many, What type, How far.

Electronic information: covalency, bond strength, type of ligands

Theoretical Correlation

Detailed Electronic Information

Structure Function Correlation
A low temperature solution intermediate: N$_2$ cleavage by a Mo(III) complex
A low temperature solution intermediate: N$_2$ cleavage by a Mo(III) complex
A low temperature solution intermediate: N$_2$ cleavage by a Mo(III) complex
Linear $\text{Mo}_2\text{N}_2$ (Mo-N=N-Mo) bond in the intermediate ~5 Å feature
Disproving small molecule crystal structure

\[
\text{Cu-Cu 2.9 Å, O-O 2.3 Å} \quad \Rightarrow \quad \text{Cu-Cu 3.6 Å, O-O 1.41 Å}
\]

catalytically important conversion

\[
\text{Cu-Cu 3.4 Å, O-O 1.66 Å}
\]

Intermediate trapped during fast interconversion of the two species?

Structurally/Functionally distinct?
Data and fits for \([\{\text{Cu(MePY2)}\}_2\text{O}_2\}]^{2+}\)

(a) the crystallographic Cu-Cu distance of 3.45 Å

(b) 100% 3.6 Å Cu-Cu

(c) a mixture of 80% 3.6 Å Cu-Cu and 20% 2.8 Å Cu-Cu.

Small molecule crystallography can be incorrect
• Sequential measurement of X-ray diffraction and X-ray absorption is possible.

• Sample requirements: Single crystals for polarized measurements: ~100 µm.

• Smaller proteins with heavier transition metals (higher than Ni) ~50 µm.

• Multiple crystals for standard XAS measurements.
Isotropic XAS
Small Sample Requirement

- Multiple crystals from small starting volume (~5 uL): solution XAS ~100 uL (~ 1mM).

Applicable to Imperfect Crystals

- Twins, multiples, poorly diffracting, cracked etc. several crystals on loop to increase signal
Small Sample Requirement

- Multiple crystals from small starting volume (~5 uL): solution XAS ~100 uL (~ 1mM).

Applicable to Imperfect Crystals

- Twins, multiples, poorly diffracting, cracked etc. several crystals on loop to increase signal

Direct Comparison to Crystallography

- Solution EXAFS may vary from crystallography due to changes in H-bonding or due to crystal packing effects. Single crystal XAS is a direct in-state comparison.

- Monitor photoreduction in single crystals and correlate to photo-damage in crystallography.
Oxyhemoglobin

Human interaction with $O_2$ Mediated by Hemoglobin (Hb)

- Fe containing $O_2$ transport protein
- Contains an Fe-porphyrin (heme)
- Present in all vertebrates
- Binds up to 4 $O_2$ molecule per Hb
Oxyhemoglobin

Fe$^{2+}$(Protoporphyrin IX)(His) + O$_2$  Fe-O$_2$ (Protoporphyrin IX)(His)

24 electrons undergo spin-pairing in the 4 subunits to form oxyhemoglobin!!
Electronic Structure

Ferrous

$\text{Fe}^{2+}$

$S=0,$

$\text{O}_2$

$S=0$

+rightharpoonup
+rightharpoonup

Ferric

$\text{Fe}^{3+}$

$S=1/2,$

$\text{O}_2^-$

$S=1/2$

+rightharpoonup
+rightharpoonup

$+$

$rightharpoonup$

$rightharpoonup$
Electronic Structure

Ferrous

Fe$^{2+}$  O$_2$
S=0,  S=0

Ferric

Fe$^{3+}$  O$_2^-$
S=1/2,  S=1/2

Is the metal center Reduced (Ferrous) or Oxidized (Ferric)?
• O-O Bond Distance indicates Reduced (Ferrous).

• Why is there a large spread in Fe-O ?
Solution Spectroscopy

- O-O Bond Distance derived from spectroscopy (rRaman) indicates Oxidized (Ferric).

Discrepancy between solution spectroscopy and x-ray crystallography??
• Crystal near-edge similar to solution.

• Structure analysis shows very similar $O_2$ bound geometry.

• Fe K-edge and pre-edge distinctly different from starting material - deoxyHb

• Curiously - Fe K-pre-edge for oxyHb in solution and crystalline forms are different.

• Since geometric structure is similar, does this point to electronic changes?
What do small molecule models with $\text{Fe}^{3+}\text{O}_2^-$ and $\text{Fe}^{2+}\text{O}_2$ look like?

Model Comparison

Fe K pre-edge XAS data and fits for low-spin ferric $[\text{Fe}^{\text{III}}(\text{TPP})(\text{ImH})_2]\text{Cl}$ and ferrous $[\text{Fe}^{\text{II}}(\text{TPP})(\text{ImH})_2]$. (A) $[\text{Fe}^{\text{III}}(\text{TPP})(\text{ImH})_2]\text{Cl}$ is well fit with two features with the lower energy representing transitions to states with $t^2_g$ hole character. (B) $[\text{Fe}^{\text{II}}(\text{TPP})(\text{ImH})_2]$ is well fit with a single feature. Analogous to $[\text{Fe}^{\text{III}}(\text{TPP})(\text{ImH})_2]\text{Cl}$, the high energy feature (~7115 eV) is part of the rising edge and not included in the pre-edge area (SI Table S3).

Model Comparison

What do small molecule models with $\text{Fe}^{3+}\text{O}_2^-$ and $\text{Fe}^{2+}\text{O}_2$ look like?
Solution & Crystal Pre-edge

\( \text{Fe}^{2+} \quad S=0 \)

\( \text{Fe}^{3+} \quad S=1/2 \)

\[ 1s \]

\[ e_g \]

\[ t_{2g} \]
DFT reproduces EXAFS distances and the differences in pre-edge.
Electronic Structure Interpretation

- Differences in Crystallography and Solution Spectroscopy Real.
- Electronic structure of oxyhemoglobin consists of both the ferrous and ferric components.
- Ferric dominates in solution and Ferrous dominates in crystal form.
Methyl-Coenzyme M Reductase catalyzes the final step of CH\textsubscript{4} formation in anaerobic archaea

\[ \text{Me-SCoM} + \text{H-SCoB} \rightarrow \text{CoM-S-S-CoB} + \text{CH}_4 \]

Very important from biological energy perspective - generates 1b tonnes of Methane annually!
An unprecedented Ni(III) site is formed during catalysis. This intermediate is proposed to have an organometallic Ni-C bond.
Crystallography - Challenging

How can we be convinced of the formation of the Ni\(^{3+}\) species?

If present what is our confidence level of the Ni-Me distance?

Is there photoreduction/differences in the crystal that can lead to an erroneous Ni-Me distance?
Solution Structure of Organometallic Intermediate

Ni K-edge data show formation of Ni$^{3+}$ in solution

Ni EXAFS data show presence of Ni-Me bond

Combination of edge and EXAFS data helped develop geometric and electronic structure
Evidence for In-Crystallo Intermediate Formation

Ni K-edge data confirm the formation of the intermediate *in-crystallo*

The data preclude significant photoreduction in the crystal

Soln and crystal data indicate similar geometric structures

2.08 Å used as benchmark
How is crystalline XAS Useful

• Biological EXAFS is a powerful technique that furnishes *atomic* resolution local structures of metalloprotein active site.

• XAS is a powerful technique to obtain valuable insights into the electronic structures of metalloproteins.

• Solution and crystalline structures of metalloproteins may vary *intrinsically*.

• Crystal structures may be mixtures, or photoreduced - XAS/EXAFS bring additional info.

• Structures can be low resolution - Complementarity with EXAFS important for electronic structure interpretation.
Limitations of the EXAFS Method

Very accurate first shell distances (+/- 0.02 Å), but “sees” the average of all of a given photoabsorber.

Ability to identify types of ligands, but only if ΔZ > 1 (for Z~6-17).

Not able to separate out contributions from different oxidations states of the same photoabsorber type (i.e. cannot separate Fe(III) EXAFS contribution from the Fe(IV) EXAFS contribution).

EXAFS range may be truncated due to the presence of Z+1 atoms (e.g. Zn in Cu EXAFS and Fe in Mn EXAFS)
Take Home Messages

What to look for in an EXAFS Paper?

- A good, high quality data set. Are the EXAFS data shown? How far out in k-space have the data been obtained? What is the fit range?

- Explanation of data processing and analysis packages used.

- Assessment of how the “best-fit” was arrived at.

- Potential discussion of correlated parameters and resolution of the data.

- Statistics

- Be suspicious of...filtered data, large changes in E₀, too many parameters.
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