SAXS and Biochemistry

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Absorption and Fluorescence spectroscopies

- Protein folding (mechanism, kinetics, thermodynamics)
- Ligand binding
- Measuring Catalytic activity and carry out mechanistic studies of enzymes
- Detect (transient) Conformational changes
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
Basic Scheme of a spectrophotometer

\[ A = \ln \left( \frac{1}{T} \right) = \varepsilon \cdot c \cdot l \]

Absorbance
For absorption spectroscopy

Scheme of a dual (double) beam spectrophotometer

<table>
<thead>
<tr>
<th>Differences:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stability</td>
</tr>
<tr>
<td>Resolution</td>
</tr>
<tr>
<td>Signal-to-noise</td>
</tr>
<tr>
<td>Linearity of response</td>
</tr>
<tr>
<td>Artifacts (e.g.: photoreactions/degradation)</td>
</tr>
<tr>
<td>Speed</td>
</tr>
</tbody>
</table>

Scheme of a (photo)diode array spectrophotometer

Figure 1. Schematic of photodiode array spectrophotometer
Basic Scheme of a spectrophotofluorometer

Intensity of emitted light at
\[ \lambda_2 > \lambda_1 \]

Detector
(\textit{with scanning capability})

Monochromator
(\textit{with scanning capability})

Lamp

Monochromator

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

Intensity of incident light
\[ \lambda_1 I_0 \]

https://history.nih.gov/exhibits/bowman/ScienSPF.htm
Several natural compounds absorb light in the UV and visible region of the spectrum. Some are also fluorescent.
- 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:

\[ A_\lambda = \varepsilon_\lambda \times c \times l \]
Intrinsic chromophors in proteins

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

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

In general: a 1 mg/ml solution will absorb 1 at 280 nm
Several prosthetic groups (coenzymes and cofactors) absorb light in the UV and visible region and are intrinsic chromophores of proteins. Used for:

Protein identification and quantitation;
Study of protein function, conformational changes, ....

Hemoglobin oxygenation
Cytochrome c reduction

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.
Cobalamins (vit B12 derivatives)

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

Pyridoxal phosphate (PLP)

Decarboxylation  Transamination  Racemization  β Elimination  Retro Aldol Cleavage  Others
The flavin coenzymes FMN and FAD are derivatives of riboflavin (vitamine b2) and participate in oxidoreduction reactions.
The flavin absorbance spectrum is sensitive to:

**Redox state**

- Flavin ox (Yellow)
- Flavin hydroquinone (leuco)

**Ionization state of isoalloxazine positions, which is in turn sensitive to environment (protein, ligands, ...)**

- Neutral semiquinone (Blue)
- Anionic semiquinone (Red)
The flavin absorption spectrum is sensitive to the «environment»:

Binding of Benzoate (an inhibitor of D-amino acid oxidase that mimicks the iminoacid intermediate) causes large absorbance changes, which can be used to calculate Kd.
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.
Excitation and emission spectra of flavin coenzymes

- The excitation spectrum reflects the absorption spectrum.
- The shape of the emission spectrum is independent from $\lambda_{ex}$.
- By comparing excitation and emission spectra, the compound may be identified.
### 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)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>inner filter effects</td>
</tr>
<tr>
<td><strong>Quantification of solute</strong></td>
<td>ε, extinction coefficient M⁻¹ cm⁻¹</td>
<td>F ∝ 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></td>
</tr>
<tr>
<td>- Solvent</td>
<td></td>
<td></td>
</tr>
<tr>
<td>- Other solutes</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Return to ground state</strong></td>
<td>Very fast</td>
<td>(Relatively) slow</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(can measure fluorescence decay over time after flash of exciting light)</td>
</tr>
</tbody>
</table>
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 their fluorescence to monitor folding/unfolding, dimerization and conformational changes (e.g.: upon ligand binding)

Figure 1.25. Emission spectra of melittin monomer and tetramer. Excitation was at 295 nm. In the schematic structure, the tryptophans are located in the center between the four helices. From [23].
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
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:

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

$\varepsilon_{280}$ calculated at pH 6.5, in 6.0 M guanidium 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 visible and UV region....
1) 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).
2) Release of the coenzyme by denaturation yields the spectrum of free flavin (to be identified as FAD or FMN).
3) 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$^{-1}$.
4) If FMN the known $\varepsilon_{446}$ of free FMN (12.2 mM$^{-1}$ cm$^{-1}$) should be used.
MICAL is a multidomain cytoplasmic protein participating in actin cytoskeleton dynamics through its N-terminal flavoprotein domain.
Take into account the presence of the chromophore, which absorbs light in the UV, to calculate the $\varepsilon_{280}$ of the protein.

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

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


1 for holo-DMGDH
determined experimentally in buffer + GuHCl

Output:

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

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

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

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

The absorbance spectrum of FAD in buffer and buffer + GuHCl shows peak absorbance at 280 nm and 448 nm. The absorbance at 280 nm is 19.95 for buffer and 24.32 for buffer + GuHCl. The absorbance at 448 nm is 11.3 for buffer and 11.9 for buffer + GuHCl.
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.
**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</th>
<th>Detection of 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>
<td>- Use 3-5 different protein quantities and check linearity. Intercept should be zero.</td>
</tr>
<tr>
<td>Lowry</td>
<td>10</td>
<td>low</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>
<td>- Pre-precipitate protein (make sure it is re-solubilized prior to assay)</td>
</tr>
<tr>
<td>BCA</td>
<td>1</td>
<td>low</td>
<td></td>
<td>- Does your protein precipitate in assay? (check effect of order of reagents addition)</td>
</tr>
<tr>
<td>660 dye</td>
<td>1</td>
<td>low(?)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

commercial formulations; see also: http://wolfson.huji.ac.il
Modified Lowry

Figure 1. Reaction schematic for the Modified Lowry Protein Assay.

Bradford

Biocinchoninic acid

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 bicinechoninic acid (BCA)-containing protein assay.
### Calibration curve

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

\[ y = 0.0455x \]
\[ R^2 = 0.9948 \]

### 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>
</thead>
<tbody>
<tr>
<td>MOCH0</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>

\[ y = 0.0278x - 0.0025 \]
\[ R^2 = 0.9966 \]
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

From Mariangela Camozzi thesis

No such problem with BCA assay
**Dimethylglycine dehydrogenase:**
FAD dependent dehydrogenase containing covalently bound FAD. Same covalent bond found in succinate dehydrogenase, fumarate reductase.
DMGDH feeds 1 carbon unit into folate-dependent cycle;
Defects of DMGDH: diseases belonging to broad class of «mitochondrial diseases»
Combine absorption and fluorescence spectroscopies to identify the coenzyme bound to the protein
Absorption spectrum of the N-terminal domain of h-MICAL and of the released flavin coenzyme

Excitation and emission spectra of flavin coenzymes

Fluorescence emission spectrum of the native MICAL N-terminal domain and of the released coenzyme shows that the fluorescence of bound flavin is quenched.
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
Anaerobic NADPH titration of glutamate synthase β subunit (β-GltS) and of its G298A variant

E-FAD + NADPH -> E-FAD\textsubscript{red} + NADP\textsuperscript{+}

**Wild-type:** bound FAD is reduced by NADPH with formation of a stable \( \text{FAD}_{\text{red}} \cdot \text{NADP}^+ \) charge-transfer complex: very low Kd

The substitution of the second G of the GXGXXA motif of the putative binding site of the adenylate portion of NADP:
- weakens NADPH binding,
- prevents the formation of the CT complex, thus it
- alters the positioning of the nicotinamide ring

E-FAD + NADPH \rightleftharpoons E-FAD \cdot \text{NADPH} \rightleftharpoons (E-FAD\textsubscript{red} \cdot \text{NADP}^+)\text{CT}

\[ \rightleftharpoons \text{E-FAD}_{\text{red}} \cdot \text{NADP}^+ \rightleftharpoons \text{E-FAD}_{\text{red}} + \text{NADP}^+ \]
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/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 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.

\[ v = - \frac{d[S]}{dt} = \frac{d[P]}{dt} \]
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 $k_{cat}$) and $K_m$ 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).
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.

The Assumptions of the steady-state model:
- \([E] \ll [S_0]\)
- Measure \(v_o\) (initial velocity) when \([P] = 0\)
- \(v = k_3*[ES]\)
- \([ES] = \text{constant}\)

\[
\begin{align*}
E + S & \rightleftharpoons ES & \text{ES} & \rightarrow E + P \\
k_1 & \quad k_3 & \quad k_2 & \\
& \quad & & \\
\end{align*}
\]

\[
V_o = \frac{V_{\text{max}}[S]}{K_m + [S]} \\
V_{\text{max}} = k_3[E_t] \\
K_m = \frac{k_2 + k_3}{k_1} \\
k_{\text{cat}} = \frac{V}{[E_t]} = \text{turnover number} \\
\frac{V}{K} = \text{catalytic efficiency}
\]
Evaluation of $k_{\text{cat}}$ and $k_{\text{cat}}/K$ values may help establishing the physiological reaction of novel enzymes.
\[ v = - \frac{d[S]}{dt} = \frac{d[P]}{dt} \]

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)oxygogenases

\[
\text{NADPH} + H^+ + O_2 \quad \xrightarrow{\text{MICAL}} \quad \text{NADP}^+ + H_2O_2
\]

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

Depending on the products several indicator rxns can be used.

\[
\text{D-alanine} + \text{O}_2 \xrightarrow{\text{D-amino acid oxidase}} \text{Pyruvate} + \text{ammonia} + \text{H}_2\text{O}_2
\]

\[
\text{L-lattato} \xrightarrow{\text{LDH}} \text{L-lactate} \xrightarrow{\text{LDH}} \text{NADH} + \text{H}^+
\]

\[
\text{NAD}^+ \xrightarrow{\text{LDH}} \text{NADH} + \text{H}^+
\]

\[
\text{H}_2\text{O} \xrightarrow{\text{HRP}} \text{X}_{\text{ox}} \xrightarrow{\text{colored}} \text{X}
\]

For consecutive reactions:

\[
A \rightarrow B \rightarrow C
\]

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

Suitable control expts need to be done to set up a reliable coupled assay.
H$_2$O$_2$ 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.

H$_2$O$_2$  $\rightarrow$  H$_2$O

Amplex red  $\rightarrow$  Resorufin

[Chemical structures and graphs]
Possible artifacts: The case of HRP-coupled assay of MICAL NADPH oxidase reaction

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 $\text{H}_2\text{O}_2$ enhance NADPH oxidation
- NADPH inhibits HRP
- NADPH lowers the amount of $\text{H}_2\text{O}_2$ 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
Assay set-up requires optimization of

- Temperature
- Buffer composition
- 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 $V/K_{\text{NADPH}}$ 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.
Solvent (glycerol) viscosity effects on the steady-state kinetic parameters of the NADPH oxidase reaction of MICAL forms

The high solvent viscosity effect on $k_{\text{cat}}/K_{\text{NADPH}}$ reveals the presence of a conformational change between NADPH binding and oxidation that is partially rate limiting.
Most enzyme reactions are well described by the Michaelis-Menten equation

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

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

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

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

\[ \frac{V}{K} = \text{catalytic efficiency} \]
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**
- Reaction too fast to measure the initial velocity;
  
  Monomer/dimer equilibrium; dimer is inactive

A tight binding inhibitor in the reaction mixture,

**Monomer/ dimer equilibrium and the monomer is inactive**
Use activity assays of HIV protease to determine the dissociation constant of the (active) dimer

\[ 2 \text{ M} \rightleftharpoons D \text{ (dimer, active)} \]
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.

\[ \text{HIV1 protease} \]

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

\[ \varepsilon \approx 0.50 \text{ mM}^{-1}\text{cm}^{-1} \]
Absorption-based assay allows to explore a broader substrate range for V and Km determinations

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

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

\[ K_d = \frac{[M]^2}{[D]} \quad [E_{tot}] = 2[D] + [M] \]

- 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 nM
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
Rapid reaction kinetics allow to directly measure rate constants to study:

**Chemical reactions**

**Folding/Unfolding**

**Protein-protein, Protein-ligand interactions**

**Conformational changes**

At equilibrium:

$$K_{eq} = \frac{[C]_{eq}[D]_{eq}}{[A]_{eq}[B]_{eq}} = \frac{k_f}{k_r}$$

Revised kinetics diagram: A multistep process
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).

\[
\begin{align*}
A &= A_0 e^{-k_1 t} \\
B &= A_0 k_1 / (k_2 - k_1) (e^{-k_1 t} - e^{-k_2 t}) \\
C &= A_0 [1 + 1/(k_1 - k_2) (k_2 e^{-k_1 t} - k_1 e^{-k_2 t})]
\end{align*}
\]

\[
\begin{align*}
I_{\lambda A} &= [A]^* \varepsilon_{\lambda A} \\
I_{\lambda B} &= [B]^* \varepsilon_{\lambda B} \\
I_{\lambda C} &= [C]^* \varepsilon_{\lambda C}
\end{align*}
\]

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

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

Isosbestic points
Requirements of rapid kinetics

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

[Substrate]: $\mu M, mM$

([S]$> 10\times [E]$ 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
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**

![Single Mixing Stopped-Flow Diagram]

- E + S →
- (1:1 or variable volume mixing)

**Double Mixing Stopped-flow**

![Double Mixing Stopped-Flow Diagram]

- E + S₁ → ES₁
- Aging time, varies
- ES₁ + S₂ (or I, or ...) →
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
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)
The upper limit of measured rates is set by:

Dead-time, Time-constant (Time Resolution), Sensitivity of detector

**Dead-time**

<table>
<thead>
<tr>
<th>Time (sec)</th>
<th>Absorbance</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.000</td>
<td>1.000</td>
</tr>
<tr>
<td>0.020</td>
<td>0.000</td>
</tr>
</tbody>
</table>

**Time-constant (Resolution)**

<table>
<thead>
<tr>
<th>Time (sec)</th>
<th>Absorbance</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.000</td>
<td>1.000</td>
</tr>
<tr>
<td>0.040</td>
<td>0.000</td>
</tr>
</tbody>
</table>

Push, 3 atm

Detector
Abs-monitored Rapid reaction measurements at one or multiple wavelengths

Measurement of the rate of reduction of the FAD coenzyme bound to glutamate synthase or its isolated β subunit by NADPH in the stopped-flow reveal the catalytic competence of FAD bound to the isolated β subunit and the formation of a FADred-NADP+ charge transfer complex.

\[
\text{E-FAD} + \text{NADPH} \overset{900/s}{\rightleftharpoons} \text{E-FAD.NADPH} \overset{\text{dead-time}}{\rightleftharpoons} (\text{E-FAD}_{\text{red}} \cdot \text{NADP}^+)_{\text{CT}} \\
\overset{\text{CT formation is too fast to tell if there is also a CT}}{\rightleftharpoons} \text{E-FAD}_{\text{red}} \cdot \text{NADP}^+ \overset{\text{CT formation}}{\rightleftharpoons} \text{E-FAD}_{\text{red}} + \text{NADP}^+
\]
Diode array set-up is slower than PMT mode but monitors entire the spectrum:

- Interesting/informative wavelengths can be selected later;
- Global analysis can be applied also combining data from rapid reaction and static experiments (starting/final spectra of Eox, Ered)
Monitoring the Reductive and Oxidative Half-Reactions of a Flavin-Dependent Monooxygenase 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 SidA. The isoalloxazine ring of the FAD cofactor is shown. The oxidized flavin (A) binds to NADPH (B) and reacts to form reduced flavin and NADP⁺ (C). After reaction with molecular oxygen and binding of ornithine, the C4a-hydroperoxylavin is formed (D). This is the hydroxylating species. After hydroxylation of ornithine, the hydroxyflavin (E) must be dehydrated to form the oxidized enzyme. NADP⁺ remains bound throughout the catalytic cycle and is the last product to be released (F).
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.
Reductive half-reaction of SidA (in the absence of the ornithine substrate and oxygen)

Figure 2. Anaerobic reduction of SidA with NADPH in the stopped-flow instrument. A) Spectral changes recorded after mixing equal volumes of 30 μM SidA and 45 μM NADPH. The first spectrum (oxidized SidA) and last spectrum (fully reduced SidA) is highlighted in blue and red, respectively. B) Absorbance trace at 452 nm recorded as a function of time.
Oxidative half-reaction of SidA

NADPH-reduced SidA + Ornithine + O₂
Reaction of reduced 3HB6H enzyme in the presence of 3HB with oxygen.
Fluorescence to monitor processes at equilibrium or in time-resolved mode (kinetics)
Absorption versus fluorescence spectroscopy

<table>
<thead>
<tr>
<th>Detection limits</th>
<th>Absorbance</th>
<th>Fluorescence</th>
</tr>
</thead>
<tbody>
<tr>
<td>μM – mM</td>
<td>≤ μM</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Linearity of signals</th>
<th>2 orders of magnitude</th>
<th>≈Narrow</th>
</tr>
</thead>
<tbody>
<tr>
<td>(e.g.: 1 – 100 μM)</td>
<td>(e.g.: 0.1 – 1 μM)</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Quantification of solute</th>
<th>ε, extinction coefficient</th>
<th>F ∝ c*l,</th>
</tr>
</thead>
<tbody>
<tr>
<td>M⁻¹ cm⁻¹</td>
<td>in arbitrary units</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Sensitivity to:</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature</td>
<td></td>
<td>Very high (can be exploited)</td>
</tr>
<tr>
<td>Solvent</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Other solutes</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Return to ground state</th>
<th>Absorbance</th>
<th>Fluorescence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Very fast</td>
<td>(Relatively) slow</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(can measure fluorescence decay over time after flash of exciting light)</td>
<td></td>
</tr>
</tbody>
</table>
The sensitivity of fluorescence emission to the environment is often exploited to study protein folding/unfolding:

Change in Trp exposure to solvent upon folding/unfolding, oligomerization, ligand binding at equilibrium or time-resolved mode
ANS fluorescence increases when it interacts with hydrophobic residues of a protein.

ANS can be used to monitor the exposure or burial of hydrophobic residues in proteins during unfolding, dissociation of oligomer, and conformational changes in general.
Exploiting an intrinsic fluorophore: e.g., the case of flavoproteins in which protein denaturation leads to loss of flavin fluorescence quenching: i.e.: fluorescence increase

Fig. 1. (A) Schematic representation of the ThermoFluor® binding assay. A solvatochromic dye (i.e., SYPRO Orange) is used as an indicator of protein unfolding. Binding of the dye to the unfolded protein results in a significant increase in its intrinsic fluorescence. (B) Schematic representation of ThermoFAD. In this case, the increase in fluorescence is generated by exposure of the flavin cofactor to the solvent upon protein unfolding. (C) Overview of fluorescence properties of flavins and comparison with RT-PCR instrumental parameters. Dashed line, flavin excitation spectrum; continuous line, flavin emission spectrum; red, wavelength range for RT-PCR fluorescence excitation; green, SYBR Green detection range; orange, SYPRO Orange detection range. Flavin fluorescence emission can be measured using the SYBR Green fluorescence filter on the RT-PCR instrument without any adaptation.

ThermoFAD, a ThermoFluor®-adapted flavin ad hoc detection system for protein folding and ligand binding

Federico Forneris, Roberto Ortu, Daniele Bonivento, Laurent R. Chiarelli and Andrea Mattevi

Department of Genetics and Microbiology, University of Pavia, Italy
FRET: Foerster (Fluorescence) resonance energy transfer occurs when the emission spectrum of the «Donor» overlaps with the absorption spectrum of the «Acceptor» without emission of photon from donor.

The efficiency of FRET depends on the distance between Donor and Acceptor. FRET can be used to monitor distances between Donor and Acceptor.
Light can be polarized allowing for (e.g.) Circular dichroism (CD) and fluorescence anisotropy measurements

Can be coupled to (stopped-flow) rapid reaction kinetics

http://www.bio-logic.info/

www.photophysics.com
Circular dichroism spectroscopy to measure:
- Folding/unfolding
- Conformational changes
- Reduction of redox center (e.g.: flavin coenzyme)

The principle: different absorption of left vs right circularly polarized light by chiral molecules as a function of environment

Warning: Signals are small; interference by air, solvents, solutes: high; but the technique is very sensitive

A few useful websites: the principle made easy?
http://www.photophysics.com/tutorials/circular-dichroism-cd-spectroscopy/1-understanding-circular-dichroism
Electromagnetic waves and circular dichroism: an animated tutorial
szilagyi.andras@ttk.mta.hu
Protein Circular Dichroism Data Bank: pcddb.cryst.bbk.ac.uk/home.php
Expected signals from secondary structure elements

<table>
<thead>
<tr>
<th>170 nm</th>
<th>Far-UV</th>
<th>250 nm</th>
<th>Near-UV</th>
<th>320 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Structural types identified:</td>
<td>Structural types identified:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alpha helix</td>
<td>Phenylalanine</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Beta turn</td>
<td>Tyrosine</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Polypeptide</td>
<td>Tryptophan</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Polyproline</td>
<td>Disulphide bonds</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Conformation**

- α-helix
  - (H-bonded)

**Molecular Shape**

- β-sheet parallel and anti-parallel
  - (H-bonded)

**CD spectrum**

- β-turn
  - (Type I, II, III....)
- γ-turns
  - (some turns not H-bonded in proteins)

- Polyproline
  - PII helix
  - 31-helix
  - (H-bonded)
  - (left hand extended helix, no intramolecular H-bonds)

- Irregular
  - (Disordered)
CD is often used to study folding – unfolding, often in conjunction with fluorescence

Example: HIV protease
Use CD to monitor activatory conformational changes in PBP2a (Penicillin Binding Protein 2a) the peptidyl transferase responsible of methicillin resistance in *Staphylococcus aureus* (MRSA), in the presence of peptidoglycan to lead to novel antibiotics.

**Figure 1.** (A) Far-UV circular dichroic spectra of compound 4 by itself (4.0 mM, —), of PBP 2a by itself (3 nM, ○), and of PBP 2a (3 nM) in the presence of 4 at 1.0 mM (△), 2.0 mM (●), 3.0 mM (□), and 4.0 mM (○). The lines connect the data points and were not fitted to any specific model. (B) Change in molar ellipticity of PBP 2a at 222 nm as a function of the concentrations of compound 4.

**How allosteric control of Staphylococcus aureus penicillin binding protein 2a enables methicillin resistance and physiological function**


---

*Fig. 1.* Domains of PBP2a and I
Fluorescence anisotropy

The principle:
Excite fluorophor with polarized light. The emitted light will be polarised or depolarised depending on speed of tumbling (which is in turn related to mass).

Applications: measure binding

Example: Characterize interaction between p53 and peptides mimicking one of its interactors to stabilize oncogenic mutants
Using synchrotrons and XFELs for time-resolved X-ray crystallography and solution scattering experiments on biomolecules
Matteo Levantino¹, Briony A Yorke², Diana CF Monteiro³, Marco Cammarata⁴ and Arwen R Pearson²

Time-resolved X-ray diffraction from protein crystals and scattering from protein solutions provide complementary information. Crystallization can yield atomic resolution information about the whole molecule, but requires that the crystal remains intact during the reaction. Solution scattering is admirably sensitive to structural changes with the advantage that crystals are not required. However, associating the scattering changes with specific structural rearrangements remains difficult.

Designing a time-resolved experiment
When planning a time-resolved experiment, a number of factors must be taken into consideration. First, the timescales of interest must be identified as these will define the X-ray source and experimental protocol required. Once the decision as to type of experiment and time-resolution required has been reached then the question of reaction initiation must be addressed.
Techniques for triggering protein conformational changes either in crystalline or solution samples. Light activation: light pulses are used to photolyze a bond, rapidly increase the temperature of the solvent surrounding the protein, or to induce the release of a photocaged compound. Rapid mixing: a solution containing either protein crystals or solubilized protein can be mixed with a solution containing a substrate or a denaturant agent and then probed with X-rays to monitor any structural change occurring as a function of time.
Time-Resolved Small-Angle X-ray Scattering Study of the Folding Dynamics of Barnase

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²Department of Biomolecular Science, Faculty of Engineering, Gifu University, Gifu, Gifu 501-1193, Japan
³RIKEN Harima Institute, SPring-8 Center, Sayo, Hyogo 679-5148, Japan
⁴Medical Research Council Centre for Protein Engineering and Department of Chemistry, Cambridge University, Hills Road, Cambridge CB2 0QH, United Kingdom
⁵Institute of Multidisciplinary Research for Advanced Materials, Tohoku University, Sendai, Miyagi 980-8577, Japan
Equilibrium fluorescence and SAXS-monitored titrations

Time-resolved SAXS

Fig. 3. (a and b) The time-dependent changes in $R_2^2$ (open circles) and $R(0)$ (open squares) obtained from the Guinier analysis of the SAXS profiles for the kinetic folding of barnase at 26°C. The continuous line represents the single-exponential fit. The filled diamond and square represent $R_2^2$ at zero and infinite time, respectively. (c) Time-resolved Kratky profiles for the same folding process of barnase. For clarification, seven representative profiles were presented, which were obtained for the static native state in the presence of 0.7 M Gdn (purple), the unfolded state in the presence of 5 M Gdn (red), and the kinetic data at 11 ms (orange), 250 ms (yellow), 560 ms (green), 1.0 s (sky blue), and 2.2 s (blue) after initiating the folding.

Fig. 4. The pair distribution functions, $P(r)$, calculated from the time-resolved scattering profiles of barnase folding. The open circles and squares correspond to $P(r)$ functions of the native state at 0.7 M Gdn and the unfolded state at 5.0 M Gdn, respectively. The filled and open diamonds correspond to $P(r)$ functions for the kinetic data at 11 ms and 2.2 s, respectively.
Use DLS in kinetic mode to follow the kinetics of (slow) association/dissociation of a protein
Apoptosis Inducing factor (AIF):
Flavoprotein anchored to the inner mitochondrial membrane, facing the intermembrane space:
- unknown catalytic activity (if any) but essential for maintenance of Complex I and II (??);
- moonlights as apoptosis inducing factor.
- The NADH-reduced form is very stable: a NADH sensor?
- The reduced form is dimeric
Monitor the dimerization of AIF upon reduction with NADH by DLS