SAXS and Biochemical Methods

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Today’s plan

- Importance of functional information to complement structural data (guide experimental design, interpret experimental results) with special reference to:
  - Equilibrium and time-resolved spectroscopic techniques focusing on:
    - Absorbance and fluorescence spectroscopies with
  - A few Examples (and some troubleshooting?)
GUEST COMMENTARY

Ten Commandments: Lessons from the Enzymology of DNA Replication

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I. RELY ON ENZYMEOLOGY TO CLARIFY BIOLOGIC QUESTIONS

II. TRUST THE UNIVERSALITY OF BIOCHEMISTRY AND THE POWER OF MICROBIOLOGY

III. DO NOT BELIEVE SOMETHING BECAUSE YOU CAN EXPLAIN IT

IV. DO NOT WASTE CLEAN THINKING ON DIRTY ENZYMES

By Efraim Racker

V. DO NOT WASTE CLEAN ENZYMES ON DIRTY SUBSTRATES
General Mode of Action of Proteins:

**Binding** to another (macro)molecule: a small molecule, another protein, a nucleic acid, the substrate if an enzyme

- Transcription
- Translation
- Signal transduction
- Allosteric activation/Inactivation
- Catalysis
Proteins may undergo conformational changes upon binding their ligand, and the ligand may be modified by the protein:
- (regulating) conformational change(s),
- chemical modification = reaction
The description of a biological process includes the description of how the energy of the system changes during the process, which requires equilibrium and kinetic studies.

\[
\begin{align*}
A + B & \underset{k_r}{\xrightarrow{k_f}} C + D \\
K_{eq} &= \frac{[C]_{eq} \cdot [D]_{eq}}{[A]_{eq} \cdot [B]_{eq}} = \frac{k_f}{k_r} \\
\Delta G^\circ &= -RT \ln K_{eq} \\
k &= K \frac{k_B T}{h} e^{-\frac{-\Delta G^\neq}{RT}}
\end{align*}
\]
For a multistep process (e.g.: an enzyme reaction): multiple stable intermediates, transition states, equilibrium constants, rate constants
Protein folding, protein-protein interaction, conformational changes can also be described through equilibrium and kinetic approaches.
The description of a biological process requires the determination of the concentrations of the reagents and products at any given time to determine rates and equilibrium constants.

\[ A + B \overset{k_f}{\underset{k_r}{\rightleftharpoons}} C + D \]
**Discontinuous methods** for the Detection and Quantitation of the reaction components are time-consuming, and they often require:

- Quenching of the reaction
- Chromatographic separation of the reaction components at different times followed by:
  - Detection and Quantitation of the reaction components by UV, Vis Abs, Fluorescence; Conductivity; Radioactivity, ....
Discontinuous methods may not allow the isolation and identification of (unstable) intermediates or products including protein-protein, protein-ligand complexes, etc.
Absorbance and fluorescence spectroscopies can allow the rapid acquisition of signals over time with high sensitivity.

The absorption and fluorescence spectra often allow to identify and quantify the chemical species in solution

\[
A + B \xrightleftarrows^{k_1} \xrightarrow{k_2} C + D
\]

The array of spectroscopic methods we can use to monitor also the kinetics of a biological process is increasing.
Today’s plan (2):

- Basic principles of absorbance and fluorescence spectroscopies
- Applications of absorbance and fluorescence spectroscopies to the study of the properties of proteins with special reference to the characterization of enzyme reactions
Absorption and fluorescence spectroscopies use a narrow region (200-800 nm) of the electromagnetic spectrum.
Basic principles of absorption and fluorescence spectroscopies

Only some electronic transitions are possible when a sample is irradiated with near UV – visible light
The set of possible transitions is typical of a given (macro)molecule depending on its precise structure and environment.
- A large number of compounds absorb in the near UV region

- Compounds with (several) conjugated double bonds yield complex absorbance spectra, with absorption also in the visible region

- The absorption spectrum of a compound (especially in the visible region) allows its identification and quantitation:

\[ A_\lambda = \varepsilon_\lambda \ast c \ast l \]
Basic Scheme of a spectrophotometer

\[ A = \ln \left( \frac{I_0}{I} \right) = \varepsilon \, c \, l \]

Absorbance
For absorption spectroscopy

Dual (double) beam spectrophotometer

( Photo ) Diode array spectrophotometer

Differences:

- Stability
- Resolution
- Signal-to-noise
- Linearity of response
- Artifacts (e.g.: photoreactions/degradation)
- Speed

Figure 1. Schematic of photodiode array spectrophotometer
Nanodrop:
Similar to diode array, but CCD (Charge Coupled Device) detector

0.5 - 1 mm lightpath

(Better to record the entire spectrum to check/avoid artifacts)
(Make sure the actual reading is not too low or too high)
Basic Scheme of a spectrophotofluorometer

Intensity of emitted light at
\( \lambda_2 > \lambda_1 \)  
I, low

Detector
(with scanning capability)

Monochromator
(with scanning capability)

Sample cuvette
with \( c \) moles/liter of absorbing species

Intensity of incident light
\( \lambda_1 I_0 \)
## Absorption versus fluorescence spectroscopy

<table>
<thead>
<tr>
<th></th>
<th>Absorption</th>
<th>Fluorescence</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Detection limits</strong></td>
<td>μM – mM</td>
<td>≤ μM</td>
</tr>
<tr>
<td><strong>Linearity of signals</strong></td>
<td>2 orders of magnitude (e.g.: 1 – 100 μM)</td>
<td>≈Narrow (e.g.: 0.1 – 1 μM) inner filter effects</td>
</tr>
<tr>
<td><strong>Quantification of solute</strong></td>
<td>ε, extinction coefficient ( M^{-1} \ \text{cm}^{-1} )</td>
<td>( F \propto c*l, ) in arbitrary units</td>
</tr>
<tr>
<td><strong>Sensitivity to:</strong></td>
<td></td>
<td>Very high (can be exploited)</td>
</tr>
<tr>
<td>- Temperature</td>
<td></td>
<td>(Relatively) slow</td>
</tr>
<tr>
<td>- Solvent</td>
<td></td>
<td>(can be exploited: measure fluorescence decay over time after flash of exciting light)</td>
</tr>
<tr>
<td>- Other solutes</td>
<td></td>
<td>May imply FRET</td>
</tr>
<tr>
<td><strong>Return to ground state</strong></td>
<td>Very fast</td>
<td></td>
</tr>
</tbody>
</table>
Other powerful spectroscopic techniques (not discussed)

Circular dichroism

Fluorescence anisotropy

Fluorescence decay measurements

FRET

.......
Several natural compounds absorb light in the UV and visible region of the spectrum.

Some are also fluorescent
Intrinsic chromophors in proteins

Amide bond (220 nm): use to detect proteins and peptides

![Amide bond structure](image)

Aromatic amino acids: Use absorbance at 280 nm to detect and quantify proteins

![Aromatic amino acids](image)

In general: a 1 mg/ml solution will absorb 1 at 280 nm
Several natural compounds absorb light in the UV and visible region of the spectrum and are fluorescent.

Aromatic amino acids:
Use absorbance at 280 nm to detect/quantify proteins but also exploit the sensitivity of their fluorescence to the environment to monitor folding/unfolding, dimerization and conformational changes (e.g.: upon ligand binding).
Several prosthetic groups (coenzymes and cofactors) absorb light in the UV and visible region and are intrinsic chromophores of proteins. Modifications of the prosthetic groups and or their environment alter the absorption spectrum providing tools to monitor changes in their ligation or redox state and of their environment.

Use for:
Protein identification and quantitation;
Study of protein function, conformational changes, ....
Cobalamins (vit B12 derivatives)

Methionine synthase (MetH): enzyme forms part of the catalytic cycle

Pyridoxal phosphate (PLP)

Decarboxylation

Racemization

Transamination

β Elimination

Retro Aldol Cleavage

Others
The flavin coenzymes FMN and FAD are derivatives of riboflavin (vitamine b2) and participate in oxidoreduction reactions.
Flavoproteins catalyze a large number of different (redox) reactions, and are among the best characterized enzymes thanks to the sensitivity of the flavin absorption (and fluorescence) spectrum to (small) changes in their state/environment.

Flavoenzymes classes: Dehydrogenases, Electrontransferases, Dioxygenases, Oxidases, Monooxygenases
Examples of some applications of absorption and fluorescence spectroscopies during protein purification and characterization
Track/Identify your protein
Absorbance-monitored gel filtration chromatography for buffer exchange, polishing removal of non specific aggregates or resolution of different oligomerization states

Figure 2 | Gel filtration profiles. Representative good (left) and bad (right) gel-filtration profiles of two different proteins purified on an ÄKTAxpress system using a HiLoad Superdex 200 column (GE Healthcare).
Use of absorbance spectroscopy for protein concentration determination:
critical for stoichiometry (cofactor content, ligand binding),
specific activity, mass/shape (by SAXS) determination

**UV absorbance** (computed/determined)
**Vis absorbance** (if chromophore is present and extinction coefficient is known)

Colorimetric methods:

- Biuret:
- Lowry
- Bradford
- BCA
- 660 dye
Figure 1. Reaction schematic for the Modified Lowry Protein Assay.

Figure 1. Reaction schematic for the Coomassie dye-based protein assays (the Coomassie (Bradford) Protein Assay and the Coomassie Plus (Bradford) Assay).

Figure 1. Reaction schematic for the bicinchoninic acid (BCA)-containing protein assay.
Protein concentration determination – Colorimetric methods exploit absorption changes of reagents in the free/protein bound form

<table>
<thead>
<tr>
<th>Method</th>
<th>Lower limit of calibration curve (μg in 1ml assay)</th>
<th>Sensitivity to Protein aa composition</th>
<th>Sensitivity to interference/Troubleshooting</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biuret</td>
<td>15</td>
<td>low</td>
<td>More or less all are sensitive to buffer, reducing agents, detergent, denaturants (guanidine)!</td>
</tr>
<tr>
<td>Lowry</td>
<td>10</td>
<td>low</td>
<td>- Use 3-5 different protein quantities and check linearity. Intercept should be zero.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>- Check effect of your buffer added in a fixed amount in Std curve and your samples</td>
</tr>
<tr>
<td>Bradford</td>
<td>1</td>
<td>high</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>- Pre-precipitate protein (make sure it is re-solubilized prior to assay)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>- Does your protein precipitate in assay? (check effect of order of reagents addition)</td>
</tr>
<tr>
<td>BCA</td>
<td>1</td>
<td>low</td>
<td></td>
</tr>
<tr>
<td>660 dye</td>
<td>1</td>
<td>low(?)</td>
<td></td>
</tr>
</tbody>
</table>

commercial formulations; see also: http://wolfson.huji.ac.il
### Calibration curve

<table>
<thead>
<tr>
<th>BSA</th>
<th>ug/ul</th>
<th>ul</th>
<th>ug</th>
<th>A595</th>
<th>retta</th>
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<tbody>
<tr>
<td>0.56</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.05</td>
<td>0.05</td>
</tr>
<tr>
<td>0.56</td>
<td>2</td>
<td>1.12</td>
<td>0.05</td>
<td>0.05</td>
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<tr>
<td>0.56</td>
<td>4</td>
<td>2.24</td>
<td>0.1089</td>
<td>0.1089</td>
<td></td>
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<tr>
<td>0.56</td>
<td>6</td>
<td>3.36</td>
<td>0.1487</td>
<td>0.1487</td>
<td></td>
</tr>
<tr>
<td>0.56</td>
<td>8</td>
<td>4.48</td>
<td>0.1842</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.56</td>
<td>10</td>
<td>5.6</td>
<td>0.226</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.56</td>
<td>12</td>
<td>6.72</td>
<td>0.2662</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

![Graph of Calibration curve](image)

### Assay

<table>
<thead>
<tr>
<th>sample</th>
<th>F</th>
<th>ul</th>
<th>A595</th>
<th>ug</th>
<th>ug/ul</th>
<th>ug/ul*F</th>
<th>Ave</th>
<th>[spetto]</th>
<th>B/S</th>
</tr>
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<tbody>
<tr>
<td>MOCH 0</td>
<td>6</td>
<td>2</td>
<td>0.053</td>
<td>1.164835</td>
<td>0.582418</td>
<td>3.494505</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.0518</td>
<td>1.138462</td>
<td>0.569231</td>
<td>3.415385</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>0.107</td>
<td>2.351648</td>
<td>0.587912</td>
<td>3.527473</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>0.113</td>
<td>2.483516</td>
<td>0.620879</td>
<td>3.725275</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>0.1607</td>
<td>3.531868</td>
<td>0.588645</td>
<td>3.531868</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>0.1663</td>
<td>3.654945</td>
<td>0.609158</td>
<td>3.654945</td>
<td>3.558242</td>
<td>3.29</td>
<td>1.081532449</td>
<td></td>
</tr>
</tbody>
</table>

![Graph of Assay](image)
The Bradford Assay with DMGDH samples in 3 M Gu/HCl (30 mM GuHCl in assay – constant) is sensitive to the **order of addition of reagents** (maybe due to protein precipitation in assay format A?).

**Assay method B**
- Bradford reagent: 1 ml
- 0.1 M NaCl: 90 ul
- 3 M Gu/HCl buffer: (10 – x) ul
- **Protein solution**: x ul

**Assay method A**
- 0.1 M NaCl: 90 ul
- 3 M Gu/HCl buffer: (10 – x) ul
- **Protein solution**: x ul
- **Bradford reagent**: 1 ml

No such problem with BCA assay

From Mariangela Camozzi thesis
Theoretical/calculated $\varepsilon_{280}$ value for a protein

http://web.expasy.org/protparam/

The principle:

$$\varepsilon_{280} = n_{\text{Tyr}} \varepsilon_{280, \text{Tyr}} + n_{\text{Trp}} \varepsilon_{280, \text{Trp}} + n_{\text{cystine}} \varepsilon_{280, \text{cystine}}$$

Where:

$$\varepsilon_{280, \text{Tyr}} = 1490 \text{ M}^{-1}\text{cm}^{-1}, \quad \varepsilon_{280, \text{Trp}} = 5500 \text{ M}^{-1}\text{cm}^{-1}, \quad \varepsilon_{280, \text{cystine}} = 125 \text{ M}^{-1}\text{cm}^{-1}$$

calculated at pH 6.5, in 6.0 M guanidinium hydrochloride, 0.02 M phosphate buffer.

Two values: one assuming that all Cys are free (no Abs), one assuming that all Cys form SS bonds (low $\varepsilon_{280, \text{cystine}}$)

Gu/HCl should have little effect on $\varepsilon_{280}$, but better check with denatured and dialyzed protein
If your protein contains a chromophor absorbing light in the UV region....
Take into account the presence of the chromophore, which absorbs light in the UV, to calculate the $\epsilon_{280}$ of the protein

Example:

Dimethylglycine dehydrogenase (DMGDH), a mitochondrial enzyme containing covalently bound FAD, participating in the metabolism of one-carbon units

\[
\epsilon_{280} = n_{\text{Tyr}} \epsilon_{280,\text{Tyr}} + n_{\text{Trp}} \epsilon_{280,\text{Trp}} + n_{\text{cystine}} \epsilon_{280,\text{cystine}} + n_{\text{FAD}} \epsilon_{280,\text{FAD}}
\]


determined experimentally in buffer + GuHCl
Submit DMGDH sequence to http://web.expasy.org/protparam/

Output:

Number of amino acids: 861
Molecular weight: 96236.7
Theoretical pI: 6.74

Two (not too different) values of the $\varepsilon_{280}$:

$\varepsilon_{280} = 143505 \text{ M}^{-1} \text{ cm}^{-1}$ assuming all pairs of Cys residues form cystines (Abs 0.1% (=1 g/l) = 1.491)

$\varepsilon_{280} = 143130 \text{ M}^{-1} \text{ cm}^{-1}$ assuming all Cys residues are reduced (Abs 0.1% (=1 g/l) = 1.487)
Determine $\varepsilon_{280, FAD}$ experimentally in buffer + GuHCl using known $\varepsilon$ at 450 nm of FAD in diluted buffer without GuHCl, pH 7.

Here are the absorbance values at different wavelengths:

- **Buffer**
  - $\varepsilon_{448, FAD}$: 11.3 mM$^{-1}$cm$^{-1}$
  - $\varepsilon_{280, FAD}$: 19.95 mM$^{-1}$cm$^{-1}$

- **Buffer + GuHCl**
  - $\varepsilon_{448, FAD}$: 11.9 mM$^{-1}$cm$^{-1}$
  - $\varepsilon_{280, FAD}$: 24.32 mM$^{-1}$cm$^{-1}$
Calculation of protein extinction coefficient taking into account the bound cofactor/coenzyme: use $\varepsilon_{280}$ of protein (from ProtParam) AND $\varepsilon_{280}$ of coenzyme in guanidine (in mM$^{-1}$cm$^{-1}$);

<table>
<thead>
<tr>
<th>$\varepsilon_{280}$-protein</th>
<th>$\varepsilon_{280}$-FAD</th>
<th>$\varepsilon_{280}$-EFAD</th>
<th>$\varepsilon_{448}$-EFAD</th>
<th>$A_{280}/A_{448}$</th>
<th>$f$(holo)</th>
</tr>
</thead>
<tbody>
<tr>
<td>143.13</td>
<td>24.32</td>
<td>167.45</td>
<td>11.9</td>
<td>14.0714286</td>
<td>1</td>
</tr>
<tr>
<td>143.13</td>
<td>21.888</td>
<td>165.018</td>
<td>10.71</td>
<td>15.4078431</td>
<td>0.9</td>
</tr>
<tr>
<td>143.13</td>
<td>19.456</td>
<td>162.586</td>
<td>9.52</td>
<td>17.0783613</td>
<td>0.8</td>
</tr>
<tr>
<td>143.13</td>
<td>17.024</td>
<td>160.154</td>
<td>8.33</td>
<td>19.2261705</td>
<td>0.7</td>
</tr>
<tr>
<td>143.13</td>
<td>14.592</td>
<td>157.722</td>
<td>7.14</td>
<td>22.089916</td>
<td>0.6</td>
</tr>
</tbody>
</table>

The $A_{280}/A_{448}$ ratio of the denatured protein solution can be used to determine the fraction of holoEnzyme in the DMGDH prep.
Combine absorption and fluorescence spectroscopies to identify the coenzyme bound to the protein.
MICAL, the novel multidomain flavoenzyme participating (not only) in actin cytoskeleton dynamics.
Absorption spectrum of the purified N-terminal domain of h-MICAL

- The spectrum of the «as isolated» MICAL indicates the presence of a flavin coenzyme forming a charge-transfer complex (with Trp400 from X-ray structure)
- Release of the coenzyme by denaturation yields the spectrum of free flavin (to be identified as FAD or FMN)
- If FAD from the known $\varepsilon_{448}$ of free FAD (11.3 mM$^{-1}$ cm$^{-1}$) we can calculate the $\varepsilon_{458}$ of MICAL-bound FAD as 8.1 mM$^{-1}$ cm$^{-1}$
- If FMN the known $\varepsilon_{446}$ of free FMN (12.2 mM$^{-1}$ cm$^{-1}$) should be used
• The excitation spectrum reflects the absorption spectrum.
• The shape of the emission spectrum is independent from $\lambda_{\text{ex}}$.
• By comparing excitation and emission spectra, the compound may be identified.
Fluorimetric Indentification of the cofactor bound to MICAL Fp domain as FAD exploiting the different fluorescence of FAD and FMN due to quenching of the isoalloxazine fluorescence by the AMP moiety.

If FAD, PDE will bring along a 10x increase of fluorescence due to conversion into FMN, and removal of internal quenching of fluorescence by the AMP moiety.
Use of absorption spectroscopy to monitor ligand binding, redox reactions at equilibrium
The flavin absorbance spectrum is sensitive to:

**Redox state**

Data are from [23].

**Ionization state of isoalloxazine positions, which is in turn sensitive to environment (protein, ligands, ...)**
Anaerobic NADPH Titration of MICAL-MO

• FAD hydroquinone is formed without formation of intermediates

• $K_{eq} = 0.591 \times 10^6$  \hspace{1cm}  $\Delta G^\circ' = -32 \text{ kJ/mol}$  \hspace{1cm}  $\Delta E^\circ' = 0.166 \text{ V}$

• $E_m$ of the FAD/FAD$_{hq}$ couple: $-0.150 \text{ V}$
Use of absorption and fluorescence spectroscopies to monitor enzyme-catalysed reactions under steady-state and pre-steady-state (rapid reaction) conditions.
Kinetic measurements aim to define the mechanism of a reaction or process and its free energy profile.
Initial velocity measurements under steady-state conditions allow to determine the kinetic parameters $V$ and $K_M$ for the substrates, which depend on the rate constants that govern the individual reaction steps.

Velocity measurements under pre-steady-state conditions allow to determine directly the values of the rate constants that govern the individual reaction steps.
Initial velocity measurements of the enzyme-catalyzed reaction under steady-state conditions are carried out, under a variety of conditions,
- to quantify the enzyme and
- to obtain information on the enzyme function, the reaction mechanism, regulatory mechanisms, the active enzyme form.

\[ v = - \frac{d[S]}{dt} = \frac{d[P]}{dt} \]
The Michaelis-Menten Equation relates the initial reaction velocity to the concentration of (active) E forms, [S] and «groups» of rate constants of elementary reaction steps.

\[ v_0 = \frac{V_{\text{max}}[S]}{K_m + [S]} \]

\[ V_{\text{max}} = k_3[E_t] \]

\[ K_m = \frac{k_2 + k_3}{k_1} \]

\[ k_{\text{cat}} = \frac{V}{[E_t]} = \text{turnover number} \]

\[ V/K = \text{catalytic efficiency} \]
Evaluation of $k_{\text{cat}}$ and $k_{\text{cat}}/K$ values may help establishing the physiological reaction of novel enzymes.
Robust assays of enzyme activity are needed to gain information on:
- The enzyme substrates/products
- Inhibitors testing
- Definition of the enzyme mechanism (also for drug design)
- Screen and analyse engineered forms (also for biotechnological applications)

Information on the enzyme are gained by correlating changes of the steady-state kinetic parameters V (or kcat) and Km as a function of (e.g.):
- substrate(s), their concentration,
- pH, ions, solvent viscosity,
- effectors (inhibitors/activators),
- temperature
- isotopic substitution of defined positions of substrates (substrate kinetic isotope effects) and solvent (solvent kinetic isotope effects).
\[ v = - \frac{d[S]}{dt} = \frac{d[P]}{dt} \]

Substrates $\rightarrow$ Products

Continuous spectrophotometric assays are very handy: no sample manipulation, direct observations, often high sensitivity, reproducibility.

Discontinuous methods for the Detection and Quantitation of the reagents are time-consuming, requiring: (Chromatographic) separation of the reaction components at different times followed by Detection and Quantitation of the reaction components by UV, Vis Absorbance, Fluorescence; Conductivity; Radioactivity; ....
Example: Monitor NAD(P)H oxidation (or NAD(P) reduction) in reactions catalyzed by dehydrogenases/reductases, oxidases, (mono)oxygenases

\[
\text{NADPH} + \text{H}^+ + \text{O}_2 \rightleftharpoons \text{NADP}^+ + \text{H}_2\text{O}_2
\]

Monitoring the entire spectrum can help troubleshoot: aggregation, precipitation of substrates/products; artifacts

Calculate initial velocity from absorbance changes at 340 nm with known extinction coefficient of NAD(P)H.
Coupling the reaction of interest with an indicator reaction with substrates/products suitable for a spectrophotometric assay is very handy.

For consecutive reactions:

\[ A \rightarrow B \rightarrow C \]

If \( v_{B \rightarrow C} >> v_{A \rightarrow B} \), then \( v_{A \rightarrow C} = v_{A \rightarrow B} \)

Depending on the products several indicator rxns can be used.


$H_2O_2$ production is often measured by coupling it to Horseradish Peroxidase in the presence of Amplex red by fluorescence, but possible artifacts may arise from the specific reaction.

\[ \text{Amplex red} \rightarrow \text{Resorufin} \]

\[ H_2O_2 \rightarrow \text{Resorufin} \]

\[ H_2O \]

[Graphs showing absorbance and emission spectra]
Possible artifacts: The HRP coupled assay of MICAL-MO

Turnover number: 15.6 or 10.4/s by monitoring NADPH oxidation vs 8 or 2.9/s by monitoring Amplex red oxidation

Figure 5, PNAS, 2005
Controls:

- HRP, Amplex red and H₂O₂ enhance NADPH oxidation
  - NADPH inhibits HRP
- NADPH lowers the amount of H₂O₂ detected at the end of the reaction

Conclusions:

- the spectrophotometric coupled assay cannot be used to assay MICAL NADPH oxidase activity
- Rather just measure NADPH oxidation at 340 nm
EGCG is a specific and potent noncompetitive inhibitor of mMICAL-MO with Ki, 0.5 mM:

MICAL controls axon growth in response to semaphorins binding to their Plexin receptor

Inhibition of MICAL may promote nerve regeneration after spinal chord injury

EGCG, as a specific inhibitor of MICAL1 could be used as a drug to promote axon regeneration

(-) epigallocatechin gallate EGCG mimics MICAL LOF mutants by acting as a specific inhibitor of MICAL-MO function

EGCG is a specific and potent noncompetitive inhibitor of mMICAL-MO with Ki, 0.5 mM:
Effect (and structure) of (-) epigallocatechin gallate (EGCG) is very similar to the effect of xanthofulvin, a potent inhibitor of Sema3A, which has been shown to promote recovery from spinal cord injury in rats.
The activity assay is critical to gather sound data.

ECGC as a catecol scavenges $H_2O_2$

EGCG causes MICAL denaturation as revealed by enzyme titration.

Quantitation of $H_2O_2$ (50 μM) with HRP/o-dianisidine in the presence of EGCG.
By monitoring NADPH oxidation at 340 nm (no HRP, no dye but with hMICAL), EGCG is a much less potent inhibitor than previously reported.

- NonCompetitive inhibition but $k_{is} = k_{ii} = 17 \mu M >> 0.5 \mu M$

- Excess inhibition at high NADPH due to enzyme denaturation?
Assay set-up requires optimization of

- Temperature
- Buffer composition (type of ions, ionic strength, viscosity)
- pH
- Added ligands, ions, coenzyme/cofactors

If there is a choice between absorbance- and fluorescence-based assay, select the assay method
MICAL NADPH oxidase reaction is sensitive to ionic strength and the type of anions.

Strong effect on V/K_{NADPH} mainly due to effect on K_m due to:

Competition between anions and NADPH

Electrostatic effects

Design mixed buffer for pH studies to minimize ions and I effects, keep I under control in expts.
pH dependence of steady-state kinetic parameters of the NADPH oxidase reaction of MICAL forms need to be studies in a mixed buffer that guarantees a constant ionic strength.
Most enzyme reactions are well described by the Michaelis-Menten equation

\[ V_o = \frac{V_{\text{max}} [S]}{K_m + [S]} \]

\[ V_{\text{max}} = k_3 [E_t] \quad K_m = \frac{k_2 + k_3}{k_1} \]

\[ k_{\text{cat}} = \frac{V_{\text{max}}}{[E_t]} = \text{turnover number} \]

\[ \frac{V_{\text{max}}}{K_m} \text{ or } \frac{k_{\text{cat}}}{K_m} = \text{catalytic efficiency} \]
Deviations from the Michaelis-Menten equation are informative.

Two enzyme forms?

Substrate inhibition?

Allosteric activation
Dependence of $v$ from $[E_T]$: deviations from the predicted linearity are informative.

Expected and Most common behavior:
- Non-enzymatic reaction
- Monomer/dimer equilibrium and the monomer is inactive
- Reaction too fast to measure the initial velocity;
- Monomer/dimer equilibrium; dimer is inactive
- A tight binding inhibitor in the reaction mixture,
Use activity assays of HIV protease to determine the dissociation constant of the (active) dimer.
Selection of the activity assay
A fluorescent substrate to measure HIV1 protease activity

Ser-Nle-Ala-Glu-pNitro-Phe-Leu-Val-Arg-Ala-Lys-His-Abz

Quenching of Abz fluorescence by nitroTyr
The alternative absorption-based assay for HIV1 protease exploits the effect of changes of nitroTyr environment during the reaction. The observed absorbance changes are smaller than fluorescence changes.

\[ \text{Ser-Nle-Ala-Glu-}p\text{Nitro-Phe-Leu-Val-Arg-Ala-Lys-His} \]

\[ \varepsilon \approx 0.50 \text{ mM}^{-1}\text{cm}^{-1} \]

Substrate Spectra: before and after incubation with HIV-1 protease

HIV1 protease

Absorbance, 310 nm vs. time (sec)
Absorption-based assay allows to explore a broader substrate range for V and Km determinations.

**Absorbance-based assay**

Actual turnover number (ε is known)

**Fluorescence-based assay**

Apparent turnover number (F in arbitrary units)

Buffer: 100 mM Na Acetate, pH 5.00, 1 mM EDTA, 1 mM DTT, 100 mM NaCl
Activity assays allow to monitor the dissociation of the HIV-1 protease dimer.

\[ v = k_{cat} \times [\text{Dimer}] \]

\[ K_d = \frac{[M]^2}{[D]} \]

\[ [E_{tot}] = 2[D] + [M] \]

- 100 mM Na Acetate buffer, pH 5.0, 1 mM EDTA, 1 mM DTT, 100 mM NaCl
- 10 mM Na phosphate buffer, pH 6.5, 1 mM EDTA, 10% glycerol, 75 mM NaCl; calculated \( K_d, 215 \text{ nM} \)
Steady-state kinetic studies to monitor binding of activating proteins
NAD(P)H → O2 → SOH + H2O → Flavinox + Flavin + Flavinred (oxidase activity) → 4a-hydroperoxy-Flavin (monooxygenase activity) → Flavinox + H2O2 → Flavinred → NAD(P)H

Epithelial-to-mesenchimal transition (MICAL2)
- Cell division
- Endocytosis and endosome recycling
- Cell viability
- Angiogenesis
- Cell junctions
- Exocytosis
- Oxidative stress in cancer cells (MICAL1)
- Viral/Pathogen invasion (MICAL1)

MICAL family proteins
- Neuronal
- Myocytes
- Skeletal development
- Neoplasia
- Non-neuronal cells

Stabilization of inactive form by dimerization???
Inactive (Autoinhibited)
NADPH → NADP+ → Flavinox
Scaffold
Generation of H2O2 as a second messenger
Modulation of biological activity of interacting proteins (unrelated to catalytic reactions of MO domain)
Rab proteins are physiological modulators of MICAL activity by binding to its C-terminal region.

MICAL1 C-term / Rab35, 1:1 complex; Kd 6-13 µM
Effect of Rab8.GDP and (active) Rab8.GppNHp on the NADPH oxidase activity of MICAL1
(no effect on truncated forms: the RBD is in the MICAL1 C-terminal region)

$K_{Rab} = 92 \, \mu M$
$K_{Rab} = 9 \, \mu M$

$\frac{k_{cat}^{\text{Rab}}}{K_{Rab}} = 0.35 \, s^{-1}$
$\frac{k_{cat}^{+\text{Rab}}}{K_{M,NADPH}} = 1.1 \, mM$

No coelution; apparent mass of MICAL1 between that of monomer and dimer
Analysis of the aggregation state of MICAL and MOCHLIM and of complex formation with Rab8.GppNHp

**SEC-SAXS**

<table>
<thead>
<tr>
<th>Construct</th>
<th>MOCH</th>
<th>MOCHLIM</th>
<th>MICAL</th>
<th>MICAL+Rab</th>
</tr>
</thead>
<tbody>
<tr>
<td>Structural parameters</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$R_e$, nm</td>
<td>3.4 ± 0.1</td>
<td>4.0 ± 0.2</td>
<td>3.7 ± 0.1</td>
<td>3.7 ± 1</td>
</tr>
<tr>
<td>$D_{max}$, nm</td>
<td>12 ± 1</td>
<td>18 ± 2</td>
<td>12 ± 1</td>
<td>12 ± 1</td>
</tr>
<tr>
<td>Porod volume estimate (nm$^3$)</td>
<td>100 ± 15</td>
<td>145 ± 20</td>
<td>212 ± 20</td>
<td>234 ± 25</td>
</tr>
<tr>
<td>Displaced volume calculated from monomer (nm$^3$)</td>
<td>81</td>
<td>116</td>
<td>157</td>
<td>180</td>
</tr>
<tr>
<td>Porod volume/1.6</td>
<td>62.5</td>
<td>90.6</td>
<td>132.5</td>
<td>146</td>
</tr>
</tbody>
</table>

**MO** 55 kDa  
**MOCH** 68.2 kDa  
**MOCHLIM** 86.3 kDa  
**MICAL** 112 kDa
Rapid reaction kinetics
Initial velocity measurements under steady-state conditions allow to determine the kinetic parameters $V$ and $K_M$ for the substrates, which depend on the rate constants that govern the individual reaction steps.

Velocity measurements under pre-steady-state conditions allow to determine directly the values of the rate constants that govern the individual reaction steps.
Rapid reaction kinetics allow to directly measure rate constants to study:

- Chemical reactions
- Folding/Unfolding
- Protein-protein, Protein-ligand interactions
- Conformational changes
To measure individual reaction steps we need to rapidly monitor changes in a signal that is related to the identity and concentration of each species that may be formed during the process (which may be fast).

\[ A = A_0 e^{-k_1t} \]
\[ B = A_0k_1/(k_2-k_1)(e^{-k_1t} - e^{-k_2t}) \]
\[ C = A_0[1 + 1/(k_1 - k_2)(k_2e^{-k_1t} - k_1 e^{-k_2t})] \]

**Green**: At a given wavelength A, B, C have the same extinction coefficient

At different wavelengths: we can distinguish A from B from C

- \( I_{\lambda,A} = [A]^* \varepsilon_{\lambda,A} \)
- \( I_{\lambda,B} = [B]^* \varepsilon_{\lambda,B} \)
- \( I_{\lambda,C} = [C]^* \varepsilon_{\lambda,C} \)

**Isosbestic points**
Requirements of rapid kinetics

[Enzyme] : $\mu$M, mM vs nM, $\mu$M for steady-state

[Substrate] : $\mu$M, mM

( [S] > 10x [E] for pseudo-first order conditions)

Measuring Times: msec-sec vs sec-min for steady-state

Thus, need:

- large amounts of enzyme/protein & substrate/ligand
- highly concentrated protein/ligand solutions
- rapid mixing device
- rapid measuring times (in continuous methods)
- rapid data acquisition
- software (expertize) for data analysis
Detection

Continuous methods:
Absorbance, Fluorescence, Circular Dichroism, Fluorescence anisotropy, conductivity, X-ray scattering (!), .....  

Discontinuous methods (coupled to continuous flow set-up)
EPR (freeze-quench)
Mossbauer (freeze-quench)
HPLC separation of reaction components and chemical analysis (chemical quench)
Continuous flow set-up for rapid reaction studies

Push, 3 atm

Tubing of different length will lead to quenching of sample at different reaction times: 1 cm = 1 msec; 2 cm = 2 msec, etc. for 10 m/s flow rate

Cold isopentane

Analysis: one «shot» / 1 datapoint
Stopped-flow set-up for rapid reaction studies

Turbulent flow to ensure constant velocity across tubings
High flow rate (e.g.: 10 m/s = 1 cm/msec)
Different instrument set-ups for stopped-flow

**Single Mixing Stopped-flow**

\[ E + S \rightarrow \quad \text{(1:1 or variable volume mixing)} \]

**Double Mixing Stopped-flow**

\[ E + S_1 \rightarrow \quad ES_1 \]

\[ \text{Aging time, varies} \]

\[ ES_1 + S_2 \text{ (or I, or ....)} \rightarrow \]
The upper limit of measured rates is set by:
Dead-time, Time-constant (Time Resolution), Sensitivity of detector
Figure 1. The stopped-flow instrument. A) Picture of the components of the Applied Photophysics SX20 stopped-flow spectrophotometer. B) Picture of the sample handling unit. C) Scheme of the flow circuit in double mixing mode.
No intermediates detected in the stopped-flow under anaerobiosis:

**Conditions:** 20 mM Hepes/NaOH, pH 7.0, 10% glycerol, 1 mM DTT, 1 mM EDTA at 25°C

*Almost definitely 5 million copies sold*

**The Secret Diary of Adrian Mole, aged 13 3/4**

*Sue Townsend*
Reductive half-reaction

\[ \text{E}_{\text{ox}} + \text{NADPH} \rightarrow \text{E}_{\text{red}} + \text{NADP}^+ \]
\[ \text{E}_{\text{red}} + \text{O}_2 \rightarrow \text{E}_{\text{ox}} + \text{H}_2\text{O}_2 \]

For an intermediate to be catalytically competent (on the reaction path): \( k_{\text{cat}} \leq k \)

\[ \tau \text{, transit time} = \frac{1}{k} \]
\[ \tau \text{ (overall)} = \tau_1 + \tau_2 \]

Steady-state (turnover)

\[ k_{\text{cat}} = 4.0 \pm 0.1 \text{ s}^{-1} \]
\[ K_M = 28 \pm 2 \mu\text{M} \]

\[ k_{\text{red}} = 3.0 \pm 0.1 \text{ s}^{-1} \]
\[ K_M = 56 \pm 7 \mu\text{M} \]
Conditions: 20 mM Hepes/NaOH, pH 7.0, 1 mM DTT, 1 mM EDTA at 25°C. 10% glycerol, in sf only

Measure steady-state kinetic parameters in buffer + 10% glycerol

<table>
<thead>
<tr>
<th>NADPH, µM</th>
<th>NADH, µM</th>
<th>NaCl, M</th>
<th>Glycerol, %</th>
<th>K_{NAD(P)H}, µM</th>
<th>k_{cat}, s⁻¹</th>
<th>k_{cat}/K_{NAD(P)H}, s⁻¹mM⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>10-300</td>
<td></td>
<td></td>
<td></td>
<td>26±4</td>
<td>3.9±0.1</td>
<td>150 ± 23</td>
</tr>
<tr>
<td>80-670</td>
<td></td>
<td></td>
<td></td>
<td>580±24</td>
<td>0.28±0.01</td>
<td>0.48 ± 0.03</td>
</tr>
<tr>
<td>40-650</td>
<td>0.1</td>
<td></td>
<td></td>
<td>499±28</td>
<td>2.6 ±0.1</td>
<td>5.2 ± 0.4</td>
</tr>
<tr>
<td>10-300</td>
<td>10</td>
<td></td>
<td></td>
<td>93±11</td>
<td>2.9±0.1</td>
<td>31.2 ± 4</td>
</tr>
</tbody>
</table>

Reductive half reaction: K_d, 56 ± 7 µM   k_{red}, 3.0 ± 0.1s⁻¹

Conclusions:
- Hydride transfer is rate limiting turnover
- Solvent viscosity effect on both k_{cat} and k_{cat}/Km
Effect of solvent viscosity on MICAL-MO NADPH oxidase rxn

Effect of viscosity on $k_{cat}$

<table>
<thead>
<tr>
<th>Viscogen</th>
<th>slope ($k_{cat}$)</th>
<th>intercept ($k_{cat}/K_{NADPH}$)</th>
<th>slope ($k_{cat}/K_{NADPH}$)</th>
<th>intercept ($k_{cat}/K_{NADPH}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycerol</td>
<td>0.97 ± 0.01</td>
<td>-</td>
<td>3.0 ± 0.1</td>
<td>3.0 ± 0.1</td>
</tr>
<tr>
<td>Sucrose</td>
<td>1.04 ± 0.03</td>
<td>-</td>
<td>4.2 ± 0.3</td>
<td>4.2 ± 0.3</td>
</tr>
<tr>
<td>PEG8000</td>
<td>0.04 ± 0.01</td>
<td>0.94 ± 0.03</td>
<td>0.1 ± 0.14</td>
<td>0.1 ± 0.14</td>
</tr>
</tbody>
</table>

Solvent viscosity effect on $k_{cat}/K_m$ indicates that a conformational change is contributing to the determination of $k_{cat}/K_m$ value.
Viscosity effects on V and V/K by microviscogens provide information on diffusion limited steps and on conformational changes taking place during catalysis:

0 < Effect<1

Effect = 0: the parameter is determined by steps other than diffusion

Effect =1 : the parameter is governed by a diffusion limited step

Effect >1: a conformational change concurs to the value of the parameter under study

The effect of viscosity is:
• 0 when \( k_3 << k_1, k_2, k_5, k_6 \)
• 1 when \( k_3 >> k_1, k_2, k_5, k_6 \)
• >1 when conformational changes occur during the catalytic cycle
Solvent viscosity effects on MICAL-MO NADPH oxidase reaction may allow us to monitor the conformational changes within the flavoprotein domain that are believed to be part of the reaction.

At least 2 conformational changes have been proposed to occur in the catalytic cycle of MICAL:

- movement of Trp400 to allow NADPH binding and oxidation
- $FAD_{out}/FAD_{in}$ transition after hydride transfer
Monitoring the Reductive and Oxidative Half-Reactions of a Flavin-Dependent Monoxygenase using Stopped-Flow Spectrophotometry

Elvira Romero, Reeder Robinson, Pablo Sobrado
Department of Biochemistry, Virginia Polytechnic Institute and State University

Correspondence to: Pablo Sobrado at psobrado@vt.edu

URL: http://www.jove.com/video/3803/
DOI: 10.3791/3803

Scheme 1. Mechanism of Af SitA. The isoalloxazine ring of the FAD cofactor is shown. The oxidized flavin (A) binds to NADPH (B) to form reduced flavin and NADP⁺ (C). After reaction with molecular oxygen and binding of ornithine, the C4a-hydroperoxoflavin is the hydroxylating species. After hydroxylation of ornithine, the hydroxylflavin (E) must be dehydrated to form the oxidized enzyme, which remains bound throughout the catalytic cycle and is the last product to be released (F).
Save the date!

A course on «Trends in Enzymology»
Rome, 27 – 30 May 2019

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Steady-state, pre-steady-state kinetics; isotope effects
Protein engineering, Bioinformatics, Bioconversions,
Ancillary techniques
NEVER EVER EVER GIVE UP!