Use of mass spectrometry in the study of enzymes

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MCLM 452

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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**
  - **Structural studies**
  - **Stopped flow**
- **BAT, my kinda 'zyme - Erin Shonsey**

A good review of this topic

- **Kelleher, NL and Hicks LM. Contemporary mass spectrometry for the direct detection of enzyme intermediates. *Current Opinion in Chemical Biology* 9: 424-430, 2005.**

Mass spectrometry and the study of enzymes

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

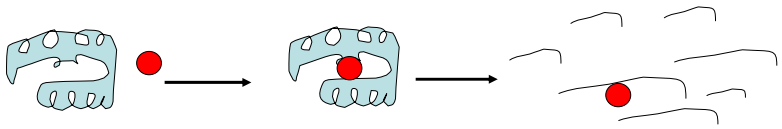
- **for example, many enzymes in the signal transduction pathways are activated by phosphorylation on serine, threonine and tyrosine residues**
- **EGF receptor (tyrosine kinase), TGF beta type I receptor (serine kinase)**
- **sites of phosphorylation can be determined by mass spectrometry because of 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

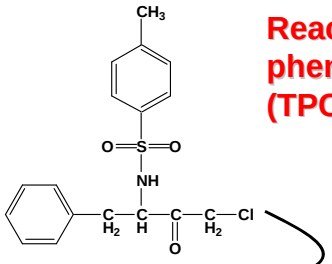
- **this could be probed by H-D exchange experiments**
- **simulation of phosphorylation may be necessary by mutating serine and threonine groups to aspartate and glutamate, respectively**

MS of enzymes



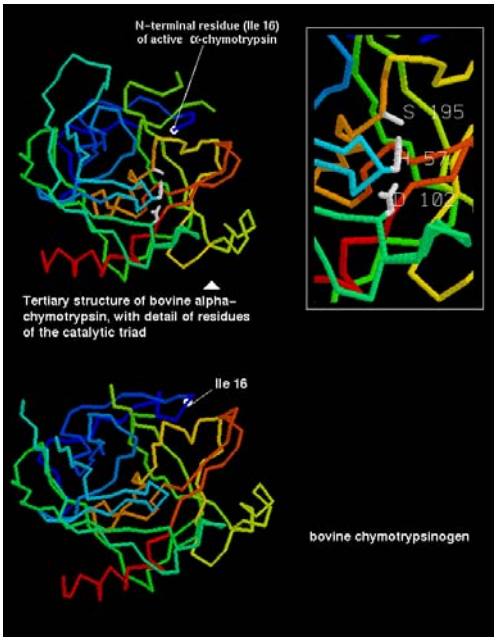
Enzymes can be inactivated by suicide substrates - these come into the active site and undergo a covalent reaction, thereby blocking the approach of other substrate molecules

- **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

```
CGVPAIQPVL SGLSRIVNGE EAVPGSWPWQ VSLQDKTGFH FCGGSLINEN
50
WVVTAAHCGV TTSDVVVAGE FDQGSSSEKI QKLKIAKVFK NSKYNSLTIN
100
NDITLLKLST AASFSQTVSA VCLPSASDDF AAGTTCVTTG WGLTRYTNAN
150
TPDRLQQASL PLLSNTNCKK YWGTKIKDAM ICAGASGVSS
CMGDSGGPLV 200
CKKNGAWTLV GIVSWGSSTC STSTPGVYAR VTALVNWVQQ TLAAN
```

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

CGVPAIQPVL SGLSRIVNGE EAVPGSWPWQ VSLQDKTGFH FCGGSLINEN
50
WVVTAAHCGV TTSDVVVAGE FDQGSSSEKI QKLKIAKVFK NSKYNSLTIN
100
NDITLLKLST AASFSQTVSA VCLPSASDDF AAGTTCVTTG WGLTRYTNAN
150
TPDRLQQASL PLLSNTNCKK YWGTKIKDAM ICAGASGVSS CMGDSGGPLV 200
CKKNGAWTLV GIVSWGSSTC STSTPGVYAR VTALVNWVQQ TLAAN

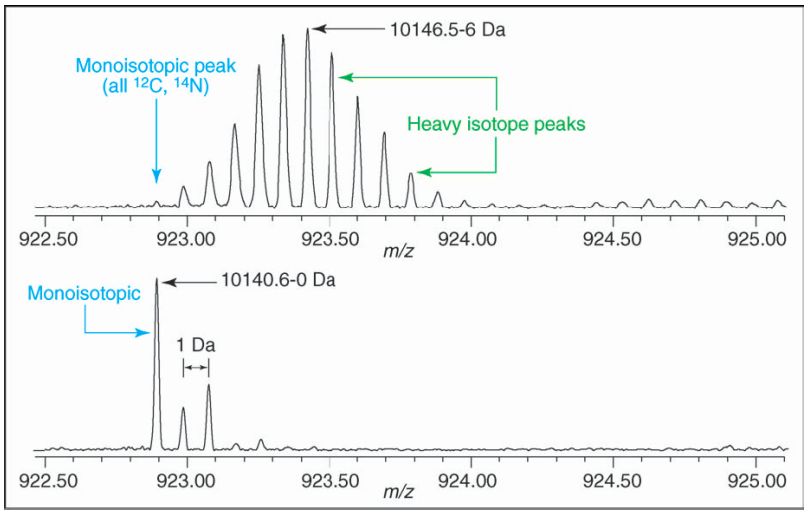
Glu-C

CGVPAIQPVL SGLSRIVNGE EAVPGSWPWQ VSLQDKTGFH FCGGSLINEN
50
WVVTAAHCGV TTSDVVVAGE FDQGSSSEKI QKLKIAKVFK NSKYNSLTIN
100
NDITLLKLST AASFSQTVSA VCLPSASDDF AAGTTCVTTG WGLTRYTNAN
150
TPDRLQQASL PLLSNTNCKK YWGTKIKDAM ICAGASGVSS CMGDSGGPLV 200
CKKNGAWTLV GIVSWGSSTC STSTPGVYAR VTALVNWVQQ TLAAN

Chymotrypsin

CGVPAIQPVL SGLSRIVNGE EAVPGSWPWQ VSLQDKTGFH FCGGSLINEN
50 WVVTAAHCGV TTSDVVVAGE FDQGSSSEKI QKLKIAKVFK
NSKYNSLTIN 100 NDITLLKLST AASFSQTVSA VCLPSASDDF
AAGTTCVTTG WGLTRYTNAN 150 TPDRLQQASL PLLSNTNCKK
YWGTKIKDAM ICAGASGVSS CMGDSGGPLV 200 CKKNGAWTLV
GIVSWGSSTC STSTPGVYAR VTALVNWVQQ TLAAN

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



 Kelleher & Hicks, 2005

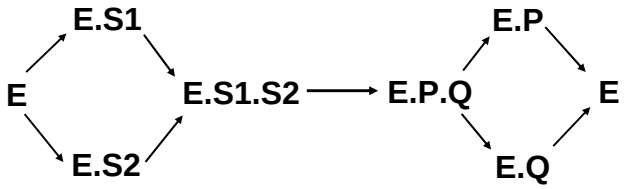
Mass spectrometry and enzyme-catalyzed reactions

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%.

Mass spectrometry and enzyme-catalyzed reactions



By measuring the molecular weights of the forms of the enzyme:substrate (product) complexes, mass spectrometry can throw enormous light on the mechanism

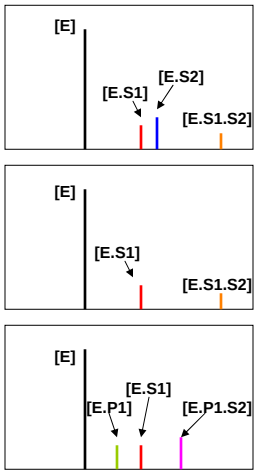
Mass spectrometry and enzyme-catalyzed reactions

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



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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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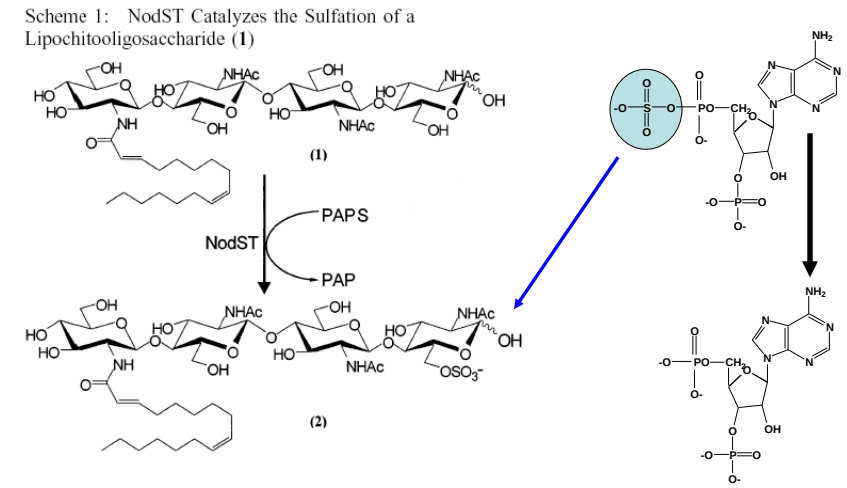
Sulfotransferase - a reaction with no absorbance or fluorescence to follow



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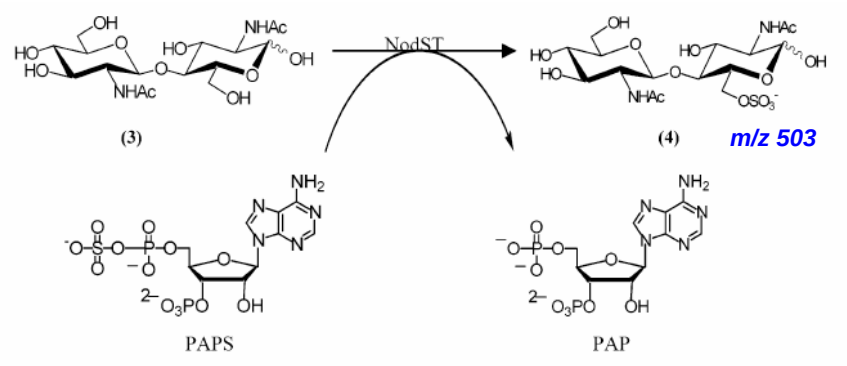
Sulfation of chitobiose



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Set up for the ST assay

Pi et al., Biochemistry 41:13283

- **NodST purified by Ni-affinity chromatography**
  - **dialyzed against 100 mM Tris-HCl, pH 8.0 - 20 mM β-ME**
  - **Diluted into 10 mM NH_4Ac 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

Pi et al., Biochemistry 41:13283

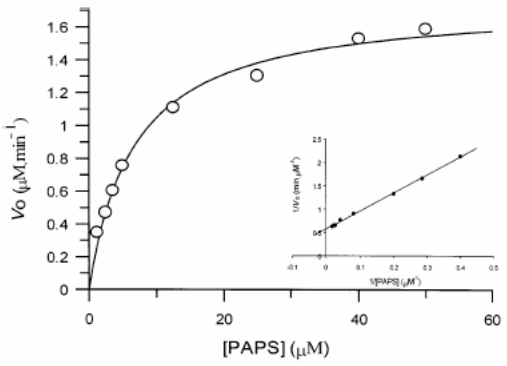

FIGURE 3: Saturation plot of V_0 vs [PAPS]. The inset is a double-reciprocal plot of $1/V_0$ vs 1/[PAPS] ([PAPS] = 1.25, 2.5, 3.5, 5, 12.5, 25, 40, and 50 μM, [chistobiose] = 1 mM, [NodST] = 90 nM, and pH 8.0).

Inhibition of ST by PAP using ESI-MS

Pi et al., Biochemistry 41:13283

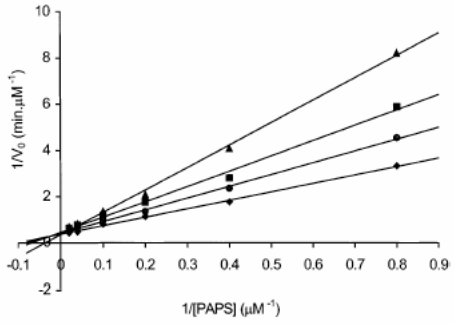

FIGURE 5: Double-reciprocal plot of $1/V_0$ vs 1/[PAPS] at different PAP concentrations: 0 μM PAP (▲), 0.75 μM PAP (■), 1.5 μM PAP (●), and 3.0 μM PAP (◆) ([PAPS] = 1.25, 2.5, 5, 10, 25, and 50 μM, [chitobiose] = 1 mM, [NodST] = 90 nM, and pH 8.0).

Protein tyrosine phosphatase kinetics and structure

QuickTime™ and a
decompressor
are needed to see this picture.

Zhou & Zhang
Methods, 2007

Global H/D exchange depends on complex formation

MKP3/C293S

ERK2/pTpY

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alone (O) or in complex (●)

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Zhou & Zhang Methods, 2007

Peptide coverage of MKP3 - red peptides have a decrease in exchange - blue ones have an increase in exchange

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Differing exchange between the peptides in MKP3

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alone (O) or in complex (●)

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Methods, 2007

Structure of the MKP3/Erk2 complex

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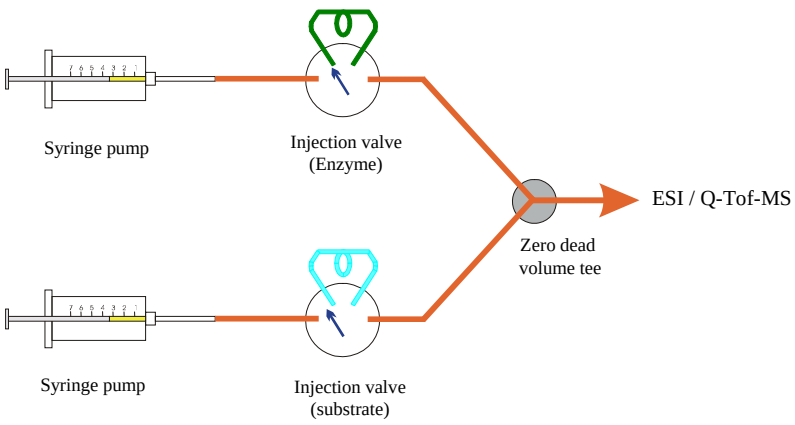
Methods, 2007

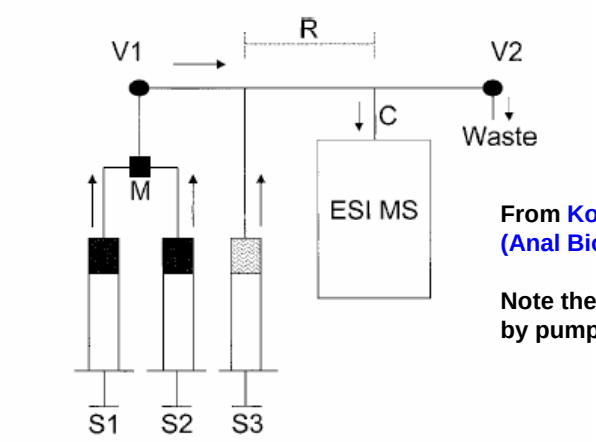
Non-covalent enzyme:substrate complexes

- **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



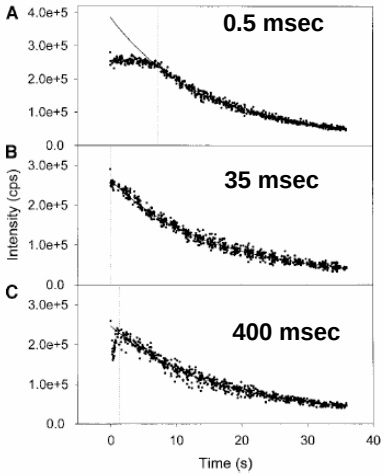
Stopped flow set up



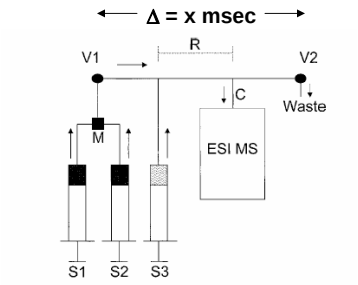
From Kolakowski and Konermann (Anal Biochem 292:107)

Note the additional flow introduced by pump S3

Effect of the delay between V1 and V2 in a stopped flow experiment

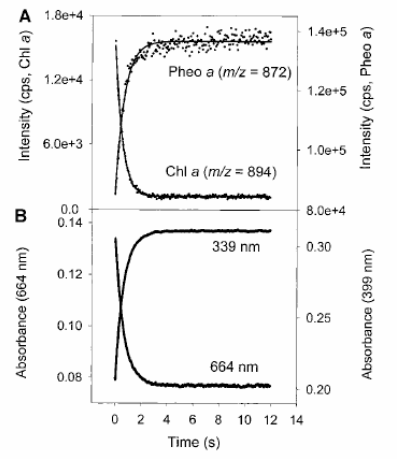


In this reaction, hydrolysis of acetylcholine in an alkaline buffer is monitored by the ion at *m/z* 146



Kolakowski and Konermann (Anal Biochem 292:107)

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.

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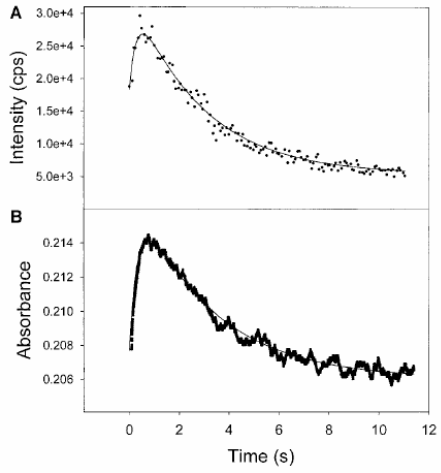
Kolakowski and Konermann (Anal Biochem 292:107)

Unfolding kinetics of myoglobin by stopped-flow ESI-MS



The upper trace (A) is the 14th charge state of holo-myoglobin $[M+14]^{14+}$ (*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

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Kolakowski and Konermann (Anal Biochem 292:107)

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**

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