Spectroscopy on photoactive proteins

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Outline
1. What do we want to know?
2. Techniques:
   a. Why ultrafast spectroscopy
   b. How to measure this
   c. Lasers and nonlinear optics
3. Data analysis
4. Examples:
   - photosynthetic reaction centers, protochlorophyllide oxidoreductase, photoactive yellow protein

1. What do we want to know?
Understand with atomic detail how:

- Enzymes catalyze reactions with enormous rate enhancements;
- \( \Delta G \)
- Electron, proton transfer, isomerization occurs;
- Subtle (and less subtle) structural changes make sure a reaction occurs.

Relation between structure – dynamic – functionality

1. Identifying electron donor and acceptors in photosynthesis

The Photosystem II core

Relation between structure – dynamic – functionality

1. Isomerization in Photoactive Yellow Protein

Groot et al., "Initial steps of signal propagation in photoactive yellow protein revealed with femtosecond mid-infrared spectroscopy," Biochim. 2011


1. Light-dependent enzyme activation and catalysis


2a. Techniques: why ultrafast spectroscopy

- Molecules move with respect to each other on a time scale of 10 fs - 1 ps (= 10.10^{-15} - 10^{-12}s)

\[ \text{O} \leftrightarrow \text{H} \]
Stretch frequency $\approx$ 3500 cm$^{-1}$
period of 10 fs

- So reactions which constitute the transfer or movement of a proton or electron or C-atoms will occur on these time scales

$\Rightarrow$ An ultrafast technique is necessary

Infrared spectroscopy, measuring the frequency of vibrational modes, is structure sensitive. For example: H-bond lengths can be determined with sub-Å resolution.

\[ X - C - O - H \quad || \quad O - X \]
\[ \omega_1 \quad \omega_2 \]
$\omega = (k/m)^{1/2}$
2b. Techniques: how to measure this?

Lambert-Beer’s law: transmission of light through a cell

\[ T = \frac{I}{I_0} = 10^{-c \cdot \varepsilon \cdot L} \]

where \( c \) (or \( \alpha \)) is the molar extinction coefficient.

Absorption (a.u.)

Wavelength (nm)

2b. Techniques: how to measure this?

Excited state absorption

Electronic transitions in molecules

Ground state

Excited State Absorption

Difference Absorption Spectrum: \( \Delta A(t) - \Delta A(t=0) \)

Stimulated emission

Heterodyne detection, observation of superposition of local oscillator field (= probe field) and signal field:

\[ I(t) = n(\omega_s) c |E_{LO}(t) + E_{S}(t)|^2 = I_{LO}(t) + I_{S}(t) + 2 n(\omega_s) c |\text{Re}[E_{LO}(t) \cdot E_{S}(t)]| \]

And solve to get

Here is used that \( \text{Im}[E_{LO}(t) \cdot P(t) \cdot E_{S}(t)] \)

The probe absorption is related to the out-of-phase component of the polarization signal. It is quadratic in both pump and probe field. And linear rather than quadratic in the weak nonlinear polarization \( P \).
2b. Techniques: how to measure this?

- In summary, we need a short laser pulse to start a reaction and we need probe light in a large as possible spectral region.

2c. Lasers and nonlinear optics

- Volg e+, H⁺ transfer via absorsptieveranderingen in elektronische overgangen:
  - 400 – 1000 nm,
  - en vibratiele overgangen:
  - 3 – 10 µm = structuur info

2c. Lasers and nonlinear optics

- Probe/excite from 350nm – 10 µm, 100.10⁻¹⁵ s – 1 ns.

2c. Lasers and nonlinear optics

- Light Amplification by Stimulated Emission Radiation:
  - Population inversion
  - Cavity
  - Gain medium -> Titanium:sapphire

- Single mode → CW laser
- Many modes with phase relation → short pulse in the cavity

2c. Lasers and nonlinear optics

- Oscillator

  - Cavity
  - Pump laser
  - Leaky mirror

  For Ti:sapphire oscillators
  - λ = 800 nm,
  - Rep rate = 80 MHz
  - Low power ~10 nJ
  - Pulses can be as short as ~10 fs

Regenerative amplification

- Amplify from nanj to millijoules
  - Peak power 20 fs pulse if focussed to 100 micrometer = 10¹² W/cm²
  - 1000 times damage threshold most materials!
Regenerative amplification

Setup

White light generation by Self Phase Modulation

Parametric generation or amplification
Noncollinear optical parametric amplification

- When using a non-collinear phase matching angle in BBO pumped at 400 nm, the phase matching angle becomes independent of wavelength over a large part of the spectrum, for an angle of 3.7° between pump and signal (Gale, Hache 1994) ⇒ large bandwidth
- The spatial walk-off (from the extraordinary pump beam) is 4.0°, with $k_p$ farther from optical axis than $k_p$. This is coincidently close to the noncollinear angle $\alpha$, ⇒ high gain
- Sub-10 fs with $\mu$J energies can be obtained (efficiency 10-30%)

Optimize bandwidth by matching the signal and idler group velocities (=degeneracy for collinear beams).

Expressed in terms of $\alpha$ and $\theta$ and solved for large bandwidths, one finds $\alpha = 3.7^\circ$ and $\theta = 32^\circ$.

400 nm pump

White light seed

Shorter pulses by
- minimizing dispersion of white light (no dispersive optics)
- or even lengthening pump pulse
- optimal compression (small apex angle prisms or gratings)

NIPA

Setup

The instrument response function

The cross- or auto correlation is given by

$$ I_{\text{autocorrelation}}(\tau) = \int \int I_0(t)I_0(t+\tau) \, dt \, d\tau $$

Rule of thumb: time from 10-90% ~ $\tau_{\text{cross}}$
Important experimental aspects:

- Repetition rate of laser must be slower than photocycle, or sample must be refreshed for every shot.
- Excitation density must be low, only when less than 10% of complexes are excited you are in a linear regime.
- Population dynamics are measured under the 'magic' angle 54.7°; at other angles orientational dynamics are measured.

\[ \text{anisotropy} = r = \frac{(\Delta D_{//} - \Delta D_{\perp})}{(\Delta D_{//} + 2\Delta D_{\perp})} \]

2b. Techniques: how to measure this?

- In summary, we need a short laser pulse to start a reaction and we need probe light in a large as possible spectral region.

Electronic transitions in molecules

- Voigt e H⁺ transfer via absorptieveranderingen in elektronische overgangen 400 – 1100 nm, en vibrationele overgangen 3 – 10 µm = structuur info.
2b. Techniques: how to measure this?

- **Ground State Absorption**
  - Time resolution ~100 fs (10^{-13}s)

- **Excited State Absorption**

- **Difference Absorption Spectrum:** \( A(t) - A(t=0) \)

Data: example

Typically 256 traces per experiment, so Nx256 traces

Global analysis

\[
y(t, \lambda) = \sum_{i=1}^{\infty} \text{exp}(-\lambda_i \Delta t) \text{DAS}_i(\lambda)
\]

Without any a priori knowledge, fit your data to a minimum number of exponential decays, convoluted with instrument response function \( \Delta t \):

\[
i(t) = \frac{1}{\Delta t/2\pi} \text{exp}(-\log(2)(2(t-\mu)/\Delta t^2))
\]

Amplitudes: Decay Associated (Difference) Spectra

Target analysis

- Time traces at 400 and 500 nm
- Time-gated spectra at 0.4 ms (squares) and 1.6 ms (triangles)

Model 1

Model 2

Concentration profiles

DADS

SADS

Global analysis

Or, if there is some a priori knowledge, set up a compartmental model. For instance, assume that the first compartment, representing component 1, is excited. Component 1 irreversibly decays with rate \( k_1 \) thereby forming component 2 with fractional yield \( \Phi_1 \). Component 2, which is represented by the second compartment, decays with rate \( k_2 \), which is smaller than \( k_1 \).
You can set up a physical realistic linear compartmental model where transitions between compartments are described by microscopic rate constants which constitute the off-diagonal elements of the transfer matrix $K$. The diagonal elements of $K$ contain the total decay rates of each compartment. With the concentration of the compartments described by the vector $c(t) = [c_1(t), ..., c_{n_{comp}}(t)]^T$.

Thus, a linear compartmental model with $n_{comp}$ compartments is described by a differential equation for these concentrations:

$$\frac{d}{dt}c(t) = Kc(t) + f(t)$$

Where $f(t) = \left( \lambda_1 x_1 + \cdots + \lambda_{n_{comp}} x_{n_{comp}} \right)^T$.

Real data: the bacterial reaction center

Look at residuals etc

Real data: the bacterial reaction center

Interpretation...

The first radical pair "P+B−" contains
• anion bands of both B and H!
• H bleaching!

→ First RP is actually a mixture. Coherent or incoherent?
Coherent or incoherent fast equilibrium between $B^-$ and $H^-$?

The decay of the $|g_H\rangle$ coherence is much faster than the decay of the $|B_H\rangle$ coherence. Considering that the dephasing of the $|g_H\rangle$ coherence is caused by the transition energy fluctuations on $H$, whereas the dephasing of the $|B_H\rangle$ coherence is due to the fluctuations on the gap between $H$ and $B$ transition energies, the transition energy fluctuations on $B$ and $H$ must be strongly correlated, because in-phase energy fluctuations do not destroy coherence.

Such a strong correlation can arise for two possible reasons: strong electronic coupling between $B$ and $H$ and/or strong correlation between nuclear modes that modulate transition frequency fluctuations of localized BChl and BPhy excitations.

Our theoretical analysis found that strong electronic coupling alone cannot reproduce the sawtooth pattern and a dephasing time as long as that observed (11). Instead, cross-correlation between nuclear modes modulating the energy levels of localized BChl and BPhy excitations is required.

Modeling: the electronic coupling $J=220\text{ cm}^{-1}$ and the gap between excitonic $H$ and $B$ states is $680\text{ cm}^{-1}$

Does this put us in the adiabatic electron transfer regime? Yes, according to:

Transmission Coefficients for Chemical Reactions with Multiple States: Role of Quantum Decoherence
Aurélien de la Lande,†,* Jan Rezac,‡ Bernard Levy,† Barry C. Sanders,§ and Dennis R. Salahub||
J. Am. Chem. Soc. 2011, 133, 3883–3894:

‘In principle, an adiabatic regime is achievable even with moderate electronic coupling provided $\tau_{\text{dec}}$ is large enough. In that regard it is important to mention recent experimental findings that have revealed cases of electronic coherences persisting over tens or hundreds of femtoseconds.’

Electron transfer in BRC is adiabatic

Charge separation in Photosystem I

500 550 600 650 700 910 980 1050

Relative $\Delta \varepsilon$

500 550 600 650 700 910 980 1050

wavelength (nm)

500 550 600 650 700 910 980 1050

Relative $\Delta \varepsilon$

500 550 600 650 700 910 980 1050

wavelength (nm)
Plant Photosynthesis

Structure of Photosystem I

Reaction center: Cofactors and charge separation

Understanding primary charge separation

Difficult...

Keto and ester C=O as redox probe

Renger, review in Current Science, 2010

Shuvalov et al BBA 2010

Renger, review in Current Science, 2010

Shuvalov et al BBA 2010

Look at changes in C=O absorption between 1800 and 1600 cm⁻¹; exact frequency depends on environment, presence of H-bonds, dimers etc, and there are very specific changes for Chl/Chl⁺ and Chl/Chl⁻.
**Excited state difference spectra**

- Chl*/Chl in THF
- Pheo*/Pheo in THF
- keto
- ester

**Vis-pump/IR probe on Photosystem I**

- Synechococcus elongatus trimers
- Excite at 700 nm (~30% direct RC excitation), P=100 nJ.
- Probe between 1750-1450 cm$^{-1}$
- Sodium ascorbate & phenozine methosulfate to keep RCs open
- Sample re-excitation after 1 minute or more

**Final spectrum compared with FTIR P$^+$/P$^{700}$**

- Final spectrum is $P^+A_1^-$ (aka $P^+Q^-$)

**Spectral evolution in time**

- SpecA $\rightarrow$ SpecB $\rightarrow$ SpecC $\rightarrow$ SpecD $\rightarrow$
- $k_1$ $k_2$ $k_3$ $k$
- $\sim$ time-gated spectra

**Comparison with P$^+$H$^-$ of Photosystem 2**

**Spectral evolution in time**

- Early RP formation
- Different electron donors
- 7 ps mixture of excited States and RP
- 40 ps RP but different from
- $k_1$P$^+A_1^-$ (aka $P^+Q^-$)
Can we model this?

- Energy transfer, also from the red pigments

![Energy transfer diagram]

Rates free fit parameters

Can we do better?

- Energy transfer, also from the red pigments

![Energy transfer diagram]

Rates free fit parameters

More complicated, but more realistic:

- The radical pairs

![Radical pairs diagram]

- RP2: P_{A2} (aka P_{Q2})
  - RP1: different electron donor & different acceptor
Charge separation: $A^+_A A^-_{0A}$

Second radical pair: $P^+_A A^-_1$ (aka $P^+Q^-$)

Kinetics
- $A^+_A A^-_{0A}$ formation: 0.3 ps
- Equilibrium $A^+_A A^-_{0A}$ and $P^+_A A^-_1$: 6 ps
- Full population of $P^+_A A^-_1$: 40 ps (known)
- Antenna decay: 1 and 10 ps (known)

In Photosystem 2:
- PheophytinD1 is the primary electron acceptor,
- ChlD1 electron donor in Photosystem II

Conclusions
- In both plant reaction centers, $P$ is not the primary electron donor
- Electron and hole transfer occur to create the radical pair $P^+_700 A^-_1$ in PSI
- Different from situation in bacterial RCs

Eliminating diffusional events
- Problem: Protein dynamics and conformational changes in enzyme catalysis ("slow") are almost impossible to distinguish from the actual reaction involving proton and hydride transfer ("fast") in most enzymes.
- Look at catalytic events in the light-driven enzyme NADPH:protochlorophyllide oxidoreductase (POR).
Light-driven step in the biosynthesis pathway of chlorophyll

POR, Short-chain alcohol dehydrogenase

Tracking PChlide to Chlide conversion

Elektronic transitions in molecules

Pump-probe experiments

Experimental setup
Measure ultrafast dynamics via absorption difference spectroscopy

Ultrafast spectroscopy on POR

Global analysis

Intermediate I675 formed in 3 and 400 ps

Nature of I675?
Single pulse ultrafast spectroscopy

- Amplified Ti:sapphire laser w/ multichannel detection
- time resolution 100 fs
- spectral range 350-1000 nm
- Line-scan sample scanner, period ~ 1 minute
- Complete dataset in 1 minute = with one laser pulse/spot

POR transient traces as function of scan nr

After additional 15 mins CW lamp illumination

POR product depends on illumination history

Number of photons / cycles through excited state
Reaction rate and product yield increase

Accumulated Chlide

How to model this?

Inactive fraction (n)

Active fraction (n)

Product (n)

Target analysis,

fit 256 time traces x 55 scans, estimate spectra, rates, e and e x QY

Not, Single, and Double excited enzymes

Inactive → Active → (I_{675}) Chlide

e

e x QY

Where
- e is the excitation rate per pulse and
- QY is the quantum yield of Chlide formation

Solve the coupled differential equations and fit to the individual scans

→ e = 0.045 per pulse & QY = 0.3 ± 0.1

(estimated excitation density = 0.03)
Activation of POR

<table>
<thead>
<tr>
<th>Quantum Yield 0</th>
<th>Quantum Yield 0.83</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inactive</td>
<td>Active</td>
</tr>
<tr>
<td>Pchlide<em>I → PChlide</em>II → PChlide*III</td>
<td>e × QY</td>
</tr>
</tbody>
</table>

Active enzymes = enzymes that have been excited once!

First photon turns the enzyme ‘on’, whereas the second photon induces catalysis.

Is activation conformational change?

Record absorption difference spectra in the midIR under similar illumination conditions, using 5-ns laser pulses at a 20 Hz repetition rate to excite and measure, while flashing, light-induced minus dark difference spectra every second, with rapid scan FTIR.

Spectral changes in midIR as a function of illumination

Wavenumber (cm⁻¹)

Number of photons (a.u.)

Time (s) / Number of photons (a.u.)

Spectral changes in midIR as a function of illumination

Wavenumber (cm⁻¹)

A → B → C

Blue only appears under continued illumination.

PChlide keto: FLN spectroscopy

Fluorescence line narrowing experiments at 4K
Refinement

First photon induces a conformational change
Only after absorption of a second photon can ultrafast coupled proton and hydride transfers proceed.

Conformational change is highly efficient (1 photon) and directive (results in high QY)
Nature of conformational change: What is the lifetime? Does it survive turnover?

Summary so far:

Mixing single turnover enzyme with fresh PChlide

Activity survives turnover

Putting an activated enzyme in the dark

Lifetime active conformation > 24 hrs

Comparing mesophilic and thermophilic POR

Estimated excitation density consistently 2x lower in thermophilic POR
Quantum yield of activation is lower

Nature of I675 state

Ultrafast 3 ps and 180 ps proton transfer from Tyr189 to PChlide
Conclusions

Protein motions control activity:
POR can be switched by a first photon in an active conformation, see midIR & thermophilic POR

In the activated state proton transfer occurs on picosecond time scale, hydride on microsecond time scale.

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