Understanding immobilised enzymes and immobilised substrates

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• Normal enzymology

• Immobilised enzymes

• Solid-phase (immobilised) substrates
Why use enzymes with their substrates attached to a solid particle?

- Applications in solid phase chemical synthesis
- Assay or screening of enzymes on substrate arrays
- Fundamental relevance to *in vivo* state

NB extensive literature on enzymes evolutionally adapted to attack solid substrates, e.g. cellulases
How will enzyme behaviour change when substrates are attached to a solid support?

- Studies for solid-phase synthesis applications often show disappointing rates and/or yields

- But little fundamental study of reasons why

- Understanding these should help improve applications
Equilibrium positions will not be affected, will they?

- Well, yes they will

Equilibrium conversion to peptide

\[ \text{Solid phase is PEGA}_{1900}, \quad \text{Rein Ulijn et al, 2003.} \]
Why should the positions of these two equilibria differ?

\[ \text{NH}_3^+ + \text{RCOO}^- \leftrightarrow \text{NHCOR} \]

\[ \text{R'NH}_3^+ + \text{RCOO}^- \leftrightarrow \text{R'NHCOR} \]

- Mutual electrostatic repulsion of charged amino groups on solid surface favours amide synthesis – protonation of free amino group important in making amide hydrolysis favourable in aqueous solution, so suppression shifts equilibrium.

- Amide synthesis on solid phase involves transfer of hydrophobic R group out of aqueous solution (to near surface and/or bead interior, where hydrophobic hydration is less unfavourable)

- With solid phase amine, easier to use (and remove) excess acid – practically useful – theoretically trivial, allowed for in comparison just shown.

- so not the only factor.
Real-time spatially resolved enzyme kinetics on solid-phase substrates

Bz-Arg-coumarin
Non-fluorescent

Trypsin

Peptide coupling

7-aminocoumaryl acetamide
Highly fluorescent
Real time images of trypsin action in PEGA bead

Optical section by two-photon microscopy. PEGA beads with attached Bz-Arg-coumarin-(Gly)$_6$- 

Joe Deere et al, 2007
How should substrate moiety be attached to surface?

• Logical to use extended linker ("spacer") to avoid interference by surface
• Analogy with affinity adsorbents and immobilised enzymes
• Commonly used, but no systematic study
Effect of spacer length: substrates used

\[ \text{Ac-Trp-(Gly)}_n \text{CO-NH-PEG}_{1900}\text{-polyacrylamide} \]

\[ \text{Ac-Trp-(Gly)}_n \text{CO-NH-Si-O-Si-O} \]

Controlled pore glass

\( n = 0 \text{ to } 8 \)

Chymotrypsin releases Ac-Trp for analysis
Effect of oligo-Gly spacer length on rate

Joe Deere, Antonia Lalaouni, Laura Solares et al, 2008
Circular dichroism on biocatalyst particles

• Far UV spectrum can give secondary structure composition
• Changes in near UV are sensitive indication of 3D structure changes

• Achiral scattering just means need more intense source – state-of-the-art bench instruments suitable
• Rotating cell to keep particles in uniform suspension
• Cell close to detector to minimise differential scattering
Effect of subtilisin immobilisation on near UV CD

Both in aqueous buffer

Ashok Ganesan et al, 2006

Increased intensity, same shape - probably means same structure but more rigid
Near UV CD shows loss of tertiary structure after immobilised subtilisin is inactivated in acetonitrile.

[Graph showing UV CD spectrum with wavelength from 250 to 310 nm and CD values from -500 to 100 deg.cm².dmol⁻¹, with different curves indicating fresh and inactive SC-Silica in various conditions.]
Near UV CD is possible with polymer supports

Green: Lewatit VPOC 1600
Blue: Novozyme 435
Both in acetonitrile (1% water).

Ashok Ganesan at al, 2009.
Absorption flattening

- CD signal (and absorbance) of a suspension is lower than if the same amount of chromophore was uniformly distributed
- Reduced by largest factor at wavelengths where absorbance greatest
- So distorted spectrum must be corrected before analysis
Cause of flattening best seen from appearance of suspension viewed along light path

Volume fraction = 0.1, pathlength = 2 X mean particle size.

Absorbance cannot exceed 0.15, however high the chromophore concentration in the particles.
Calculation of flattening effects by simulation

• Place particles randomly within a simulated volume, to give required volume fraction
• For array of light paths through the simulated volume, calculate transmission along each, based on extent to which it meets particles
• Hence calculate average transmissions, and thus absorbance and CD signals

• Accurate answers in a few seconds on desktop computer
How to correct experimental data: relationship of $Q_{CD}$ to measurable absorbance of suspension ($A_{sus}$)

Correction of far UV CD for absorption flattening

Aqueous buffer with dissolved free subtilisin (——) or suspended silica-immobilised enzyme (·····), and after correction (- - - -).  

Ashok Ganesan et al, 2006
Far UV CD is significantly changed on inactivation

Subtilisin in aqueous solution (——).
Silica-immobilised enzyme: in aqueous (⋯⋯), in ACN (- - - -), inactivated ((-⋯⋯⋯-)).
Corrected for absorption flattening.

Inactivation is accompanied by increase in beta structure

<table>
<thead>
<tr>
<th>Sample</th>
<th>Helix</th>
<th>Sheet</th>
<th>Other</th>
<th>NRMSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aqueous solution</td>
<td>0.26</td>
<td>0.14</td>
<td>0.60</td>
<td>0.062</td>
</tr>
<tr>
<td>Immobilised in aqueous</td>
<td>0.29</td>
<td>0.11</td>
<td>0.59</td>
<td>0.095</td>
</tr>
<tr>
<td>Immobilised in ACN</td>
<td>0.31</td>
<td>0.11</td>
<td>0.58</td>
<td>0.183</td>
</tr>
<tr>
<td>Inactivated in ACN</td>
<td>0.30</td>
<td>0.22</td>
<td>0.49</td>
<td>0.218</td>
</tr>
</tbody>
</table>

IR spectrum little effected by immobilisation or medium, but suggests shift to beta structure on inactivation.

Aqueous solution (—), silica-subtilisin (−−−−−), after brief ACN exposure (-----), and inactivated (--.--.--).

Fluorescence lifetime distribution

Aqueous solution

Immobilised in aqueous

Immobilised in ACN

Inactivated in ACN

Time-resolved decay curves analysed by maximum entropy method

Ashok Ganesan et al, 2009
Prominence of 297 nm CD band correlates well with catalytic activity in octane

Various chymotrypsin preparations

Kusum Solanki, Munishwar Gupta, Peter Halling, unpublished
CONCLUSIONS

• Improving understanding of effects on equilibrium and kinetics during enzyme action on immobilised substrates
• Better understanding of the state of enzyme molecules in immobilised preparations using spectroscopic techniques, especially circular dichroism
ACKNOWLEDGEMENTS

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• Sabine Flitsch (Manchester)
• Rein Ulijn (now back at Strathclyde)
• Nick Price, Sharon Kelly (CD facility, Glasgow Univ)
• Kusum Solanki, Munishwar Gupta (Chemistry, IIT Delhi)
Far UV CD is masked by signal from support

Green: Lewatit VPOC 1600
Blue: Novozyme 435
In ACN 1% water.

Ashok Ganesan et al, unpublished
Strong CD signal from support is totally unexpected and theoretically almost impossible!

- Shows that this synthetic polymer has significant chirality
- Reproducible, even different batch numbers
- Polymer has plenty of chiral centres, but generation from achiral monomers (methyl and butyl methacrylates, divinylbenzene) should generate equal amounts of R and S
- Presumably not the Nobel Prize for discovering parity violation – perhaps some stray chiral impurities during synthesis
- Found also in Duolite and an anion exchange Lewatit
For kinetic studies, it would be ideal to resolve sites of reaction in bead

- Needs some kind of imaging method
Two-photon microscopy allows spatially resolved kinetics on solid phase substrates.

Thermolysin catalysed hydrolysis of Fmoc-Phe-Phe-PEGA, examined by dansyl labelling of released amino groups and two-photon microscopy, giving optical sections.

Release of Ac-Trp from CPG

Joe Deere, Antonia Lalaouni, Laura Solares et al, 2008
Spacer length effects

- In absence of any spacer, see enzyme preference for amine leaving group structure
- \( \text{Gly}_1 \) or \( \text{Gly}_2 \) sufficient for maximal rate with PEGA, probably because of flexibility of PEG chains
- Optimum at \( \text{Gly}_4 \) with CPG
- Decline with longer spacers may reflect known structure change in oligoglycines
Enzyme reactions on solid-phase substrates

- Can get large and synthetically useful shifts in equilibrium compared to equivalent reaction in solution
- Methods being developed to study effects on kinetics with spatial resolution
- Spacers are important for optimal rate, but best length depends on support type
Most physico-chemical techniques used to study dissolved proteins suffer problems with suspensions

- Light scattering affects/prevents optical methods
- May need mixing to keep in suspension
- Molecular scale physics altered (e.g. NMR relaxation)
Mathematical models of absorption or CD flattening

• Series of literature treatments (since Duysens, 1956!)
• All require some approximations or assumptions, e.g.
  – spherical particles
  – uniform size
  – very low volume fraction
• Give flattening coefficient, observed divided by unflattened signal – e.g. $Q_{CD}$
Flattening coefficient for CD estimated by simulation

Simulations for pathlength twice particle size, volume fraction 0.01 (triangles) or 0.1 (squares). $A_{\text{part}}$ is the absorbance along a diameter through one particle. Lines are for literature analytical models. Halling (2009).
Simulation is readily adapted to handle particle size range

<table>
<thead>
<tr>
<th>Particle volume fraction</th>
<th>Pathlength/Particle size</th>
<th>$Q_{CD}$ monodisperse</th>
<th>$Q_{CD}$ size range</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.01</td>
<td>2</td>
<td>0.213 ± 0.014</td>
<td>0.174 ± 0.010</td>
</tr>
<tr>
<td>0.01</td>
<td>200</td>
<td>0.200 ± 0.009</td>
<td>0.186 ± 0.007</td>
</tr>
<tr>
<td>0.1</td>
<td>2</td>
<td>0.253 ± 0.004</td>
<td>0.207 ± 0.004</td>
</tr>
</tbody>
</table>

Simulation results for absorbance = 1 through particle diameter, with either monodisperse particles, or standard deviation 20% of mean size. Halling (2009).
Fluorescence of endogenous Trp greatly enhanced in inactivated biocatalyst escapes intramolecular quenching

Excitation at 295 nm. Aqueous solution (—), silica-subtilisin in aqueous (····), in ACN (· · · ·), and inactivated (———)

Pressing discs for transmission IR may perturb protein structure, can be avoided by DRIFT

Spectra of lyophilised subtilisin, measured in pressed KBr discs by transmission (—) or DRIFT (……), in mix with KBr by DRIFT (- - -).

Ashok Ganesan et al, 2009
Experimental requirements for proper CD flattening correction

• Volume fraction of particles – usually should be known
• Particle size distribution – should be measurable
• Correct treatment of scattering contribution to measured absorbance – wide angle collection used anyway to reduce differential scattering contribution
• Parallel measurement of absorbance of CD sample suspension – should be obtainable from data for photomultiplier voltage