Kinetic and equilibrium
Absorbance and fluorescence spectroscopies
My Research Interests:

- enzyme mechanisms
- regulation of activity in enzymes

Main projects

- Structure-function relationships of flavin- and iron-sulphur-dependent oxidoreductases.

Other/Collaborative projects

- *Mycobacterium tuberculosis* enzymes acting on tRNA’s as potential drug targets: Glu-tRNA synthetase & Glu-tRNA reductase.
- Folding Inhibitors of HIV-1 protease
Our approach:

gene cloning, engineering & expression
• protein (over)production & purification
  • structure-function studies
    – steady-state & pre-steady-state kinetics
      – mechanistic studies
    – absorbance & fluorescence spectroscopies

  – EPR, NMR (D. Edmondson, Atlanta; W.R. Hagen, Delft)
    – X-ray crystallography (A. Mattevi, Pavia)
  – Small-angle X-ray scattering (Dmitri Svergun et al., EMBL-Hamburg)
    – Cryoelectron microscopy (Nicolas Boisset et al., Paris VI)
  – Molecular dynamics (V. Coiro, A. Di Nola, Rome; E. Fois et al., Como)
NEVER EVER
EVER
GIVE UP!
General Mode of Action of Proteins:

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

2) Effect

- 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 (conformational change, 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 (steady-state) and kinetic studies.

\[
A + B \quad \underset{k_r}{\overset{k_f}{\rightleftharpoons}} \quad C + D
\]

At equilibrium:

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

\[
\Delta G^\circ = -RT \ln K_{eq}
\]
For a multistep process (e.g.: an enzyme reaction):
Protein folding and conformational changes can 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 reagents 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 Absorbance, Fluorescence; Conductivity; Radioactivity, ....
Discontinuous methods may not allow the isolation and identification of (unstable) intermediates or products including protein-protein, protein-ligand complexes, etc.
Continuous methods that do not require sample manipulation are:

- less time consuming,
- less expensive (a part from the equipment?) and more precise (lower number of steps where experimental error can be introduced)

Spectroscopic methods may allow the direct observation of the species present in solution IF

- the signals (spectra) of A, B, C, etc. can be distinguished from each other
- the intensity of the signals can be related to the concentrations of A, B, C, etc.
- the signals can be acquired very rapidly to monitor changes over time
Absorbance and fluorescence spectroscopies can allow the rapid acquisition of signals with high sensitivity.

The absorption and fluorescence spectra often allow to identify and quantify the chemical species in solution.
Today’s plan:

- 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
UV-Vis Absorbance and fluorescence spectroscopies use a narrow (200-800 nm) region of the electromagnetic spectrum
Basic principles of absorbance and fluorescence spectroscopies

Only some electronic transitions are possible when a sample is irradiated with near UV – visible light.
- A light absorbing compound is called a «chromophore».

- 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.
Several natural compounds absorb light in the visible region of the spectrum.
Several biological compounds are also fluorescent

Figure 3.2. Absorption and emission spectra of the fluorescent amino acids in water of pH 7.0.

Figure 3.4. Emission spectra from intrinsic tissue fluorophores. Revisited from [25].
How to measure absorbance and fluorescence
The relation between absorbance at a given $\lambda$ and concentration of the absorbing species is given by the Lambert-Beer Law:

$$A = \varepsilon c l$$

$A$, absorbance = $\log \left( \frac{I_o}{I} \right)$

$\varepsilon$, molar extinction coefficient; $c$, concentration, $l$, optical path.
Scheme of a dual (double) beam spectrophotometer
Scheme of a (photo)diode array spectrophotometer

Figure 1. Schematic of photodiode array spectrophotometer
Basic scheme of a spectrophotofluorimeter

Detector → Monochromator

$\lambda_2 > \lambda_1$
Excitation and emission fluorescence spectra

Excitation spectrum,
\( \lambda_{\text{em}}: 523 \text{ nm} \)

Emission spectrum,
\( \lambda_{\text{ex}}: 450 \text{ nm} \)

FMN
## Absorption versus fluorescence spectroscopy

<table>
<thead>
<tr>
<th>Detection limits</th>
<th>Absorbance</th>
<th>Fluorescence</th>
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<tbody>
<tr>
<td>μM – mM</td>
<td>≤ μM</td>
<td>≤ μM</td>
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<table>
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<tr>
<th>Linearity of signals</th>
<th>Absorbance</th>
<th>Fluorescence</th>
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<tbody>
<tr>
<td>2 orders of magnitude (e.g.: 1 – 100 μM)</td>
<td>≈ Narrow(er) (e.g.: 0.1 – 1 μM)</td>
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<tr>
<th>Quantification of solute</th>
<th>Absorbance</th>
<th>Fluorescence</th>
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<tbody>
<tr>
<td>ε, extinction coefficient (M^{-1} \text{ cm}^{-1})</td>
<td>F ( \propto c^*l), in arbitrary units</td>
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<th>Sensitivity to:</th>
<th>Absorbance</th>
<th>Fluorescence</th>
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<tr>
<td>Temperature</td>
<td>≈</td>
<td>Very high</td>
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<tr>
<td>Solvent</td>
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<td>Other solutes</td>
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<th>Return to ground state</th>
<th>Absorbance</th>
<th>Fluorescence</th>
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<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>
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</table>
Intrinsic Chromophores in proteins

Amide bond (220 nm)

- Use to detect peptides and proteins when concentration is low
- Several other compounds absorb at 220 nm
Intrinsic Chromophores in proteins

Aromatic amino acids, which are also fluorescent

Use absorbance at 280 nm to detect proteins.

If the number of aromatic amino acids (especially Trp) is limited, monitoring their fluorescence changes can help study:
- protein folding/unfolding;
- protein-ligand and protein-protein interactions;
- conformational changes.
Otherwise, the data can be difficult to interpret.
Protein unfolding exposes Trp to solvent causing changes of fluorescence intensity and $\lambda_{em}$.

Protein oligomerization may bury a Trp residue.
Fluorescence quenchers by collision such as acrylamide can be used to monitor the location of a Trp residue:

- If buried: no effect of quencher;
- If solvent exposed: effect of quencher
Intrinsic Chromophores in proteins with absorbance in the visible region: several coenzymes and cofactors.

Some are also fluorescent.

Modifications of the compounds or of their environment alter the absorption (and fluorescence) spectrum.

Hemoglobin oxygenation  Cytochrome c reduction
Pyridoxal phosphate (PLP, vitamin B6)
Cobalamins (vit B12 derivatives)

Methionine synthase (MetH)

Base-on wt

Base-off wt

Base-off D757E

Base-on wt

Base-on D757E

Wavelength (nm)
The flavin coenzymes FMN and FAD are derivatives of Riboflavin (vitamine b2) and participate to oxidoreduction reactions.
NAD(P)$^+$ and NAD(P)H, derivatives of vitamin B3 (PP), substrates/products of dehydrogenases/reductases, have different absorbance and fluorescence properties.

Excitation and emission spectrum of NAD(P)H
Several synthetic chromophores or fluorophores can be used to study protein properties
ANS (2-Anilinonaphthalene 6-sulfonic acid) fluorescence increases when it interacts with hydrophobic residues.

ANS is used to monitor the exposure or burial of hydrophobic residues in proteins during folding/unfolding, dissociation of oligomer, conformational changes in general.

**Figure 8.** ANS emission spectra at pH 4.7 and 25 °C ($\lambda_{ex} = 376$ nm): PR (0.35 μM) was incubated in the absence of inhibitor (a), or in the presence of 0.1 μM acetylpepsatin (b), 3.75 μM 23 (c), or 1.66 μM 32 (d). The fluorescence intensity was corrected from the fluorescence due to the same concentration of ANS (30 μM) without enzyme (a) and without enzyme and inhibitor (b–d).
Examples of the use of absorbance and fluorescence to characterize enzyme mechanisms:

- Enzyme activity assays
  - Binding studies
  - Redox titrations
- Rapid reaction kinetics
Enzymes catalyze chemical reactions with:
- High (stereo)specificity with respect to the substrates and products
- High acceleration rates (versus the uncatalysed reaction)

Enzymes do not alter the reaction equilibrium

The high acceleration rates are achieved by combining:
- Orientation and proximity effects
- Acid-base catalysis
- Electrostatic catalysis
- Covalent catalysis
- Metal-assisted catalysis
- Preferential stabilization of the transition-state
Goal of kinetic studies is to determine the reaction mechanism, the energetics, and the rates of individual steps to:

- Understand the origin of the catalytic power of enzymes
- Carry out protein and metabolic engineering
- Drug design (most potent inhibitors are transition-state analogs.)
Initial velocity measurements under steady-state conditions allow to determine the kinetic parameters $V_{\text{max}}$ and $K_M$ for the substrates, which depend on the rate constants that govern the individual reaction steps.

Kinetic measurements under pre-steady-state conditions allow to determine directly the values of the rate constants that govern the individual reaction steps and to identify intermediates.
Initial velocity measurements of the enzyme-catalyzed reaction are carried out under a variety of conditions (T, pH, inhibitors; isotope substituted substrates, solvent viscosity, ionic strength),

- to quantify the enzyme
- to obtain information on the reaction mechanism, on regulatory mechanisms, which is the active enzyme form, etc.

\[
v = - \frac{d[S]}{dt} = \frac{d[P]}{dt}
\]

Substrates $\rightarrow$ Products

\([P], \mu M \text{ or mM}\)

Time, sec or min
The assumptions of the model:
- \([E_{\text{tot}}] \ll [S_0]\)
- Measure \(v\) (initial velocity) when \([P]\) = 0
- \(v = k_3[ES]\)
- \([ES]\) = constant

The expression of \(v\):
\[
v = \frac{k_3 [E_{\text{tot}}] * [S]}{k_2 + k_3} + [S]
\]

The kinetic parameters \(V_{\text{max}}\) and \(K_m\):
\[
V_{\text{max}} = k_3[E_t]
\]
\[
K_m = \frac{k_2 + k_3}{k_1}
\]

\(k_{\text{cat}} = \frac{V_{\text{max}}}{[E_{\text{tot}}]}\)
Use of absorbance and fluorescence methods with natural or synthetic substrates to monitor substrate consumption or product formation under steady-state conditions.

\[ v = - \frac{d[S]}{dt} = \frac{d[P]}{dt} \]
A simple case:
Monitor NAD(P)H oxidation (or NAD(P) reduction) in reactions catalyzed by dehydrogenases/reductases

Alcohol dehydrogenase (ADH)

Acetaldehyde + NADH + H⁺ ↔ Ethanol + NAD⁺

\[ \epsilon_{340} = 6.23 \text{ mM}^{-1}\text{cm}^{-1} \]
When S or P cannot be observed directly it is possible to couple the reaction of interest with an «indicator reaction» with substrates/products suitable for a spectrophotometric assay.

D-alanine + O₂ → Pyruvate + ammonia + H₂O₂

D-amino acid oxidase

L-lactate → NADH + H⁺

LDH

NAD⁺

H₂O

X

X_ox colored

H₂O

LDH, lactate dehydrogenase; HRP, horse radish peroxidase
For consecutive reactions:

\[ A \rightarrow B \rightarrow C \]

If \( v_{B \rightarrow C} \gg v_{A \rightarrow B} \), then \( v_{A \rightarrow C} = v_{A \rightarrow B} \)
When S or P cannot be observed directly it is possible to use synthetic (non-physiological) substrates suitable for a spectrophotometric assay.

$$\text{Amplex Red} \xrightarrow{\text{H}_2\text{O}_2, \text{H}_2\text{O}} \text{Resorufin}$$

$$\varepsilon \approx 50 \text{ mM}^{-1}\text{cm}^{-1}$$
Use of synthetic substrates to study protease activity

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

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

HIV 1 Protease Activity
Use of synthetic substrates to study protease activity:
A FRET-based fluorescent assay for HIV1 protease

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

\[ \lambda_{\text{ex}}, 320 \text{ nm}; \quad \lambda_{\text{em}}, 420 \text{ nm}; \]
Fluorescence resonance energy transfer (FRET) occurs when the emission spectrum of a «Donor» molecule overlaps with the absorption spectrum of the «Acceptor» molecule without emission a photon from the «Donor».

Emission of the «Donor» will be quenched by the «Acceptor».

- The Acceptor may or may not emit light at a longer wavelength.

- The Efficiency of FRET depends on the distance between Donor and Acceptor. Thus, FRET can be used to monitor distances between Donor and Acceptor.
With the absorption-based assay of HIV1 protease we can cover a broader range of substrate concentrations due to the high starting fluorescence of Substrate III.

 Conditions: 100 mM NaAcetate, pH 5.0, 1 mM EDTA, 1 mM DTT, 100 mM NaCl; 25°C

<table>
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<tr>
<th>Parameter</th>
<th>Value</th>
<th>Std. Error</th>
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<tr>
<td>Vmax</td>
<td>118.5736</td>
<td>3.2182</td>
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<tr>
<td>Km</td>
<td>25.8217</td>
<td>2.4562</td>
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<tr>
<th>Parameter</th>
<th>Value</th>
<th>Std. Error</th>
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<tbody>
<tr>
<td>Vmax</td>
<td>195.2216</td>
<td>12.3678</td>
</tr>
<tr>
<td>Km</td>
<td>20.1679</td>
<td>3.2118</td>
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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 Kd, 215 nM
**Determination of equilibrium (or dissociation) constants**

At equilibrium:

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

\[ \Delta G^\circ = -RT \ln K_{eq} \]

\[ K_d = \frac{[A]_{eq}[B]_{eq}}{[C]_{eq}[D]_{eq}} \]
Flavin-dependent enzymes contain FMN or FAD as the coenzyme, a useful intrinsic spectroscopic probe.
The flavin cofactor acts as an intermediate electron acceptor between the substrate/product couple.

Thus, the reductive and the oxidative half-reactions can be studied separately by absorbance and fluorescence spectroscopies under equilibrium or time resolved conditions for kinetic and mechanistic work.
The flavin absorbance (and fluorescence) spectrum is sensitive to the redox state, chemical modifications of the isoalloxazine ring, the «environment» (hydrophobicity, protonation state of ionizable groups, complexes, ....)

![Flavin spectra and structures](image-url)
Influence of the protein on the absorbance spectrum of bound FAD

1) The spectrum of the «as isolated» MICAL indicates the presence of a flavin coenzyme, but the spectrum differs from that of authentic flavin, FAD or FMN.
2) Release of the coenzyme by denaturation yields the spectrum of authentic FAD or FMN
   - A charge-transfer complex between the flavin and Trp400 from X-ray structure
   - Need to determine if the coenzyme is FAD or FMN
   - Use the information to determine the stoichiometry and the extinction coefficient of the bound coenzyme
Influence of the protein on the fluorescence of bound FAD

1) The fluorescence of the bound coenzyme is quenched by the protein
Fluorimetric Identification of the cofactor bound to MICAL as FAD

If the coenzyme is FAD, PDE will cause a 10x increase of fluorescence due to conversion into FMN, and removal of internal quenching of fluorescence by the AMP moiety.
Some flavoproteins can form a Flavin-N(5)-sulfite adduct

The formation of the flavin-N(5)-sulfite adduct gives information on the electronic distribution of the **bound** flavin coenzyme (e.g. Degree of electrophilicity of N(5)).
Use of the study of sulfite reactivity to distinguish between the flavins of glutamate synthase (GltS)
Glutamate synthase (GltS)

L-Glutamine + 2-oxoglutarate + NADPH $\rightarrow$ 2 L-Glutamate + NADP$^+$

Bacterial NADPH-GltS

1 x 50 kDa + 1 x 150 kDa
1 x FAD, 1 FMN,
1 x [3Fe-4S]; 2 x [4Fe-4S]

Eukaryotic NAD(P)H-GltS

1 x 150 kDa
1 FMN,
1 x [3Fe-4S];
Initial Scheme of bacterial NADPH-GltS reaction

- Site 1:
  - NADPH \rightarrow \text{Flavin 1} \rightarrow \text{NADP}^+

- Site 2:
  - L-Glu \rightarrow 2-IG \rightarrow \text{Flavin 2} \rightarrow \text{L-Glu}
  - L-Gln \rightarrow NH_3
  - 2-OG
The study of the reactivity of the flavin coenzymes of GltS with sulfite allowed us to distinguish between the flavin at the synthase site and the flavin at the NADPH oxidizing site.

Sulfite reacts with only one of the GltS flavins; sulfite does not interfere with the reduction of the other flavin by NADPH.

2-OG displaces sulfite from GltS in a competitive fashion.
Conclusions:
- Flavin 2, the flavin at the synthase (2-OG) site reacts with sulfite,
- 2-OG binds near the sulfite-reacting flavin;
- From the backtitration of the GltS-sulfite complex with 2-OG we can calculate the $K_d$ of the GltS-2-OG complex
- the flavin at the NADPH oxidizing site does not react with sulfite

- We can use sulfite reactivity to monitor the state of site 2 with respect to flavin environment and 2-OG binding
Production and characterization of the isolated $\alpha$ and $\beta$ subunits and of the homologous Ferredoxin-dependent GltS

L-Glutamine + 2-oxoglutarate + NADPH $\rightarrow$ 2 L-Glutamate + NADP$^+$

Bacterial NADPH-GltS

- 1 x 50 kDa + 1 x 150 kDa
- 1 x FAD, 1 FMN,
- 1 x [3Fe-4S]; 2 x [4Fe-4S]

Eukaryotic NAD(P)H-GltS

- 1 x 150 kDa
- 1 FMN, 1 x [3Fe-4S];

$\alpha$ and $\beta$ subunits

- GAT
- FMN

- FeS
- FAD
- NAD(P)

Production and characterization of the isolated $\alpha$ and $\beta$ subunits and of the homologous Ferredoxin-dependent GltS
The FMN coenzyme bound to the isolated α subunit and to the homologous ferredoxin-dependent GltS reacts with sulfite that is displaced by 2-OG.
Conclusions:
- Site 2, the synthase (2-OG) site is on the GltS α subunit and on the homologous Fd-GltS,
- The flavin is FMN (also 1 x [3Fe-4S] cluster)
- We can use sulfite titrations and backtitrations with 2-OG to study the state of the synthase site in mutants (even inactive mutants).

- The β subunit should contain FAD and the NADPH oxidizing site
The equations for the binding curves

\[ M + L \rightleftharpoons ML \]

High Kd: \( L_{\text{free}} \approx L_{\text{tot}} \)

\[ ML = \frac{M_{\text{tot}} \times L_{\text{tot}}}{K_d + L_{\text{tot}}} \]

Tight binding: \( L_{\text{free}} = L_{\text{tot}} - ML \)

\[ ML = \frac{-(L_{\text{tot}} + M_{\text{tot}} + K_d) \pm [(L_{\text{tot}} + M_{\text{tot}} + K_d)^2 - 4 \times M_{\text{tot}} L_{\text{tot}})]^{1/2}}{2} \]
(A_o – A_x) → [E-sulfite]
(A_o – A_fin) → [E_{tot}]

E + Sulfite$\xrightleftharpoons{K_{Sulfite}}$ESulfite

\[
\frac{(A_o - A_x)}{(A_o - A_{\text{fin}})} = \frac{[E\text{-sulfite}]}{[E_{\text{tot}}]}
\]

\[
\frac{[\text{ESulfite}]}{[E_{\text{tot}}]} = \frac{[\text{Sulfite}]}{(K_{\text{Sulfite}} + [\text{Sulfite}])}
\]

E + 2OG$\xrightleftharpoons{K_{2OG}}$E - 2OG

\[
\frac{[E\text{-2OG}]}{[E_{\text{tot}}]} = \frac{[2OG]}{(K_{2OG} + [2OG])}
\]

ESulfite + 2OG$\xrightleftharpoons{K_{2OG_{\text{app}}}}$E - 2OG + Sulfite

K_{2OG_{\text{app}}} = K_{2OG} \cdot \left(1 + \frac{[S]}{K_S}\right)
Use of spectrophotometric titrations to study the properties of GltS Site 1, the NADPH oxidising site on NADPH-GltS β subunit

1) Express, purify and characterize the GltS β subunit
2) Identify the NADPH-binding site by characterizing the G298A variant
Glutathione reductase (GR) as the model of GltS β subunit on the basis of sequence similarities

- The adenylate portions of FAD and NADP bind to Rossmann-folds
- The consensus sequence is centered on $GXGXXG/A/S$ motifs
- In GR, the G-to-A substitution of the second G of the motif is diagnostic to identify the nucleotide that binds to the site.
Anaerobic NADPH titration of β-GltS and the G298A variant

As expected, the FAD coenzyme bound to the isolated β subunit does not react with sulfite.

Bound FAD is reduced by NADPH with formation of a stable FAD$_{\text{red}}$-NADP$^+$ charge-transfer complex.

The substitution of the second G of the GXGXXA motif of the binding site of the adenylate portion of NADP(H)
- weakens NADPH binding,
- prevents the formation of the E$_{\text{red}}$-NADP$^+$ CT complex.

\[
\text{E-FAD + NADPH} \rightleftharpoons \text{E-FAD.NADPH} \rightleftharpoons (\text{E-FAD}_{\text{red}}\cdot\text{NADP}^+)_{\text{CT}}
\]

\[
\rightleftharpoons \text{E-FAD}_{\text{red}}\cdot\text{NADP}^+ \rightleftharpoons \text{E-FAD}_{\text{red}} + \text{NADP}^+
\]
AADP titration of β-GltS and the G298A variant

The absorbance spectrum of βGltS is perturbed by **2-amino pyridine dinucleotide phosphate** a non-reducible analog of NADP⁺ and a mimic of NADPH.

The G298A substitution in the binding site of the adenylate portion of NADP: -weakens also AADP binding, -decreases the amplitude of absorbance changes indicating an altered positioning of the nicotinamide ring

- Use AADP to determine the effect of bound NADPH on the redox potential without the complication of reduction.

- Which are the consequences of the G298A mutation on the rate of hydride transfer from NADPH to the bound FAD?
Determination of the redox potential of flavin coenzymes
The $E^\circ$ ($E_m$) of a redox couple indicates the tendency of the species to accept/donate electrons

$$E_{A^{\text{ox}}/A^{\text{red}}} = E_A^\circ - \frac{(RT/nF)}{\ln(A_{\text{red}}/A_{\text{ox}})}$$

$$E_A = E_A^\circ (E_m) \text{ when } A_{\text{red}} = A_{\text{ox}}$$

In redox reactions electrons flow from species with lower $E_m$ to species with higher $E_m$
The Nernst equation

\[ A^{n+}_{\text{ox}} + B_{\text{red}} \rightleftharpoons A_{\text{red}} + B^{n+}_{\text{ox}} \]

\[
\begin{align*}
A^{n+}_{\text{ox}} + ne^- & = A_{\text{red}} \quad E_A = E_A^\circ - \frac{(RT/nF) \ln(A_{\text{red}}/A_{\text{ox}})}{nF} \\
B_{\text{red}} & = B^{n+}_{\text{ox}} + ne^- \quad E_B = E_B^\circ - \frac{(RT/nF) \ln(B_{\text{red}}/B_{\text{ox}})}{nF}
\end{align*}
\]

\[ \Delta E = E_A - E_B = (E_A^\circ - E_B^\circ) - \frac{RT}{nF} \ln \frac{A_{\text{red}} * B_{\text{ox}}}{A_{\text{ox}} * B_{\text{red}}} \]

\[ \Delta E = \Delta E^\circ - \frac{2.303RT}{nF} \log \frac{[A_{\text{red}}][B_{\text{ox}}]}{[A_{\text{ox}}][B_{\text{red}}]} \]

\[ \Delta G = -nF \Delta E \]

At equilibrium \( \Delta E = 0 \) \( E_A = E_B \)

\[ E_A^\circ - \frac{2.303RT}{n_AF} \log \frac{[A_{\text{red}}]}{[A_{\text{ox}}]} = E_B^\circ - \frac{2.303RT}{n_BF} \log \frac{[B_{\text{red}}]}{[B_{\text{ox}}]} \]

\( n = \) no. transferred electrons; \( F = \) Faraday constant = 96494 JV\(^{-1}\); \( R, 8.341 \text{ Jmol}^{-1}\text{K}^{-1}; T, 20^\circ \text{C} = 293 \text{ K}; \) \( \log N = 2.303 \ln N; 2.302RT/nF \) with \( n = 2 = 0.029 \text{ V at } 20^\circ \text{C} \)
Example: Spectrophotometric determination of the \( E_m \) of FMN:
- reductive titration of \textbf{FMN} under anaerobiosis, in the presence of an \textbf{indicator dye} (known \( E_m \)) and \textbf{a mediator} (to ensure equilibrium is reached after each addition of \textbf{reductant}).
- Assume FMN and Dye exchange the same no. of electrons (2)

\[
\begin{align*}
\text{FMN}_{ox} + 2e^- \ &= \text{FMN}_{red} \\
\text{Dye}_{ox} + 2e^- \ &= \text{Dye}_{red}
\end{align*}
\]

\[
\begin{align*}
E_{FMN} &= E_{m,FMN} - \frac{RT}{n_{FMN}F} \ln \left( \frac{\text{FMN}_{red}}{\text{FMN}_{ox}} \right) \\
E_{Dye} &= E_{m,Dye} - \frac{RT}{n_{Dye}F} \ln \left( \frac{\text{Dye}_{red}}{\text{Dye}_{ox}} \right)
\end{align*}
\]

\[\Delta E = E_{FMN} - E_{Dye}\]

At equilibrium \( \Delta E = 0 \)

\[
\begin{align*}
E_{m,FMN} - \frac{2.303RT}{n_{FMN}F} \log \frac{\text{FMN}_{red}}{\text{FMN}_{ox}} &= E_{m,Dye} - \frac{2.303RT}{n_{Dye}F} \log \frac{\text{Dye}_{red}}{\text{Dye}_{ox}} \\
\log \frac{\text{FMN}_{ox}}{\text{FMN}_{red}} &= (E_{m,Dye} - E_{m,FMN}) \frac{n_{FMN}F}{2.303RT} + \frac{n_{FMN}}{n_{Dye}} \log \frac{\text{Dye}_{ox}}{\text{Dye}_{red}}
\end{align*}
\]
Spectrophotometric determination of the $E_m$ of FMN:

1. Anaerobic reductive titrations of FMN; Dye; FMN+Dye
2. Determine $\lambda$ and $\varepsilon$ useful to measure $\text{FMN}_{\text{ox}}/\text{FMN}_{\text{red}}$ and $\text{Dye}_{\text{ox}}/\text{Dye}_{\text{red}}$

Conditions: 2 $\mu$M Benzyl viologen, 1 mM Xanthine, 2.5 mU xanthine oxidase in 25 mM Hepes/KOH, pH 7.0, 10% glycerol. 25 °C. Record spectra every 5 min.
Spectrophotometric determination of the midpoint potential of FMN

$$\log \frac{\text{FMN}^{\text{ox}}}{\text{FMN}^{\text{red}}} = (E_{m,Dye} - E_{m,FMN}) \cdot \frac{n_{FMN} F}{2.303RT} + \frac{n_{FMN}}{n_{dye}} \log \frac{\text{Dye}^{\text{ox}}}{\text{Dye}^{\text{red}}}$$

Calculation of $[\text{FMN}^{\text{red}}]$:
$$\frac{(A_{408,0} - A_{408,x})}{(A_{408,0} - A_{408,\text{fin}})} = \frac{[\text{FMN}^{\text{red}}]}{[\text{FMN}^{\text{tot}}]}$$

Calculation of $[\text{FMN}^{\text{ox}}]$:
$$[\text{FMN}^{\text{tot}}] - [\text{FMN}^{\text{red}}]$$

Calculation of $[\text{Dye}^{\text{red}}]$:
$$\frac{(A_{521,0} - A_{521,x})}{(A_{521,0} - A_{521,\text{fin}})} = \frac{[\text{Dye}^{\text{red}}]}{[\text{Dye}^{\text{tot}}]}$$

Calculation of $[\text{Dye}^{\text{ox}}]$:
$$[\text{Dye}^{\text{tot}}] - [\text{Dye}^{\text{red}}]$$
Spectrophotometric determination of the midpoint potential of FMN

\[
\text{Slope} = 0.9 \rightarrow 1: \quad n_{\text{dye}} = n_{\text{FMN}} = 2
\]

\[
\text{Intercept} = -0.99
\]

\[
-0.99 \times \frac{2.303RT}{nF} = -29.8 \text{ mV} = (E_{m,\text{Dye}} - E_{m,\text{FMN}}) = -252 \text{ mV} - E_{m,\text{FMN}}
\]

\[
E_{m,\text{FMN}} = -252 \text{ mV} + 30 \text{ mV} = -222 \text{ mV}
\]
Redox potential determination of the GltS β subunit and of the β GltS-AADP complex: modulation of the potential to favor reduction by NADPH

\[
E_{ox} + n \, e^- \rightarrow E_{red}
\]

βGltS subunit
\(n = 2, -340 \, \text{mV}\)

βGltS-AADP complex
\(n = 2, -307 \, \text{mV}\)

\[
E_{ox} \cdot \text{AADP} + n \, e^- \rightarrow E_{red} \cdot \text{AADP}
\]
- The protein destabilizes $\text{FAD}_{\text{red}}$ with respect to free coenzyme.

- Bound AADP (the substrate analog) favors reduction by NADPH.

\[
\begin{align*}
\text{FAD}_{\text{ox}} + 2e^- & \underset{\text{E}_{\text{ox}} \cdot \text{AADP}}{\xrightleftharpoons{\text{E}_{\text{red}} \cdot \text{AADP}}} \text{FAD}_{\text{red}} \\
\text{NADP}^+ + 2e^- & \underset{\text{E}_{\text{ox}} + 2e^-}{\xrightleftharpoons{\text{E}_{\text{red}}}} \text{NADPH}
\end{align*}
\]
From the Anaerobic NADPH titration of β-GltS and the G298A variant

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

$\rightleftharpoons$ E-FAD$_{\text{red}}$$\cdot$NADP$^+$ $\rightleftharpoons$ E-FAD$_{\text{red}}$ + NADP$^+$

The G298A substitution in the binding site of the adenylate portion of NADP weakens NADP binding and alters the positioning of the nicotinamide ring

Which are the consequences of the rate of hydride transfer from NADPH to the bound FAD?
Does GltS β subunit contain the fully active NADPH oxidising site of GltS?

$k_{cat}$ values:
Overall glutamate synthase reaction: 60 s$^{-1}$
NADPH-INT oxidoreductase reaction: 200 s$^{-1}$

$k_{cat}$ values:
NADPH-INT oxidoreductase reaction: 40 s$^{-1}$

INT, iodonitrotetrazolium salt; synthetic electron acceptor, becomes red on reduction
Rapid Reaction Kinetics.

\[ E + S \xrightleftharpoons[k_2]{k_1} ES \xrightarrow[k_3]{k_4} EP \xrightarrow[k_5]{k_6} E + P \]

\[ k_1 \quad k_2 \quad k_3 \quad k_4 \quad k_5 \quad k_6 \]
Rapid Reaction Kinetics - Pre-Steady-State Kinetics

\([E]: \mu M, mM \text{ vs } nM, \mu M\) for steady-state

\([S]: \mu M, mM\)

\(([S] > 10 \times [E]\) 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 (competence) for data analysis
What can be measured by RR Kinetics?

- Chemical reactions
- Folding/Unfolding
- Protein-protein, Protein-ligand interactions
- Induced conformational changes
Rapid Mixing device

Turbulent flow to ensure constant velocity across tubing

High flow rate (e.g.: 10 m/s = 1 cm/msec)
Detection

**Continuous methods:**

Absorbance, Fluorescence, Circular Dichroism, Fluorescence anisotropy, conductivity, X-ray scattering (?)

**Discontinuous methods:**

EPR (freeze-quench)

Mossbauer (freeze-quench)

HPLC separation of reaction components and chemical analysis (chemical quench)
Stopped-flow set-up for rapid reaction studies and absorbance or fluorescence detection

Push, 3 atm

Drive syringes

Stop syringe

Trigger

Detector

Detector
The upper limit of measured rates is set by Dead-time, Time-constant (Time Resolution), Sensitivity of detector.

**Dead-time**

![Graph of absorbance vs time for different rates with a dead-time of 2 msec.]

**Time-constant (Resolution)**

![Graph of absorbance vs time for different rates with a time constant.]

**Detector**

![Diagram of a detector with labels for push, 3 atm.]
To directly measure the rates of formation/decay of the various species we need to identify suitable wavelengths

\[
A = A_0 \ e^{-k_1t}
\]

\[
B = A_0 \frac{k_1}{(k_2-k_1)}(e^{-k_1t} - e^{-k_2t})
\]

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

\[
I_{\lambda,A} = [A]^* \ \varepsilon_{\lambda,A}
\]

\[
I_{\lambda,B} = [B]^* \ \varepsilon_{\lambda,B}
\]

\[
I_{\lambda,C} = [C]^* \ \varepsilon_{\lambda,C}
\]

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

At different wavelengths, we can distinguish A from B from C.
Diode array set-up vs PMT mode
First order reaction

\[ A \xrightarrow{k_1} B \quad A = A_0 e^{-k_1 t} \]

Pseudo-First order reaction

\[ A + B \xrightarrow{k_1} C \quad B_0 >> A_0 \]

\[ A = A_0 e^{-k_{1,\text{obs}} t} \]

\[ k_{1,\text{obs}} = f(B) \]

Conditions for a simple sf experiment

\[ E \xrightleftharpoons[k_2][k_1][A] EA \xrightarrow{k_3} EP \xrightleftharpoons[k_5][k_4][P] E \]

\[ A_0 >> E_0 \]

\[ E = E_0 e^{-k_{1,\text{obs}} t} \]

\[ k_{1,\text{obs}} = f(A) = (k_1 \cdot A)/(K_d + A) \]
Determination of the Catalytic competence of the NADPH oxidising site of the isolated GltS β subunit:

$k_{\text{cat}}$ (turnover) values:
Overall glutamate synthase reaction: $60 \text{ s}^{-1}$
NADPH-INT oxidoreductase reaction: $200 \text{ s}^{-1}$

$k_{\text{cat}}$ values:
NADPH-INT oxidoreductase reaction: $40 \text{ s}^{-1}$

Does GltS β subunit contain the fully active NADPH oxidising site of GltS?
For consecutive reactions, the rate of formation of an intermediate that is on the catalytic pathway must be greater or equal to the rate of the overall reaction:

\[
A \rightarrow B \rightarrow C
\]

\[
v_{A \rightarrow B} \text{ AND } v_{B \rightarrow C} \geq v_{A \rightarrow C}
\]

\[
\text{NADPH} + E_{\text{ox}} \overset{k_{\text{red}}}{\longrightarrow} E_{\text{red}} + \text{NADP}^+ + H^+
\]

\[
E_{\text{red}} + \text{INT}_{\text{ox}} \overset{k_{\text{ox}}}{\longrightarrow} E_{\text{ox}} + \text{INT}_{\text{red}}
\]

\[
\text{NADPH} + \text{INT}_{\text{ox}} \overset{k_{\text{cat}}}{\longrightarrow} \text{NADP}^+ + H^+ + \text{INT}_{\text{red}}
\]
Catalytic competence of the NADPH oxidising site of the isolated GltS β subunit

Measurement of the rate of FAD reduction by NADPH in the stopped-flow

\[
\text{E-FAD} + \text{NADPH} \rightleftharpoons \text{E-FAD.NADPH} \rightleftharpoons (\text{E-FAD}_{\text{red}} \cdot \text{NADP}^+)_{\text{CT}}
\]

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

\[900/s\]

Is there a charge-transfer complex between \(E_{\text{ox}}\) and NADPH prior to electron transfer? Enzyme reduction is too fast to detect such a CT.
Effect of the G298A substitution in βGltS from equilibrium titration
The mutation abolishes the $E_{\text{red}}$-NADP$^+$ CT complex

Which is the effect on $k_{\text{red}}$?

$E$-FAD + NADPH $\leftrightarrow$ E-FAD·NADPH $\leftrightarrow$ (E-FAD·NADPH)$_{\text{CT}}$ $\leftrightarrow$ (E-FAD$_{\text{red}}$·NADP$^+$)$_{\text{CT}}$ $\leftrightarrow$ E-FAD$_{\text{red}}$·NADP$^+$ $\leftrightarrow$ E-FAD$_{\text{red}}$ + NADP$^+$

Can a charge-transfer complex between $E_{\text{ox}}$ and NADPH be detected? If formation of the CT complex prior to reduction is essential, enzyme reduction is expected to be significantly slowed down in the mutant
Effect of the G298A substitution in βGltS on the reaction with NADPH as studied in the stopped-flow

**Results:**
- 10-fold weakened NADPH binding;
- very little Ered/NADP CT formed;
- ONLY 5-fold slower NADPH-to-FAD electron transfer:

**Conclusions:**
A CT complex between E_{ox} and NADPH should not be formed prior to ET in GltS.

\[
\begin{align*}
E\text{-FAD} + \text{NADPH} & \rightleftharpoons E\text{-FAD.NADPH} \rightleftharpoons (E\text{-FAD}_{\text{red}} \cdot \text{NADP}^+)_{\text{CT}} \\
\rightleftharpoons E\text{-FAD}_{\text{red}} \cdot \text{NADP}^+ & \rightleftharpoons E\text{-FAD}_{\text{red}} + \text{NADP}^+
\end{align*}
\]
Overall conclusions:

- The NADPH-GltS β subunit contains the fully active GltS site 1;

- NADP(H) binds to the C-terminal Rossmann-fold of the β subunit

- In contrast with GR and other Fp a CT between E_{ox} and NADPH is not needed for hydride transfer.

- The redox potential of bound FAD is modulated by the protein and the bound pyridine nucleotides

\[ E\text{-FAD} + \text{NADPH} \rightleftharpoons E\text{-FAD.NADPH} \rightleftharpoons (E\text{-FAD}_{\text{red}}\cdot\text{NADP}^+)_{\text{CT}} \rightleftharpoons E\text{-FAD}_{\text{red}}\cdot\text{NADP}^+ \rightleftharpoons E\text{-FAD}_{\text{red}} + \text{NADP}^+ \]
Any structural information on GltS?