The structure of molybdenum and tungsten enzyme active sites from X-ray absorption spectroscopy

or ..... What use is EXAFS if we know the crystal structure?

Graham N. George

Stanford Synchrotron Radiation Laboratory
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Overview

1. Introduction to the Molybdenum and Tungsten Enzymes.

2. X-ray absorption spectroscopy.

3. Formaldehyde Ferredoxin oxidoreductase.

4. DMSO reductase active site structure.

5. Conclusions.
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**Molybdenum and Tungsten Enzymes.**

- Mo and W are the only second and third period transition elements with well defined biological functions.
- In most (but not all cases) they catalyze two-electron redox reactions involving the transfer of an oxygen from the metal to the substrate, or the reverse reaction.

\[
(\text{Enzyme})^-\text{Mo}^{VI} \sim\text{O} + \text{R} \leftrightarrow (\text{Enzyme})^-\text{Mo}^{IV} + \text{R}\sim\text{O}
\]

**Examples:**

**DMSO reductase**

\[
(\text{Enzyme})^-\text{Mo}^{IV} + (\text{CH}_3)_2\text{S}=\text{O} \rightarrow (\text{Enzyme})^-\text{Mo}^{VI}=\text{O} + (\text{CH}_3)_2\text{S}
\]

\[
(\text{Enzyme})^-\text{Mo}^{VI}=\text{O} + 2\text{e}^- + 2\text{H}^+ \rightarrow (\text{Enzyme})^-\text{Mo}^{IV} + \text{H}_2\text{O}
\]

**Xanthine oxidase**

\[
(\text{Enzyme})^-\text{Mo}^{VI}=\text{OH}_2 + \text{R}^-\text{H} \rightarrow (\text{Enzyme})^-\text{Mo}^{IV} + \text{R}^-\text{OH} + 2\text{H}^+
\]

\[
(\text{Enzyme})^-\text{Mo}^{IV} + \text{H}_2\text{O} \rightarrow (\text{Enzyme})^-\text{Mo}^{VI}=\text{OH}_2 + 2\text{e}^-
\]
Molybdenum and Tungsten Enzymes.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>metal</th>
<th>mpt</th>
<th>Reaction</th>
<th>Crystal structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mo-containing Hydroxylases:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Xanthine Oxidase</td>
<td>Mo</td>
<td>1</td>
<td>$\text{RH} + \text{H}_2\text{O} \rightarrow \text{ROH} + 2\text{H}^+ + 2\text{e}^-$</td>
<td>×</td>
</tr>
<tr>
<td>Xanthine Dehydrogenase</td>
<td>Mo</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Purine Hydroxylase</td>
<td>Mo</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aldehyde Oxidase</td>
<td>Mo</td>
<td>1</td>
<td></td>
<td>×</td>
</tr>
<tr>
<td>Nitrate Reductases:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Respiratory</td>
<td>Mo</td>
<td>2</td>
<td>$\text{NO}_3^- + 2\text{H}^+ + 2\text{e}^- \rightarrow \text{NO}_2^- + 2\text{H}_2\text{O}$</td>
<td>×</td>
</tr>
<tr>
<td>Assimilatory</td>
<td>Mo</td>
<td>1</td>
<td>$\text{SO}_3^{2-} + 2\text{H}_2\text{O} \rightarrow \text{SO}_4^{2-} + 2\text{H}^+ + 2\text{e}^-$</td>
<td>×</td>
</tr>
<tr>
<td>Sulfite Oxidase</td>
<td>Mo</td>
<td>1</td>
<td></td>
<td>×</td>
</tr>
<tr>
<td>Formate Dehydrogenase</td>
<td>Mo</td>
<td>2</td>
<td>$\text{HCOO}^- \rightarrow \text{CO}_2 + \text{H}^+ + 2\text{e}^-$</td>
<td>×</td>
</tr>
<tr>
<td>Carbon Monoxide Oxidase</td>
<td>Mo</td>
<td>1</td>
<td>$\text{CO} + \text{H}_2\text{O} \rightarrow \text{CO}_2 + 2\text{H}^+ + 2\text{e}^-$</td>
<td>×</td>
</tr>
<tr>
<td>Biotin Sulfoxide Reductase</td>
<td>Mo</td>
<td>2</td>
<td>$\text{Biotin-S}=\text{O} + 2\text{H}^+ + 2\text{e}^- \rightarrow \text{Biotin-S} + \text{H}_2\text{O}$</td>
<td></td>
</tr>
<tr>
<td>DMSO Reductase</td>
<td>Mo</td>
<td>2</td>
<td>$(\text{CH}_3)_2\text{S}=\text{O} + 2\text{H}^+ + 2\text{e}^- \rightarrow (\text{CH}_3)_2\text{S} + \text{H}_2\text{O}$</td>
<td>×</td>
</tr>
<tr>
<td>Tetrathionate Reductase</td>
<td>Mo</td>
<td>2</td>
<td>$\text{S}_4\text{O}_6^{2-} + 2\text{e}^- \rightarrow 2\text{S}_2\text{O}_3^{2-}$</td>
<td></td>
</tr>
<tr>
<td>Trimethylamine N-oxide Reductase</td>
<td>Mo</td>
<td>2</td>
<td>$(\text{CH}_3)_3\text{N}=\text{O} + 2\text{H}^+ + 2\text{e}^- \rightarrow (\text{CH}_3)_3\text{N} + \text{H}_2\text{O}$</td>
<td>×</td>
</tr>
<tr>
<td>Arsenite Oxidase</td>
<td>Mo</td>
<td>2</td>
<td>$\text{H}_3\text{AsO}_3 + \text{H}_2\text{O} \rightarrow \text{H}_2\text{AsO}_4^- + 3\text{H}^+ + 2\text{e}^-$</td>
<td>×</td>
</tr>
<tr>
<td>Aldehyde ferredoxin oxidoreductase</td>
<td>W</td>
<td>2</td>
<td>$\text{RH} + \text{H}_2\text{O} \rightarrow \text{ROH} + 2\text{H}^+ + 2\text{e}^-$</td>
<td>×</td>
</tr>
<tr>
<td>Formaldehyde ferredoxin oxidoreductase</td>
<td>W</td>
<td>2</td>
<td>$\text{HCHO} + \text{H}_2\text{O} \rightarrow \text{HCOOH} + 2\text{e}^- + 2\text{H}^+$</td>
<td>×</td>
</tr>
</tbody>
</table>
Molybdenum and Tungsten Enzymes.

- Mo and W enzymes share a common "molybdopterin" cofactor.

- One or two cofactors are present, depending on the enzyme.
*Alcaligenes fcalis* arsenite oxidase crystal structure

Alcaligenes fecalis arsenite oxidase active site crystal structure

What is X-ray Absorption Spectroscopy?

- Scan the energy (wavelength) of an X-ray beam; monitor the absorption of X-rays by a sample as a function of incident X-ray energy.

\[
\text{Absorbance} = \log_e \left( \frac{I_0}{I_1} \right)
\]
**X-ray Absorption Spectroscopy - Basic Physics.**

- X-ray Absorption Spectra arise from ejection of a core electron.

---

(a) not enough energy to eject a core electron

(c) Just enough X-ray energy to eject a core electron: a low energy photo-electron is generated (long DeBroglie $\lambda$).

(b) "threshold" or absorption-edge X-ray energy, $E_0$

(d) core electron easily ejected: a higher energy photo-electron is generated (short DeBroglie $\lambda$).

**EXAFS**

**EXAFS = Extended X-ray Absorption Fine Structure**

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EXAFS - Basic Physics.

- EXAFS can be thought of as arising from interference between outgoing and backscattered DeBroglie photo-electron waves.

  Schematic diagram of the final-state wave function used to calculate the EXAFS

  - The absorption $\mu$ is a maximum when the backscattered wave is in phase with the outgoing wave at the absorber (constructive interference).
  - The absorption $\mu$ is a minimum when the backscattered wave is out of phase with the outgoing wave (destructive interference).
  - As the X-ray energy $E$ increases, the DeBroglie wavelength decreases and the observed EXAFS oscillations are due to successive periods of constructive and destructive interference.
EXAFS Data Reduction Methods - the Fourier transform.

The EXAFS Fourier transform of \([\{(C_6H_5)_4As\}]Cl\).

The different contributions to the EXAFS can be clearly seen as Fourier transform peaks approximately at a distance corresponding to the atomic separation. The Fourier transform has been phase-corrected for carbon back-scattering.
**Structural parameters that are available from EXAFS analysis:**

- Average bond-lengths, $R$
- Coordination Numbers, $N$
- Debye-Waller factors, $\sigma^2$

$\sigma^2$ is the mean-square displacement of the bond-length from the average value $R$. It has components from atomic vibration and disorder. Debye-Waller factors can be thought of as being similar to a crystallographic temperature factor. They are different in that they are due to relative displacements of atoms.

- Geometric information is generally not available. Sometimes the presence of *multiple scattering* allows bond-angle determination.
**X-ray Absorption Spectroscopy – Strengths and Limitations:**

- **Near-edge spectra are sensitive to oxidation state.**

- **Very accurate values for bond-lengths.**
  Bond lengths are typically determined with an accuracy of better than ±0.02 Å. Precisions are smaller than this, and normally reflect the accuracy.

- **Bond-length resolution is poor.**
  For similar scatterers (e.g. two different Mo–S) this is governed by the $k$–range of the data. The resolution limit $\Delta R \approx \pi/2k$, where $k$ is the extent of the data in Å$^{-1}$, and for a typical data set $\Delta R$ will be in the range 0.10 – 0.15 Å.

- **Coordination numbers are only moderately well determined.**
  Coordination numbers and Debye-Waller factors are determined to ca. ±25%. The uncertainty arises mostly from their high mutual correlation in the curve-fitting analysis.

- **EXAFS of mixtures provides an average radial structure.**
  This is both a limitation and a strength. It is a limitation when compared to techniques such as EPR which is very good at resolving mixtures.

- **EXAFS analysis can sometimes be problematic.**
Complementing Protein Crystallography with XAS.

A comparison of EXAFS Fourier transforms computed using crystallographic data and experimental EXAFS.

**A**: A low molecular weight complex, for which the structure is accurately known from crystallography. The computed EXAFS and the experimental data are in excellent agreement.

**B**: A molybdenum enzyme (*E. coli* formate dehydrogenase), solved to 2.8 Å resolution vs. a solution of (apparently) the same form of the enzyme. There is considerable discrepancy between experimental and computed EXAFS Fourier transforms.
Experimental Aspects:

A typical X-ray Absorption Spectroscopy experiment on SSRL’s beamline 6-2. The setup shown uses a low-temperature liquid helium cryostat, a Canberra 13 element Germanium X-ray fluorescence detector (on the left) and a Stern-Heald-Lytle X-ray fluorescence detector (right hand side). The X-ray beam enters the experiment from the beampipe on the right hand side of the photograph, and exits at the bottom left of the picture.
Pyrococcus furiosus Formaldehyde Ferredoxin Oxidoreductase.

- Catalyses the two-electron oxidation of formaldehyde to formate:
  \[ \text{HCHO} + \text{H}_2\text{O} \rightarrow \text{HCOOH} + 2e^- + 2\text{H}^+ \]
- Contains two molybdopterin cofactors, plus one Fe$_4$S$_4$ cluster.

**Pyrococcus furiosus** Formaldehyde Ferredoxin Oxidoreductase.

- 1.85 Å active site crystal structure
  

- 1 W–O at 2.1 Å, 4 W–S at 2.5 Å

- *Van der Waals radius of active site oxygen overlaps with one sulfur.*

- *Unusual asymmetric geometry around the tungsten.*

- *Un-assigned electron density present on one site of tungsten.*
**Pyrococcus furiosus** Formaldehyde Ferredoxin Oxidoreductase

*Perspective from EXAFS.*

*Anaerobically isolated oxidized enzyme.*


- **EXAFS unambiguously indicates a dioxo** $W^{VI}$ **active site.**
**DMSO reductase** - published active site crystal structures prior to 2000


3. McAlpine, A. S.; McEwan, A. G.; Shaw, A. L.; Bailey, S.  

4. McAlpine, A. S.; McEwan, A. G.; Bailey, S.  

- All structures have nearly identical polypeptide folds.
- All have totally different molybdenum active site structures.
**DMSO reductase** - published active site crystal structures:

- **Oxidized Mo**\(^{VI}\) **DMSO reductase**

  *The different crystal structures suggest different active site structures.*

  A mono-oxo 5 coordinate Mo site with one pterin dithiolene coordinated, and the other bound by only one sulfur.
**DMSO reductase** - published active site crystal structures:

- **Oxidized Mo\(^{VI}\) DMSO reductase**

  *The different crystal structures suggest different active site structures.*

  A di-oxo 5-coordinate Mo site with one pterin dithiolene coordinated.
**DMSO reductase** - published active site crystal structures:

- Oxidized Mo$^{VI}$ DMSO reductase

*The different crystal structures suggest different active site structures.*

A di-oxo 7-coordinate Mo site with both pterin dithiolenes bound.
**DMSO reductase** - published active site crystal structures:

- **All** reported crystal structures contain some chemically unexpected features.
- *e.g.* 7-coordinate oxidized DMSO reductase structure:

*The proposed enzyme active site structure contains several supposedly non-bonded atoms with overlapping Van der Waals radii (shown as wire-frame spheres).*
Di-oxo Molybdenum (VI) bond angles

- Very extensive chemical literature indicates that a typical O=Mo=O angle is 106°.
- There are no reported small molecule structures for which this angle is less than 95°.

The O=Mo=O angle in MoO$_2$(tetra-p-tolylporphrinate) is 95.1° [3]. It is a highly strained structure, as illustrated by the large deviations of the porphyrin ring from planarity.

- Values reported from protein crystallography of 80° for TMAO reductase [1] and 70° for DMSO reductase [2] are chemically unexpected.

DMSO reductase active site structure - the perspective from EXAFS.

DMSO reductase active site structure - the perspective from EXAFS.

- **No reported crystal structures are in agreement with the XAS analysis.**

- **Resonance Raman spectroscopy supports the XAS structures.**

- **Mo(V) EPR spectroscopy (\(^{17}\)O labeling) supports the XAS structures.**
DMSO reductase active site structure - the perspective from EXAFS.

- The recombinant as-isolated enzyme is different from the oxidized wild type. It is changed to a form identical to the wild-type by a cycle of reduction and re-oxidation, called "redox conditioning".

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Crystallography of mixtures provides an average picture:

- With small molecule crystallography fractional occupancy of sites would in most cases be detectable, but with proteins this might not be the case.

- With EXAFS usually just the radial structure is detected. With a mixture of similar species the correct number and (probably) the type of ligands should be determined.
**X-ray photo-reduction**

- Intense synchrotron X-ray beams can photo-reduce, or sometimes photo-oxidize samples.
- With XAS this is easy to see as the near-edge spectrum shifts.

With crystallography photo-reduction is more difficult to see.
- With crystallographic experiments the sample typically receives ~300 fold greater dose of X-rays than with EXAFS.
- The metal oxidation state can be uncertain in crystallographic data sets.
**EXAFS vs. Protein Crystallography**

- For directly coordinated ligands to a metal EXAFS can be more accurate than protein crystallography.
- EXAFS accuracy is generally better than ± 0.02 Å
- Protein crystallography accuracy ± 0.3 Å

Mo=O bond-length 1.74 Å, thermal factor for both Mo and O assumed to be 10 Å²
Crystallographic Refinement

The $R$-value is used as an index of accuracy of the structure.

- dates from the pre-computer era.

\[
R = \frac{\sum |F_o(h) - F_c(h)|}{\sum |F_o(h)|}
\]

- For small molecules a typical $R$ would be about 0.02, for proteins $R$ is almost never less than about 0.15, with a more typical value of about 0.25.

$F_o(h)$ – Observed structure factor.

$F_c(h)$ – Calculated structure factor from the current model.
Crystallographic Refinement

What exactly is refined in a protein crystal structure refinement?

\[ R' = \sum_{all \ h} (F_o(h) - F_c(h))^2 + \sum_{all \ g} (g_o - g_c)^2 \]

- \( F_o(h) \) – Observed structure factor.
- \( F_c(h) \) – Calculated structure factor from the current model.
- \( g_c \) – Calculated value of a geometrical parameter (e.g. a bond length) in the current model.
- \( g_o \) – Value of the same geometrical parameter as determined in small molecule crystal structures.

- If restraints are not used then unreasonable geometries can be obtained.
- If restraints are used then the crystal structure solution is biased towards the values of the restraints.
DMSO reductase active site structure

Recently a 1.3 Å crystal structure of *Rhodobacter sphaeroides* DMSO reductase shows a mixture of two different structures in the crystal.

Li, H-K.; Temple, C.; Rajagopalan, K.V.; Schindelin, H. *J. Am. Chem. Soc.* 2000, 122, 7673-7680. This study confirms our previous conclusion that earlier crystallographic analysis of the active site are in error.
**DMSO reductase active site structure**

- *Crystal Structure of mixture component B is very similar to that derived from EXAFS*

But - The new structure still contains chemically unreasonable features!

*Non-bonded atoms (serine O and molybdopterin S) have overlapping Van der Waals radii (shown as wire-frame spheres).*
**DMSO reductase** - published active site crystal structure of DMS-reduced form.

The proposed enzyme active site structure contains several supposedly non-bonded atoms with overlapping Van der Waals radii.

- Crystallography indicates an unusually long Me₂S=O bond (1.7 vs. 1.4 Å).
- EXAFS cannot detect the distant sulfur from DMSO.
DMSO reductase forms a substrate complex with trimethylarsine.

- $\text{Me}_2\text{S}$ and $\text{Me}_3\text{As}$ form complexes with very similar UV-visible spectra.
- Both are $\text{Mo(V)}$ EPR silent
- Both form with near-stoichiometric enzyme and $\text{Me}_3\text{As} / \text{Me}_2\text{S}$.

- Near-edge spectra indicate Mo is reduced, and As is oxidized.
Trimethylarsine reduced DMSO reductase:

- Mo and As K-edge EXAFS give a detailed active site structure.
Trimethylarsine reduced DMSO reductase:

- Mo and As K near-edge spectra indicate $\text{Mo}^{IV}$ and $\text{As}^V$ oxidation states.
- Mo and As K-edge EXAFS indicate no unusual structural features.
- The unusually short $\text{Me}_2\text{S}=\text{O}$ bond in the crystal structure of DMS-reduced enzyme may be an artifact.
Conclusion: What use is EXAFS if we know the crystal structure?

Without spectroscopy our view of the DMSO reductase active site would be very different.

- The conclusions from earlier spectroscopic studies were mostly ignored in the first crystal structure studies.
- Protein crystallography refinement alone did not provide accurate active site structures.
- To obtain an accurate structural picture the crystallography needed to be supplemented by spectroscopic information (i.e. from EXAFS, EPR and Resonance Raman).

- Caveat: One EXAFS study analyzed their data starting from the crystallographic results. This work yeilded an erroneous interpretation with physically unreasonable Debye-Waller factors.