Use of mass spectrometry in the study of enzymes

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Overview of class

- Modification of the enzyme to regulate its activity
- Examining the chemistry of enzyme:substrate intermediates
  - Locating the site of inactivation of suicide inhibitors
- Reaction mechanism
  - Measuring all substrates and products
  - Enzyme kinetics
  - Stopped flow
- BAT, my kinda ‘zyme
A good review of this topic


Mass spectrometry and the study of enzymes

Enzymes often undergo posttranslational modifications in order to be active under the conditions in a cell

- for example, many enzymes in the signal transduction pathways are activated by phosphorylation on serine, threonine and tyrosine residues

- EGF receptor (tyrosine kinase), TGF beta type I receptor (serine kinase)

- sites of phosphorylation can be determined by mass spectrometry because of the increase in mass of m/z 80 of peptides containing each phosphate group
Enzymes and Mass Spec

Enzymes may undergo changes in structure once activated (see above) or during the reaction they catalyze

- this could be probed by H-D exchange experiments

- simulation of phosphorylation may be necessary by mutating serine and threonine groups to aspartate and glutamate, respectively

MS of enzymes

Enzymes can be inactivated by suicide substrates - these come into the active site and undergo a covalent reaction, thereby blocking the approach of other substrate molecules

- to locate the region of the enzyme to which the suicide substrate is bound, carry out a trypsin digest and look for a peak that has undergone a molecular weight change (consistent with the structure of the suicide inhibitor)
Reaction of chymotrypsin with tosyl phenylalanyl chloromethylketone (TPCK) in His57

Chymotrypsin has a catalytic triad consisting of Asp102, His57 and Ser195

His57 is the site of reaction of chymotrypsin with TPCK to form a stable covalent bond, thereby acting as a suicide inhibitor
Possible proteases for locating TPCK-peptide

**Trypsin**

CGVPAIQPVLSGLSRIVNGE EAVPGSPWQ VSLQDKTGPH FCQGSINEN 50
WVVTAAHCVTSTSDVVVAGE FDQSSSEKEI QKLKIAKVFK NSKYNSLTIN 100
NDITLLKLSTAASFSQTVSA VCLPASDDDF AAGTCCVTGG WGLTRYTNNAN 150
TPDRLQQASLPILLSNTNCCK YWGTKIKDAM ICAGASGVSSEMGSQGPLV 200
CKKNGAWTLVGIVSWGSGTC STSTPGVYAR VTALVNWVQQLAN

**Glu-C**

CGVPAIQPVLSGLSRIVNGE EAVPGSPWQ VSLQDKTGPH FCQGSINEN 50
WVVTAAHCVTSTSDVVVAGE FDQSSSEKEI QKLKIAKVFK NSKYNSLTIN 100
NDITLLKLSTAASFSQTVSA VCLPASDDDF AAGTCCVTGG WGLTRYTNNAN 150
TPDRLQQASLPILLSNTNCCK YWGTKIKDAM ICAGASGVSSEMGSQGPLV 200
CKKNGAWTLVGIVSWGSGTC STSTPGVYAR VTALVNWVQQLAN

**Chymotrypsin**

CGVPAIQPVLSGLSRIVNGE EAVPGSPWQ VSLQDKTGPH FCQGSINEN 50
WVVTAAHCVTSTSDVVVAGE FDQSSSEKEI QKLKIAKVFK NSKYNSLTIN 100
NDITLLKLSTAASFSQTVSA VCLPASDDDF AAGTCCVTGG WGLTRYTNNAN 150
TPDRLQQASLPILLSNTNCCK YWGTKIKDAM ICAGASGVSSEMGSQGPLV 200
CKKNGAWTLVGIVSWGSGTC STSTPGVYAR VTALVNWVQQLAN

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**Advantage of growing recombinant bacteria on $^{12}$C/$^{14}$N labeled substrates - protein is close to monoisotopic mass**

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Kelleher & Hicks, 2005
Mass spectrometry and enzyme-catalyzed reactions

In the simplest case, an enzyme (E) reacts with a substrate (S) - an intermediate complex is formed (ES) and it is converted to an enzyme: product complex (E:P) before the product dissociates.

$$E + S \rightarrow ES \rightarrow EP \rightarrow E + P$$

First order reaction - some second order reactions behave like a first order reaction when there is an excess of one substrate and the conversion of the other is <10%.

By measuring the molecular weights of the forms of the enzyme:substrate (product) complexes, mass spectrometry can throw enormous light on the mechanism.
Mass spectrometry and enzyme-catalyzed reactions

More typical reactions involve two substrates (S1 and S2) and two products (P1 and P2). The problem in this case is the order of addition

- is it a random mechanism? If so, both E.S1 and E.S2 exist
- is it an ordered mechanism? In this case, S1 has to bind first. So, there will be E.S1 and E.S1.S2, but no E.S2
- is it a Ping-Pong mechanism? In this case, E.S1**E.P1 before S2 binds to form E.P1.S2

Mass spectrometry and substrates and products of enzyme reactions

- Most enzyme reactions are studied by measuring the appearance of a product or (more rarely) the disappearance of a substrate
- If the substrate or product has a unique absorbance or fluorescence, the reaction can be followed in real time
- Some substrates have no usable absorbance or fluorescence - these can be measured using a radiolabeled substrate - the product is isolated by a solvent extraction procedure, or by HPLC or TLC. These reactions cannot be observed in real time
- Mass spectrometry has the advantage that it is capable of measuring all substrates and products, as well as the enzyme itself

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**Sulfotransferase - a reaction with no absorbance or fluorescence to follow**

Scheme 1: NodST Catalyzes the Sulfation of a Lipochitooligosaccharide (1)

![Chemical structures](image1)

**Sulfation of chitobiose**

Scheme 2: NodST Catalyzes the Sulfation of Chitobiose (3) to (4) with m/z 503

![Chemical structures](image2)
Set up for the ST assay

- NodST purified by Ni-affinity chromatography
  - dialyzed against 100 mM Tris-HCl, pH 8.0 - 20 mM β-ME
  - Diluted into 10 mM NH₄Ac buffer, pH 8.0
- Incubate (25 μl) quenched with 100 μl of MeOH containing internal standard
- Diluted incubate (40 μl) introduced into ESI source at 20 μl/min
- MS on a ThermoFinnigan LCQ monitoring m/z 503 and m/z 468 (internal standard)

Kinetics of chitobiose ST by ESI-MS

Figure 3: Saturation plot of $V_0$ vs [PAPS]. The inset is a double-reciprocal plot of $1/V_0$ vs 1/[PAPS] ([PAPS] = 1.25, 2.5, 3.5, 5, 12.5, 25, 40, and 50 μM, [chitobiose] = 1 mM, [NodST] = 90 nM, and pH 8.0).
**Inhibition of ST by PAP using ESI-MS**

Pi et al., Biochemistry 41:13283

![Graph showing double-reciprocal plot of 1/v vs 1/[PAPS] at different PAP concentrations: 0 μM PAP (▲), 0.75 μM PAP (■), 1.5 μM PAP (●), and 3.0 μM PAP (○). ([PAPS] = 1.25, 2.5, 5, 10, 25, and 50 μM, [NodST] = 90 nM, and pH 8.0).](image)

**Non-covalent enzyme:substrate complexes**

- Shifting the enzyme from neutral pH conditions to the acidity of the spraying solution may break down the complex
- Spraying at neutral pH will increase the observed m/z values (the protein is less charged with protons)
- The larger m/z ions can be observed with an electrospray-TOF or a Qq TOF
Schematic diagram of a stop-flow system

Stopped flow set up

From Kolakowski and Konermann (Anal Biochem 292:107)

Note the additional flow introduced by pump S3
Effect of the delay between V1 and V2 in a stopped flow experiment

In this reaction, hydrolysis of acetylcholine in an alkaline buffer is monitored by the ion at m/z 146.

Following a reaction using substrate and products ions in stopped flow ESI-MS

These data are from the conversion of chlorophyll A to pheophytin A (loss of Mg and gain of two protons).

The upper traces (A) are from the ESI-MS analysis. The lower traces (B) are from absorbance changes.

Kolakowski and Konermann (Anal Biochem 292:107)
Unfolding kinetics of myoglobin by stopped-flow ESI-MS

The upper trace (A) is the 14th charge state of holo-myoglobin $[\text{M+14}]^{14+}$ ($m/z = 1255.9$)

The reaction is created by a pH jump from 6.0 to 3.0. The lower trace (B) is the absorbance at 441 nm.

The estimated time constants for the bi-exponential process are 0.29/2.8 sec for A and 0.33/3.1 sec for B

Summary of the use of (real time) ESI-MS to follow enzyme reactions

- **The pros:**
  - All the substrates and products (as well as the enzyme itself) can be studied simultaneously
  - It’s applicable to compounds with no absorbance or fluorescence

- **The cons:**
  - The buffer for the reaction has to be chosen very carefully
  - Ammonium salts are the best candidates, but they may have an effect on the reaction rates
A practical example of use of MS in enzymology - the enzyme BAT

- Falany et al., J Lip Res, 38: 86-95, 1997 (mouse - cDNA cloning and expression)
- He et al., J Lip Res, 44: 2242-2249, 2003 (rat - cDNA cloning, expression and localization)

First let’s remind ourselves of some basic biochemistry

\[ \text{Acetate (C}_2\text{) to HMG CoA (C}_6\text{)} \]
\[ \text{HMG CoA to squalene (C}_30\text{)} \]
\[ \text{Squalene to cholesterol (C}_27\text{)} \]
\[ \text{Vitamin D (C}_27\text{)} \quad \text{Bile acids (C}_24/C_27\text{)} \]
\[ \text{Steroids (C}_18/C_21\text{)} \]

20+ Nobel prizes
Evolution of bile acid conjugation

Neutral pathway
7α-hydroxylase

Acidic pathway
27-hydroxylase

24-hydroxylase
7α-hydroxylase

C24 BA

Evolution of bile acid conjugation

Taurine C27 BA

C27 BA sulfate

Taurine C24 BA

Glycine C24 BA
**Bile acid N-acylamidate formation (in hepatocytes)**

\[
\text{Bile acid + CoASH} \xrightarrow{\text{BAT, ATP, Mg}^{2+}} \text{Bile acid-SCoA}
\]

\[
\text{Bile acid-SCoA + amino acids} \xrightarrow{\text{CoASH}} \text{Bile acid amidate}
\]

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**Characterization of BAT**

- Purified from human liver cytosol 465-fold to a single protein band - retained the same ratio of glycine:taurine activity during purification
- Partial amino acid sequence and specific polyclonal antibody led to isolation of \( \lambda \text{gt11} \) clone from human liver cDNA library
- hBAT is a 418-aa protein; when expressed using a pKK233-2 vector in bacteria, it makes both glycine and taurine conjugates (and FBAL)
Sequence comparisons of mouse, rat and human BATs

r 65%  SLLASGFATLALAYWGYDDLSRLKEKVDLEYFEEGVFEEFLHRLHPKVLGPGVGIGSAG 238
m 63%  SLLASRGFATLALAWYDDLSRLKEKVDLEYFEEGVFEEFLHRLHPKVLGPGVGIGSAG 237
h 100% SLLASRGFATLALAYWDDLSRLKEKVDLEYFEEGVFEEFLHRLHPKVLGPGVGIGSAG 238

r EIGLSMAILKQTATVLINGFHVSHPVRYGKVQPTFCSEEFVTNNALGFLVEFYRT 298
m EIGLSMAILKQTATVLINGFHVSHPVRYGKVQPTFCSEEFVTNNALGFLVEFYRT 297
h QIGLSMAIYKLQVTATVLINGTHFPFFGIPQYHQLPGPSLQAQLNSTNALGFLVEFYRT 288

r FEETAKDOSYCFPIEKAGHFLFVVEDEKLNLSKVRHAKQAIAQLMKSQGKNNWLTLP 358
m FQETADCKDSCFPIEKAAGHFLFVVEDEKLNLSKVRHAKQAIAQLMKSQGKNNWLTLP 357
h FETTQGVASQYYLFPIEEEAQGQFLVGEKTIKSHAKAQIQLKLRHGKNNWLTLP 358

r GAGLIEFFYSPHLQASRMHPFVIPSINWGGEVIPH−AA 395
m GAGLIEFFYSPHLQASRMHPFASWGEVIPH−AQ 395
h GAGLIEFFYSPHLQASTTHDLR−LHGGGEVIPH−AA 393
Site-specific Cys mutations

- Mutations were prepared for the two conserved Cys residues (C235 and C372) in BATs
- C235Y hBAT had no enzyme activity
- C372A hBAT had low activity

![Graph A: Specific activity vs. Cholyl CoA concentration](image)

![Graph B: Specific activity vs. Cholyl CoA concentration](image)

ESI-mass spectrum of hBAT products

- wt-hBAT
  - m/z 514
- C372A hBAT
  - m/z 512
**LC-MS of C372A hBAT product**

- **Relative Intensity (%)**
  - Taurocholate: 514/124
  - [TC-2H]⁻: 512/124

**Time (min)**
- 4.00
- 6.00
- 8.00
- 10.00
- 12.00

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**Metabolism in E. coli expression system**

- **wt-hBAT**:
  - C372A hBAT
  - 7α-hydroxysteroid dehydrogenase

- **m/z 514**
- **m/z 512**

Sfakianos et al.

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The Protein Structure Modeling of hBAT

Asp328

Cys325


Courtesy of Alexey Murzin, Center for Protein Engineering, MRC Cambridge, UK

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Cys235→Ser, what will C235S-BAT be, transferase or thioesterase?

<table>
<thead>
<tr>
<th>Gene</th>
<th>sequence</th>
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<tr>
<td>kan-1</td>
<td>SLLASHGFATLALAYWGDLSRLPSLEKVEPVEGVEFLRHPKVLYLPGLPGVGLSVCIGA</td>
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<tr>
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<td>SLLASSGFATLALAYWDDPELPSLEKVEPVEGVEFLRHPKVLYLPGLPGVGLSVCIGA</td>
</tr>
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<td>hBAT</td>
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<td>CTE-I</td>
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? Nucleophile

Cys235→Ser

Charge Relay Mechanism shared by hBAT, thioesterases, and a large group of hydrolases

Michaelis complex

Tetrahedral intermediate

Acyl-enzyme intermediate

Active enzyme

Sfakianos, JBC 277:47270
Purification of hBAT with 6x His-tag

- Impure hBAT
- Inactivated by imidazole in elution buffer

Purification of hBAT with Avi-tag

- Impure hBAT

Sfakianos et al.

Sfakianos et al.

Shonsey et al.
Kinetics of transferase and thioesterase activities of wild-type hBAT with glycine

Specific activity (μmol/min/g) vs. cholyl CoA (μM)

- Transferase activity
- Thioesterase activity
Kinetics of transferase and thioesterase activities of C235S hBAT variant with glycine

![Kinetics graph](image)

LC-ESI-MS-MRM Analysis of Reaction Products

![LC-ESI-MS-MRM graph](image)
Bile acid CoA:amino acid N-acyltransferase

- Has a ping-pong reaction mechanism

- Bile acid CoA undergoes a thioester interchange with Cys235-BAT

- Ser can replace Cys, but the complex is less stable
  - This can either lead to lowered activity, or increased turnover

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