

# 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
  - Stopped flow
- **BAT, my kinda 'zyme**

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## **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.

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## **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

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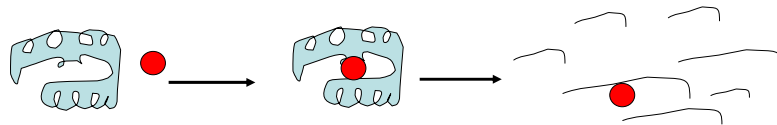
## Enzymes and Mass Spec

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

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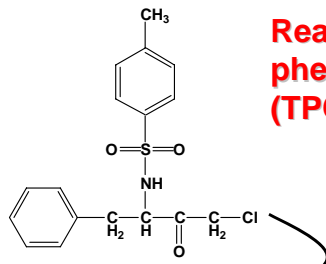
## MS of enzymes



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)

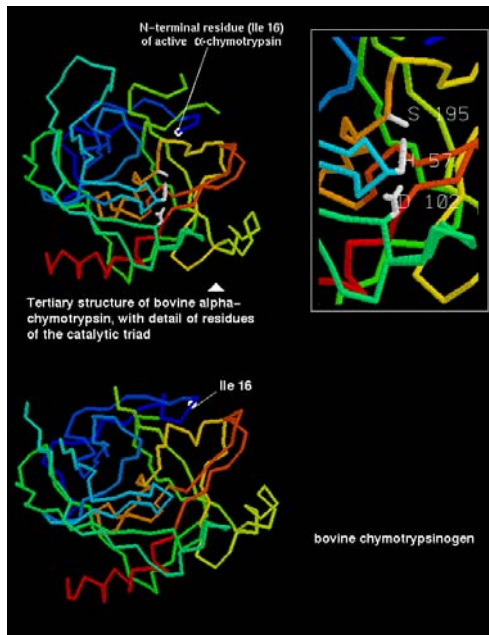
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## Reaction of chymotrypsin with tosyl phenylalanyl chloromethylketone (TPCK) in His57

CGVPAIQPVL SGLSRIVNGE EAVPGSWPWQ VSLQDKTGFH FCGGSLINEN  
 50  
 WVVTAAH**CGV** TTSDVVVAGE FDQGSSEKI QKLKIAKVKF NSKYNSLTIN  
 100  
 NDITLLKLST AASFSTVSA VCLPSASDDF AAGTTCVTTG WGLTRYTNAN  
 150  
 TPDRLQQASL PLLSNTNCKK YWGTKIKDAM ICAGASGVSS  
 CMGDSGGPLV 200  
 CKKNGAWTLV GIVSWGSSSTC STSTPGVYAR VTALVNWVQQ TLAAN

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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

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## Possible proteases for locating TPCK-peptide

### Trypsin

CGVPAIQPVL SGLSRIVNGE EAVPGSWPWQ VSLQDKTGFH FCGGSLINEN  
50

WVVTAAHCGV TTSADVAGE FDQGSSEKI QKLKIAKVKF NSKYNSLTIN  
100

NDITLLKLST AASFSQTVSA VCLPSASDDF AAGTTCVTTG WGLTRYTNAN  
150

### Glu-C

TPDRLQQASL PLLSNTNCKK YWGTKIKDAM ICAGASGVSS  
CGVPAIQPVL SGLSRIVNGE EAVPGSWPWQ VSLQDKTGFH FCGGSLINEN  
50 CMGDSGGPLV 200

CKKNGAWTLV GIVSWGSSSTC STSTPGVYAR VTALVNWVQQ TLAAN  
WVVTAAHCGV TTSADVAGE FDQGSSEKI QKLKIAKVKF NSKYNSLTIN  
100

NDITLLKLST AASFSQTVSA VCLPSASDDF AAGTTCVTTG WGLTRYTNAN  
150

### Chtymotrypsin

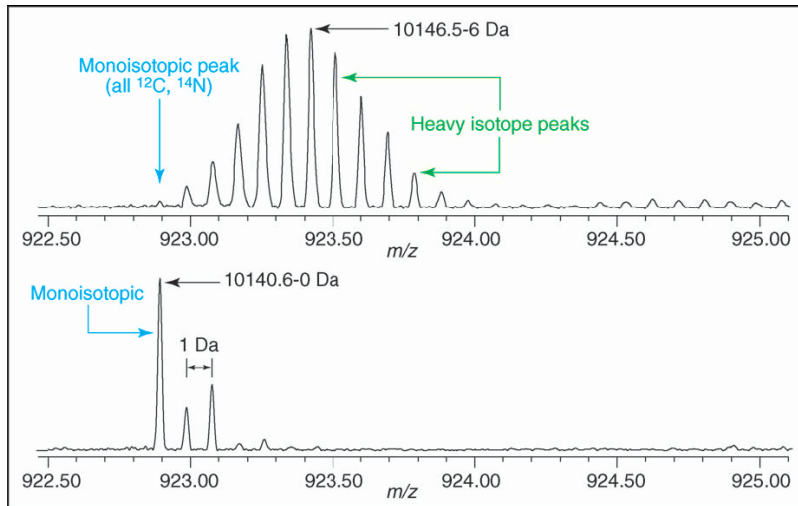
TPDRLQQASL PLLSNTNCKK YWGTKIKDAM ICAGASGVSS  
CGVPAIQPVL SGLSRIVNGE EAVPGSWPWQ VSLQDKTGFH FCGGSLINEN  
50 CMGDSGGPLV 200

WVVTAAHCGV TTSADVAGE FDQGSSEKI QKLKIAKVKF  
NSKYNSLTIN 100 NDITLLKLST AASFSQTVSA VCLPSASDDF  
CKKNGAWTLV GIVSWGSSSTC STSTPGVYAR VTALVNWVQQ TLAAN  
150

AAGTTCVTTG WGLTRYTNAN 150 TPDRLQQASL PLLSNTNCKK  
YWGTKIKDAM ICAGASGVSS CMGDSGGPLV 200 CKKNGAWTLV  
GIVSWGSSSTC STSTPGVYAR VTALVNWVQQ TLAAN

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



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## Mass spectrometry and enzyme-catalyzed reactions

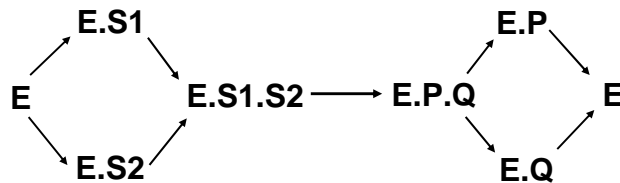
In the simplest case, an enzyme (E) reacts with a substrate (S) - an intermediate complex is formed (ES) and it is converted to an enzyme: product complex (E:P) before the product dissociates.



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%.

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## Mass spectrometry and enzyme-catalyzed reactions



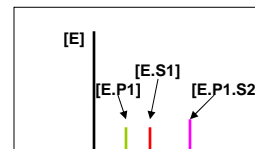
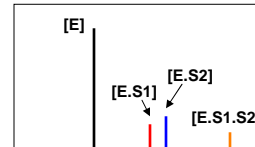
By measuring the molecular weights of the forms of the enzyme:substrate (product) complexes, mass spectrometry can throw enormous light on the mechanism

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## 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,  $E.S1 \rightarrow E.P1$  before S2 binds to form E.P1.S2



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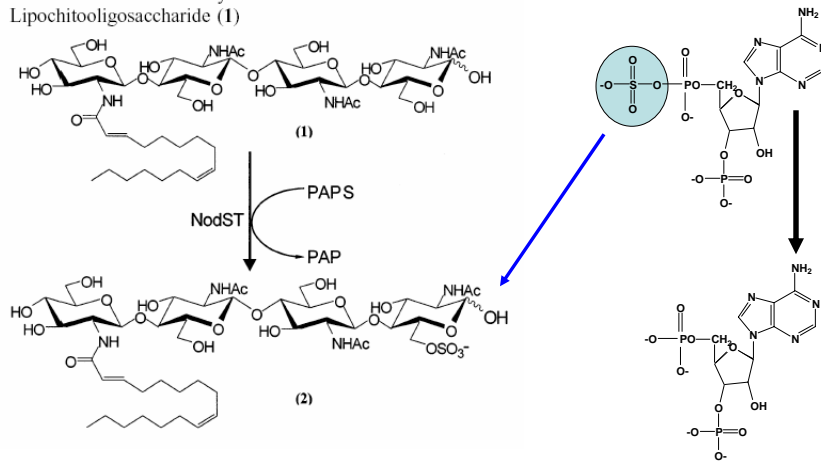
## Mass spectrometry and substrates and products of enzyme reactions

- 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

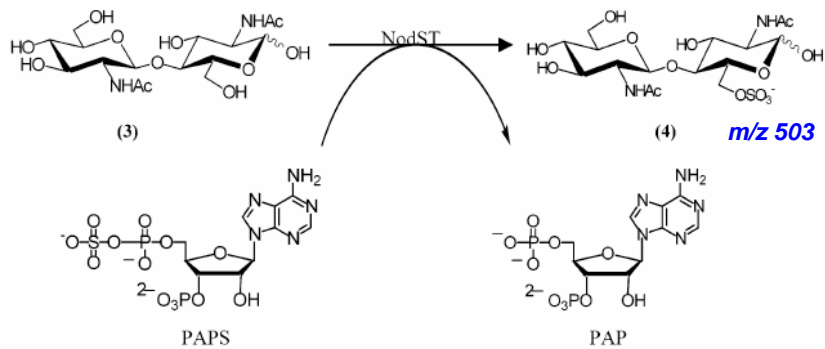
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## Sulfotransferase - a reaction with no absorbance or fluorescence to follow

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



## 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  $\beta$ -ME
  - Diluted into 10 mM  $\text{NH}_4\text{Ac}$  buffer, pH 8.0
- Incubate (25  $\mu\text{l}$ ) quenched with 100  $\mu\text{l}$  of MeOH containing internal standard
- Diluted incubate (40  $\mu\text{l}$ ) introduced into ESI source at 20  $\mu\text{l}/\text{min}$
- MS on a ThermoFinnigan LCQ monitoring  $m/z$  503 and  $m/z$  468 (internal standard)

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# Kinetics of chitobiose ST by ESI-MS

Pi et al., *Biochemistry* 41:13283

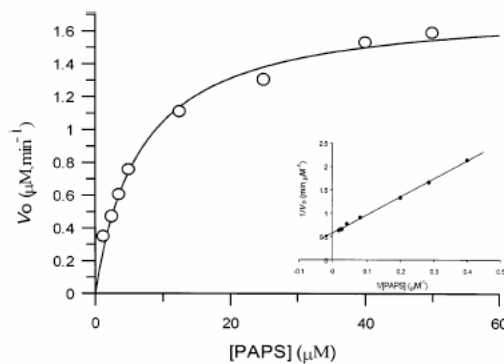


FIGURE 3: Saturation plot of  $V_0$  vs [PAPS]. The inset is a double-reciprocal plot of  $1/V_0$  vs  $1/[\text{PAPS}]$  ([PAPS] = 1.25, 2.5, 3.5, 5, 12.5, 25, 40, and 50  $\mu\text{M}$ , [chitobiose] = 1 mM, [NodST] = 90 nM, and pH 8.0).

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## Inhibition of ST by PAP using ESI-MS

Pi et al., *Biochemistry* 41:13283

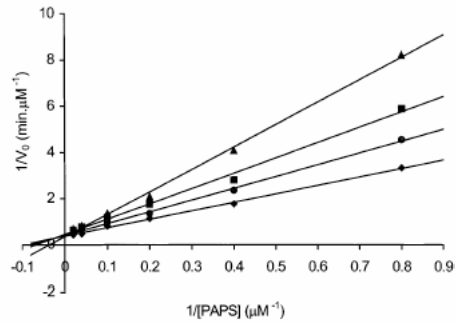


FIGURE 5: Double-reciprocal plot of  $1/V_0$  vs  $1/[PAPS]$  at different PAP concentrations:  $0 \mu\text{M}$  PAP (▲),  $0.75 \mu\text{M}$  PAP (■),  $1.5 \mu\text{M}$  PAP (●), and  $3.0 \mu\text{M}$  PAP (◆) ( $[PAPS] = 1.25, 2.5, 5, 10, 25,$  and  $50 \mu\text{M}$ ,  $[\text{chitobiose}] = 1 \text{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

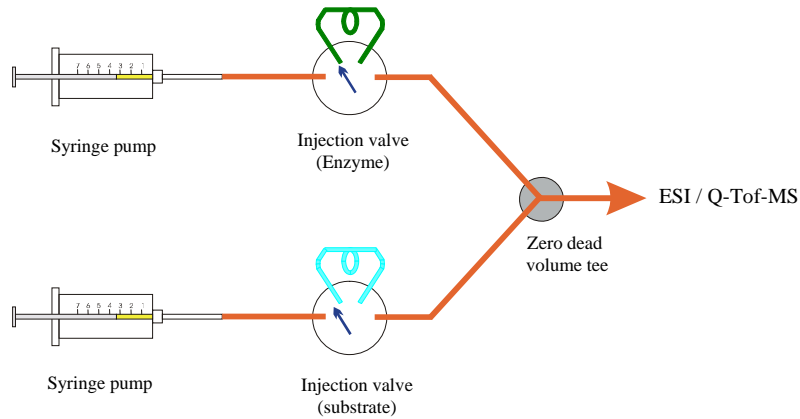
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## Non-covalent enzyme:substrate complexes

- 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

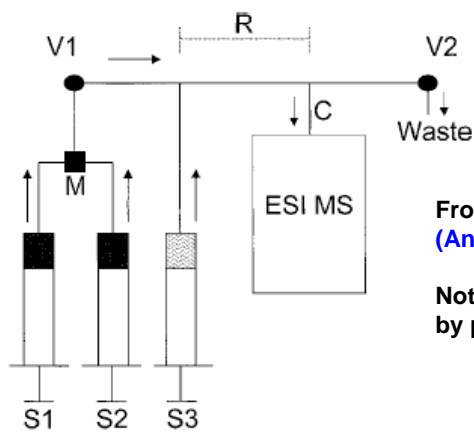
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## Schematic diagram of a stop-flow system



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## Stopped flow set up

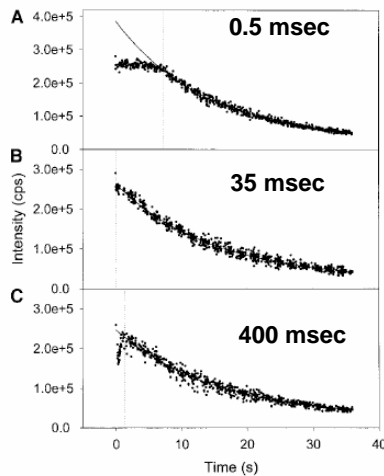


From Kolakowski and Konermann  
(Anal Biochem 292:107)

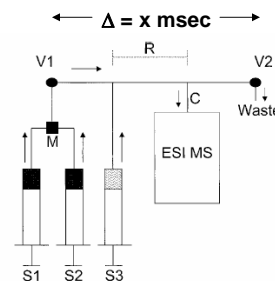
Note the additional flow introduced  
by pump S3

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



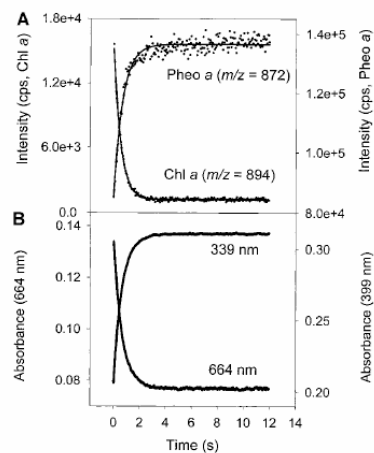
In this reaction, hydrolysis of acetylcholine in an alkaline buffer is monitored by the ion at  $m/z$  146



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Kolakowski and Konermann  
(Anal Biochem 292:107)

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



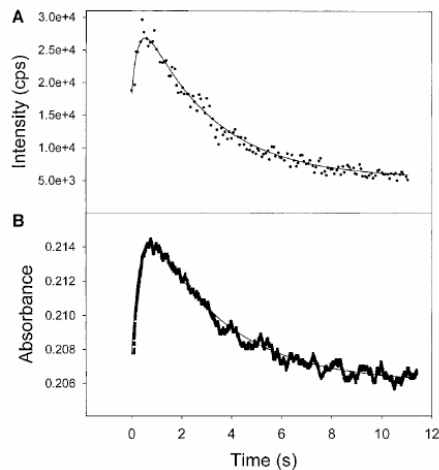
These data are from the conversion of chlorophyll A to pheophytin A (loss of Mg and gain of two protons).

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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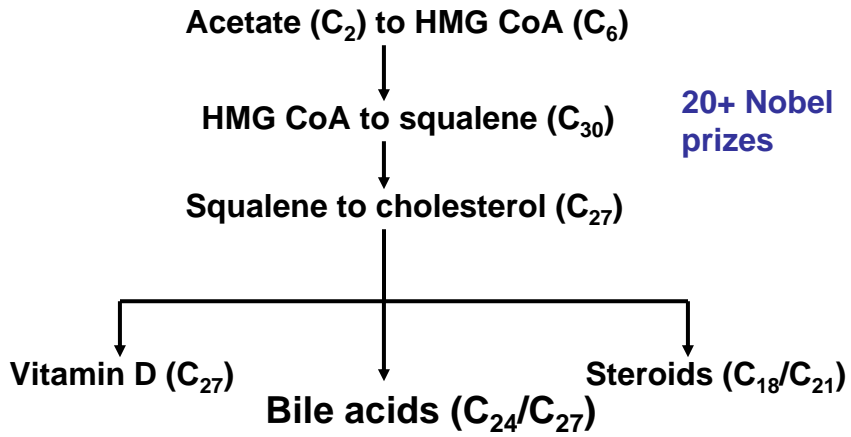
Kolakowski and Konermann  
(Anal Biochem 292:107)

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

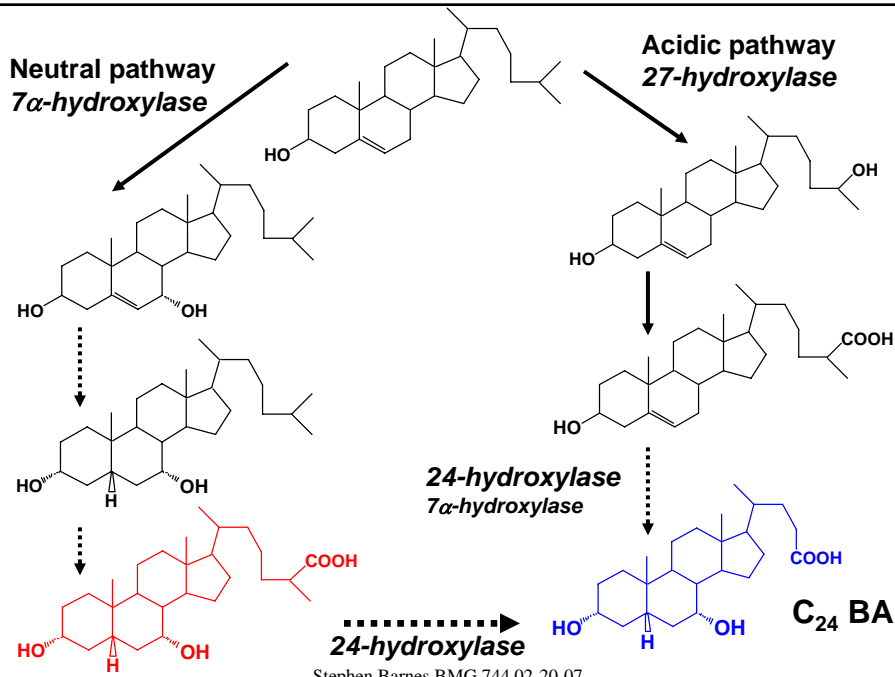
- **The pros:**
  - All the substrates and products (as well as the enzyme itself) can be studied simultaneously
  - It's applicable to compounds with no absorbance or fluorescence
- **The cons:**
  - The buffer for the reaction has to be chosen very carefully
  - Ammonium salts are the best candidates, but they may have an effect on the reaction rates

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# First let's remind ourselves of some basic biochemistry

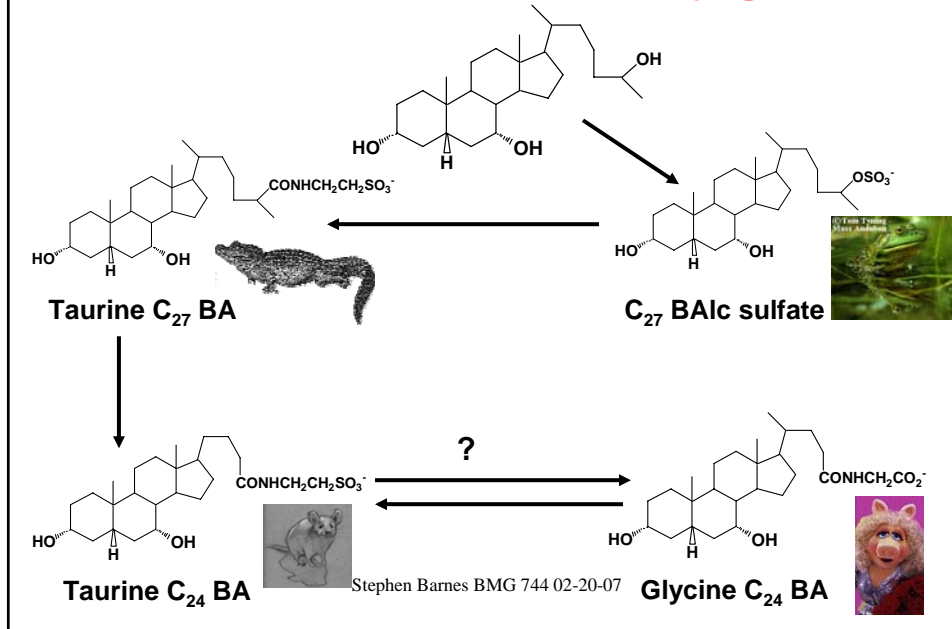


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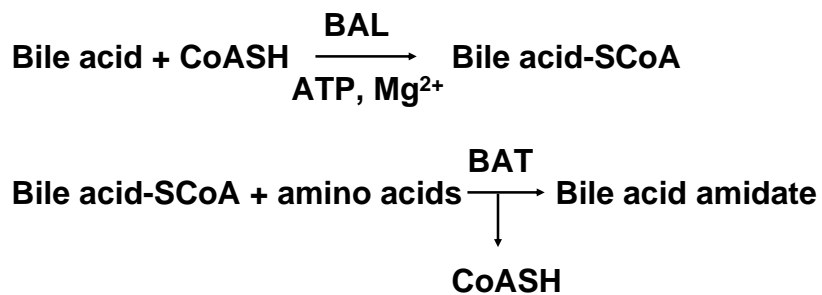


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## Evolution of bile acid conjugation



## Bile acid N-acylamidate formation (in hepatocytes)

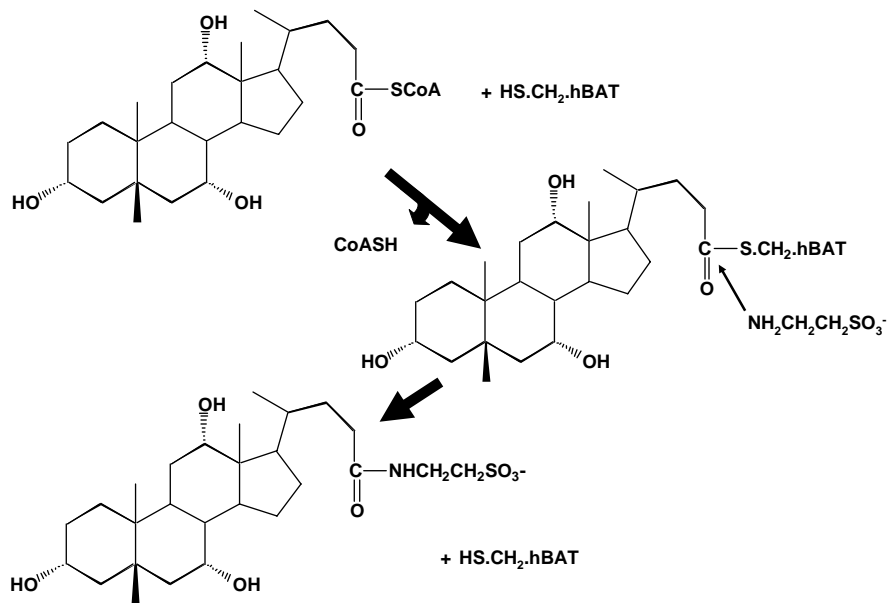


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## Characterization of BAT

- 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  $\lambda$ 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)

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## Sequence comparisons of mouse, rat and human BATs

```

                                                    *235
r 65% SLLASHGFATLALAYWGYDDLPSRLEKVDLEYFEEGVFLLRHPKVLGPGVGILSVCIGA 238
m 63% SLLASRGFATLALAYWNYDDLPSRLEKVDLEYFEEGVFLLRHPKVLGPGVGILSVCIGA 237
h 100% SLLASRGFASLALAYHNYEDLPRKPEVTDLEYFEEAANFLLRHPKVFGSGVGVVSVCQGV 238

r      EIGLSMAINLKQITATVLINGPNFVSSNPVYRGKVFQPTPCSEEFVTTNALGLVEFYRT 298
m      EIGLSMAINLKQIRATVLINGPNFVSQSPHVYHGQVYPPVPSNEEFVVTNALGLVEFYRT 297
h      QIGLSMAIYLKQVTATVLINGTNFPFGIPQVYHGQIQHQLPHSAQLISTNALGLLELYRT 288

                                                    *328
r      FEETADKDSKYCFPIEKAHGHFLFVVGEDDKKNLNSKVHAKQAIQALMKSGKKNWTLSYP 358
m      FQETADKDSKYCFPIEKAHGHFLFVVGEDDKKNLNSKVHANQAIQALMKNGKKNWTLSYP 357
h      FETTQVGASQYLFPIEEAQQGQFLFIVGEGDKKTINSKAHAEQAIGQLKRHGKKNWTLSYP 358

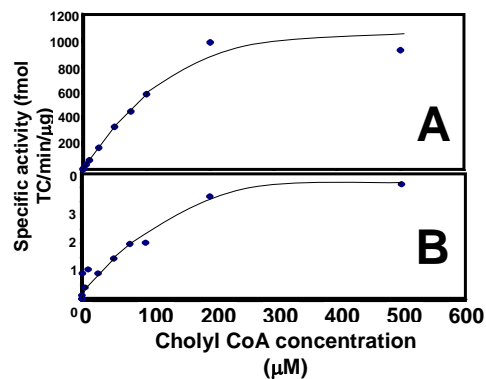
      *362      *372
r      GAGLLIEPPYSPLCSASRMPFVIPSINWGGEVIPH-AA 395
m      GAGLLIEPPYTPLCQASRMPILIPSLSWGGEVIPHSQA 395
h      GAGLLIEPPYSPLCCASTTHDLR--LHWGGEVIPH-AA 393

```

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## Site-specific Cys mutations

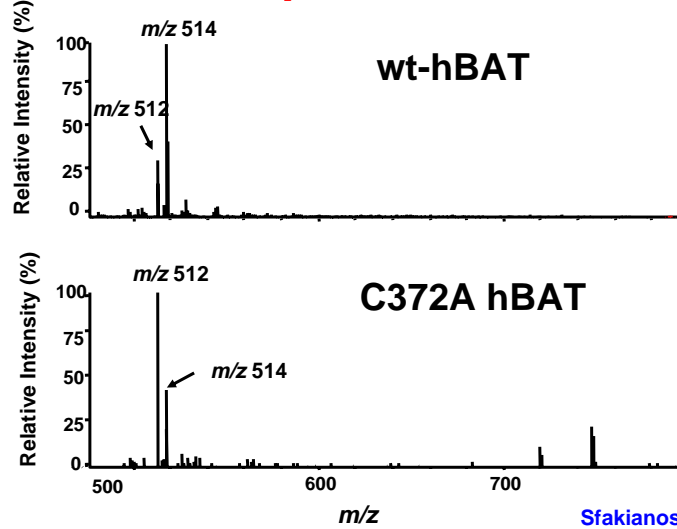
- Mutations were prepared for the two conserved Cys residues (C235 and C372) in BATs
- C235Y hBAT had no enzyme activity
- C372A hBAT had low activity



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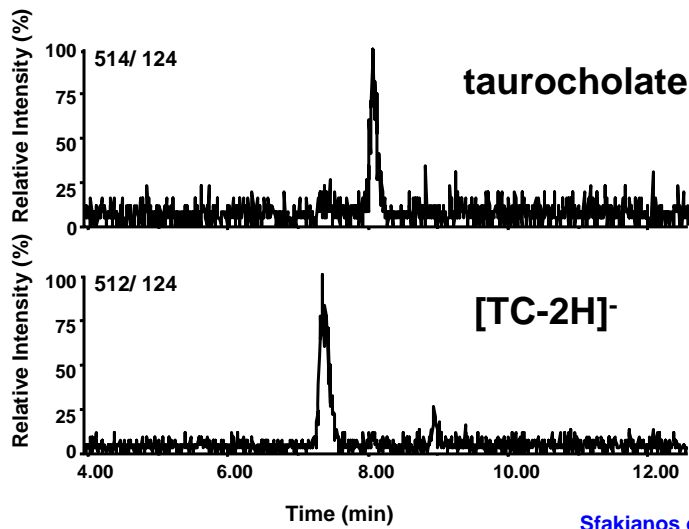
Sfakianos et al.

## ESI-mass spectrum of hBAT products



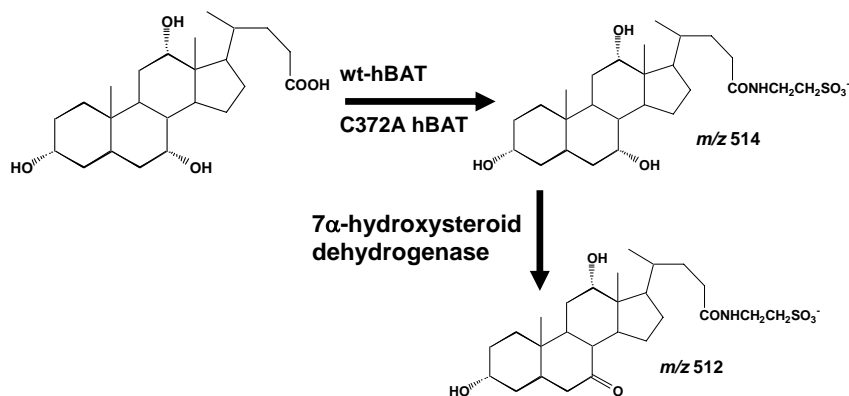
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## LC-MS of C372A hBAT product



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## Metabolism in *E. coli* expression system



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Sfakianos et al.

## hBAT Related Proteins from BLAST Search (Courtesy of Alexey Murzin, MRC lab)

```

235
kan-1  SLLASHGFATLALAYWGYDDLPSRLEKVDLEVFEEGVFLLRHPKVLGPGVGLSVCIQA 238
mBAT  SLLASRGFATLALAYWYDDLPSRLEKVDLEVFEEGVFLLRHPKVLGPGVGLSVCIQA 237
hBAT  SLLASRGFASLALAYHNYDDLPRKPEVTDLEVFEEAANFLRHPKVFSGVGVVSVCCQGV 238
MTE-I  SLLAGKGFVAMALAYNYDDLPKTMTMRIEYFEEAVNYLRGHPPEVKGPGLLGI SKGG 276
CTE-I  SLLAGKGFVAMALAYNYDDLPKTMTMRIEYFEEAVNYLRGHPPEVKGPGLLGI SKGG 235
CLCTE  SLLAGKGFVAMALAYNYDDLPKTMTMRIEYFEEAVNYLRGHPPEVKGPGLLGI SKGG 235
PLCTE  SLLAGKGFVAMALAYNYDDLPKTMTLHLEYFEEAMNYLLSHPEVKGPGLLGI SKGG 235
PTE-Ia SLLAGKGFVAMALAYNYEDLPKMDI IHLEYFEEAVTYLLSHPQVTGSGVGVGLGI SKGG 246
DLHp  KPFAEQGYAVLALSIFYAAPGLPATAEELPLEYFDRAVAWLAAPSDPKAIGVYVSKGA 138

328
kan-1  ALGLVEFYR--TFEETAD-KDSKYCFPIEKAHGHFLEFVVGEDDKNLNSKVHAKQAIQLM 345
mBAT  ALGLVEFYR--TFQETAD-KDSKYCFPIEKAHGHFLEFVVGEDDKNLNSKVHANQAIQLM 344
hBAT  ALGLLELYR--TFETTQV-GASQYLFFIEEAQQQLFIVGEGDKTINSKAHAEQAIGQLK 345
MTE-I  KDGLLDVVE--ALQSPL--VDKSFIPVERSDTTFLELVGQDDHNWKSEFYAREASKRLQ 382
CTE-I  KDGLKDVID--ALQSPL--VEQKSFIPVERSDTTFLELVGQDDHNWKSEFYANEISKRLQ 341
CLCTE  KDGLKDVID--ALQSPL--VEQKSFIPVERSDTTFLELVGQDDHNWKSEFYANEISKRLQ 341
PLCTE  KDGYDIVD--VLNSPLEGPDQKSFIPVERAESTFLELVGQDDHNWKSEFYANEACKRLQ 343
PTE-Ia KDGLKDVID--LLNPLEGPDQKSLIPVERSDTAFLELVGQDDHNWKSEFYAREASKRLQ 354
DLHp  SNYMAFYGLYDTGLKAADHPQA AIFVEKI HGPVMLISGRADAMWSSSAMSDAVVARLK 258

362
kan-1  KSGKK-NWTL LSYPGAGHLIEPPYPLCSASRMPFVIPSINWGGEVIPH-AA 395
mBAT  KNGKK-NWTL LSYPGAGHLIEPPYPLCQASRMPILIPSLSWGGEVIPHSQA 395
hBAT  RHGKN-NWTL LSYPGAGHLIEPPYPLCCASTHDLR--LHWGGEVIPH-AA 393
MTE-I  AHGKE-KPQI ICYPEAGHYIEPPYPLCSAGMHLVGANITFGGEPKPH-SV 432
CTE-I  AHGKE-KPQI ICYPEAGHYIEPPYPLCSAGMHLVGANITFGGEPKPH-SV 391
CLCTE  AHGKE-KPQI ICYPEAGHYIEPPYPLCSAGMHLVGANITFGGEPKPH-SV 391
PLCTE  AHGRR-KPQI ICYPETGHIIEPPYPLCRASLHALVGSPIWGGEPRAH-AM 393
PTE-Ia AHGKE-KPQI ICYPETGHIIEPPYPLCKASLNSLVGGPVIWGGEPRAH-AM 404
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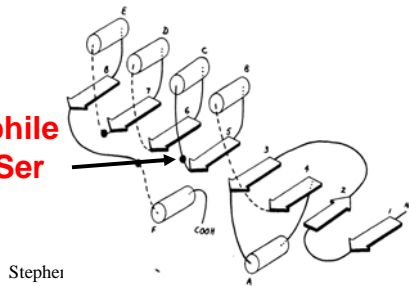
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## Cys235→Ser, what will C235S-BAT be, transferase or thioesterase?

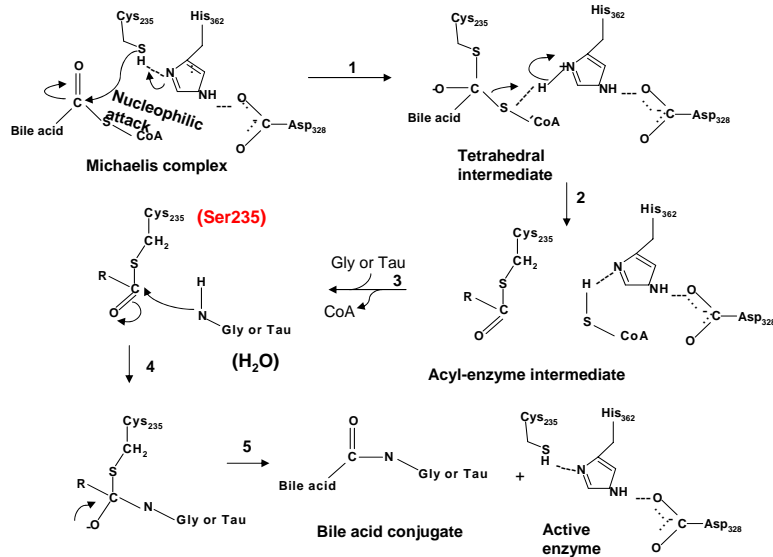
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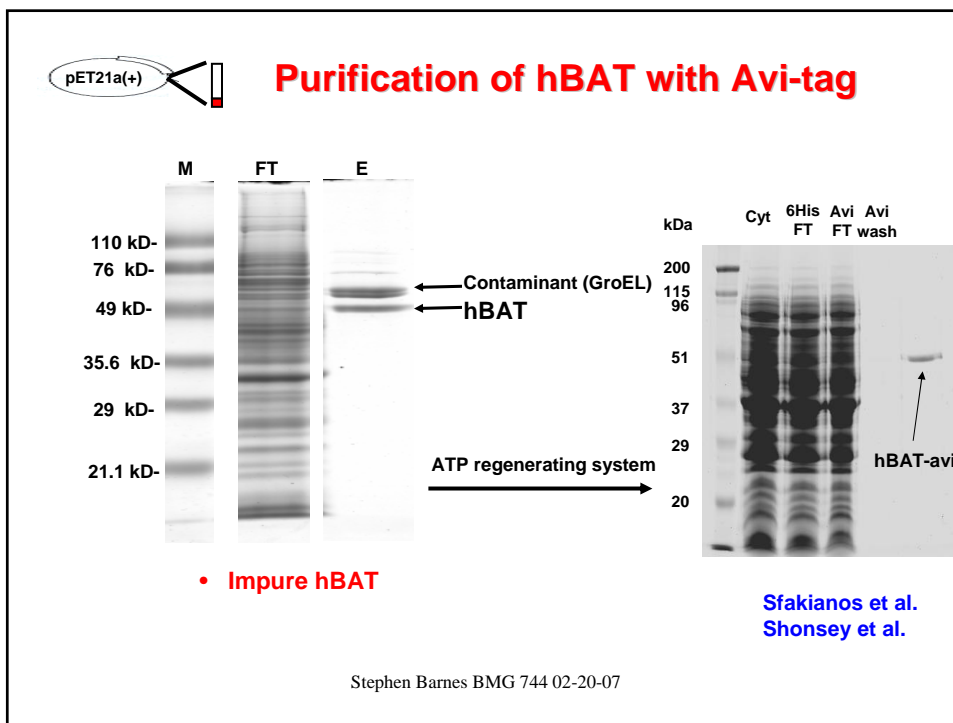
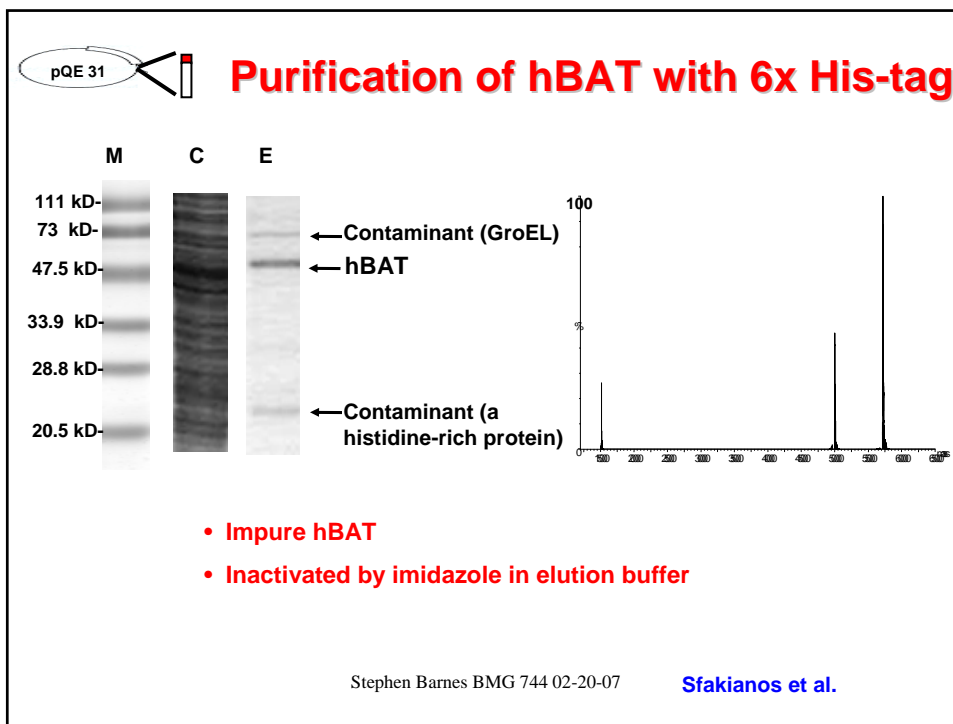
kan-1	SLLASHGFATLALAYWGYDDLPSRLEKVDLEYFEEGVFLLRHPKVLGPGVGLLSV	CIGA	238
mBAT	SLLASRGFATLALAYWNYDDLPSRLEKVDLEYFEEGVFLLRHPKVLGPGVGLLSV	CIGA	237
hBAT	SLLASRGFASLALAYHNYEDLPRKPEVTDLEYFEEANFLLRHPKVPFGSGVGVVSV	CQGV	238
MTE-I	SLLAGKGFVLMALAYNYDDLPKTMTMRIEYFEEAVNYLRGHPEVKGPGIGLLGI	SKGG	276
CTE-I	SLLAGKGFVLMALAYNYDDLPKTMTMRIEYFEEAVNYLRGHPEVKGPGIGLLGI	SKGG	235
CLCTE	SLLAGKGFVLMALAYNYDDLPKTMTMRIEYFEEAVNYLRGHPEVKGPGIGLLGI	SKGG	235
PLCTE	SLLAGKGFVLMALAYNYEDLPKTMTLHLEYFEEAMNYLLSHPEVKGPGVGLGI	SKGG	235
PTE-Ia	SLLAGKGFVLMALAYNYEDLPKDMDI IHLEYFEEAVTYLLSHPQVTGSGVGVGLGI	SKGG	246
DLHp	KPFAEQGYAVLALSIFYAAGPLPATAEELPLEYFDRAVWLAQAQPSVDPKAIGVYGV	SKGA	138

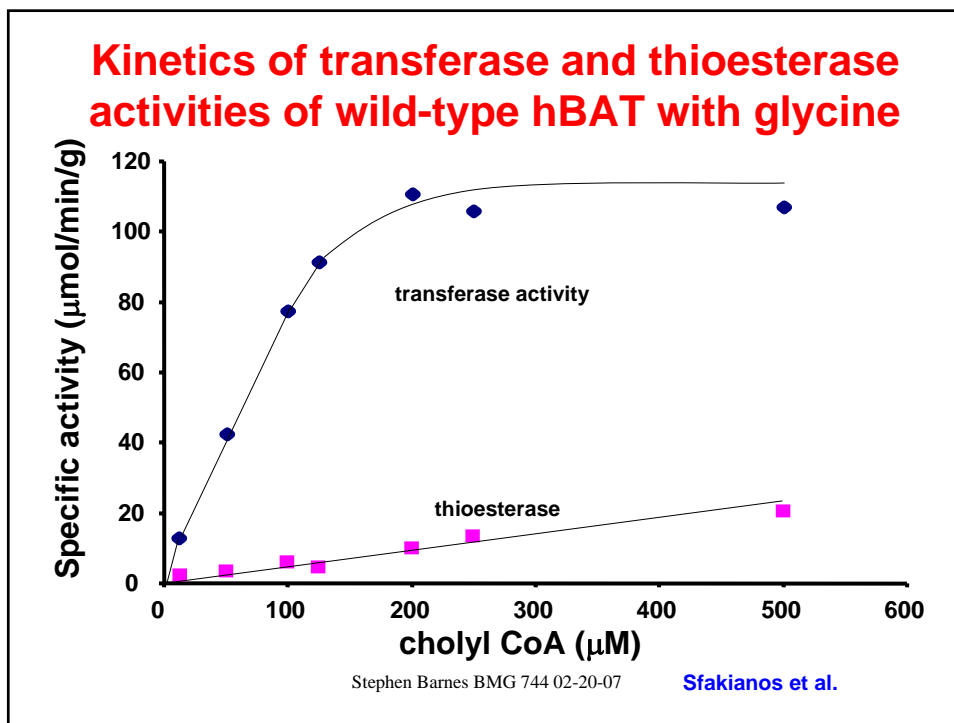
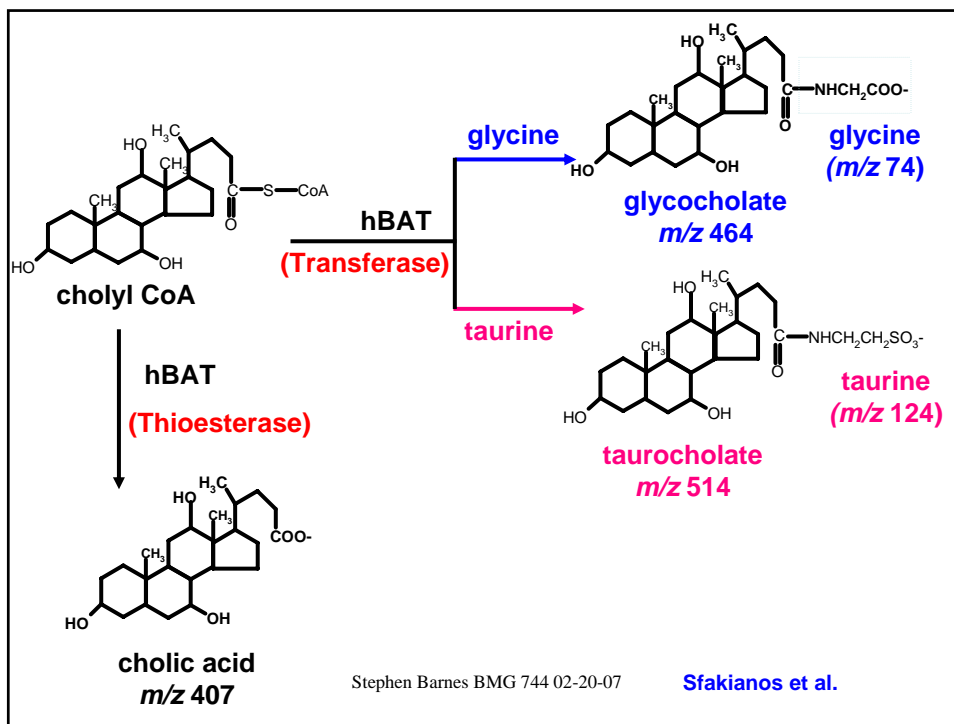
? Nucleophile  
Cys235→Ser



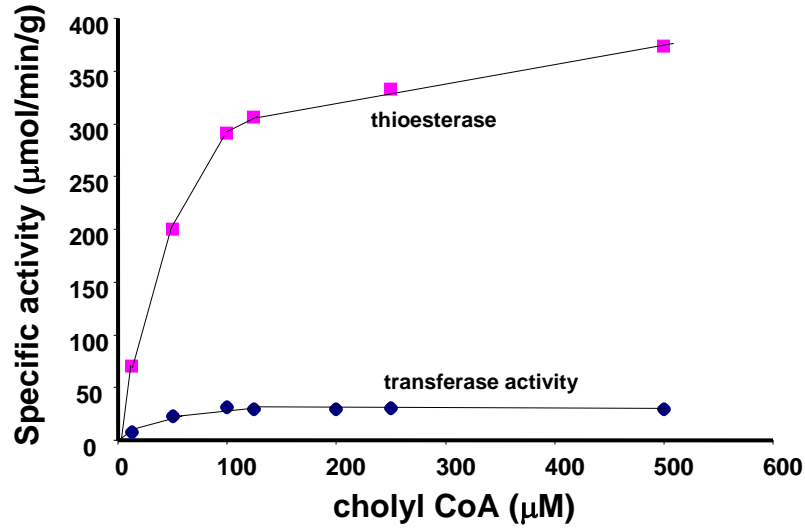
## Charge Relay Mechanism shared by hBAT, thioesterases, and a large group of hydrolases



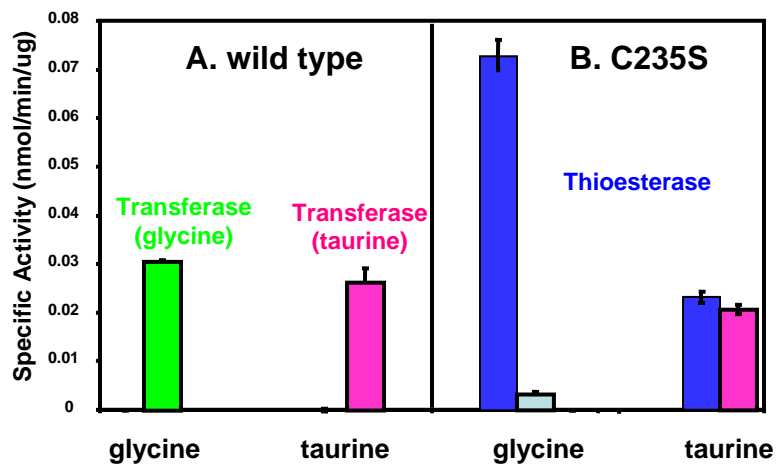




## Kinetics of transferase and thioesterase activities of C235S hBAT variant with glycine



## LC-ESI-MS-MRM Analysis of Reaction Products



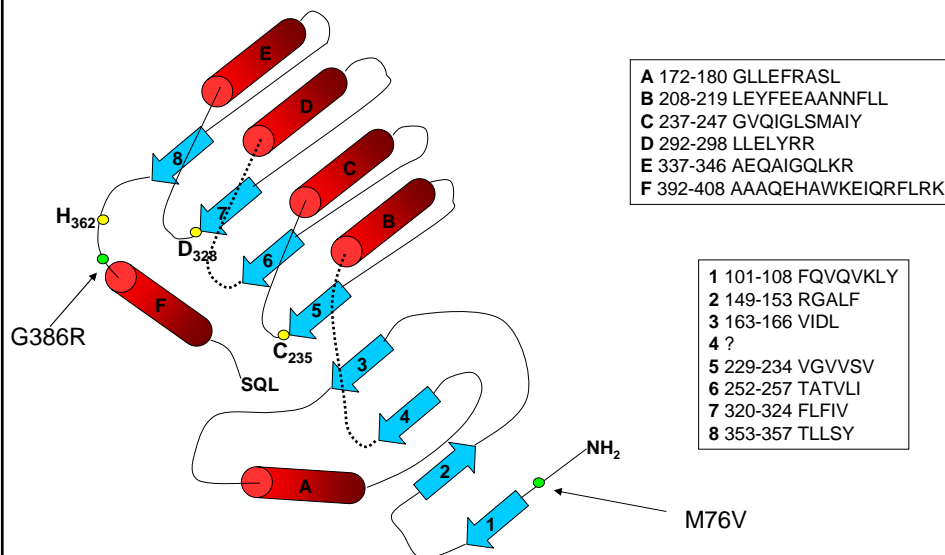
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## Bile acid CoA:amino acid N-acyltransferase

- 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

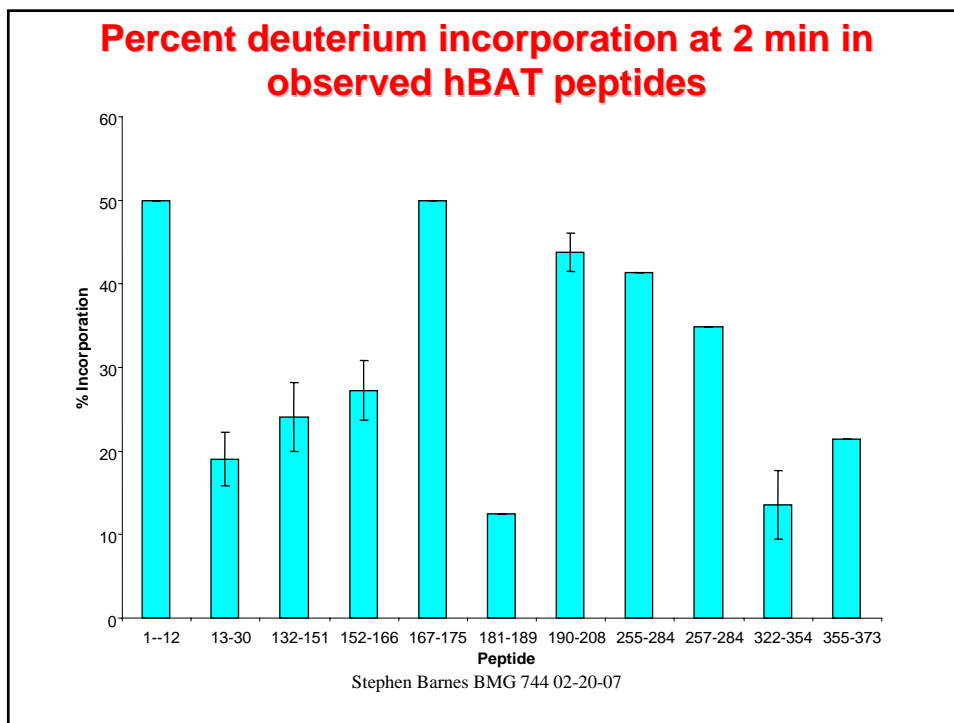
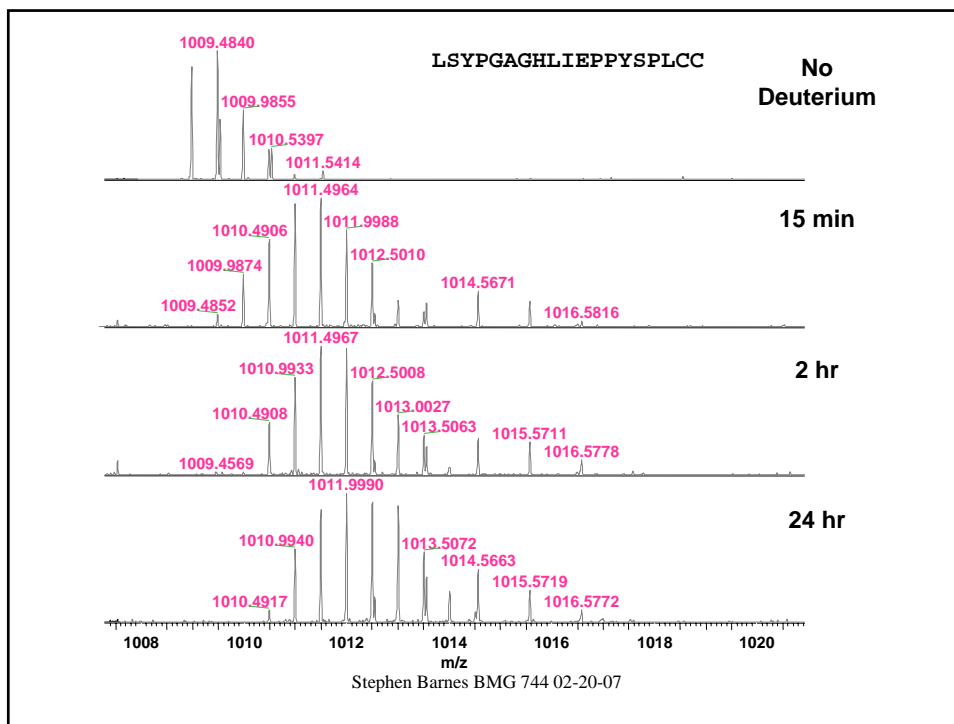
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## Revised BAT structure

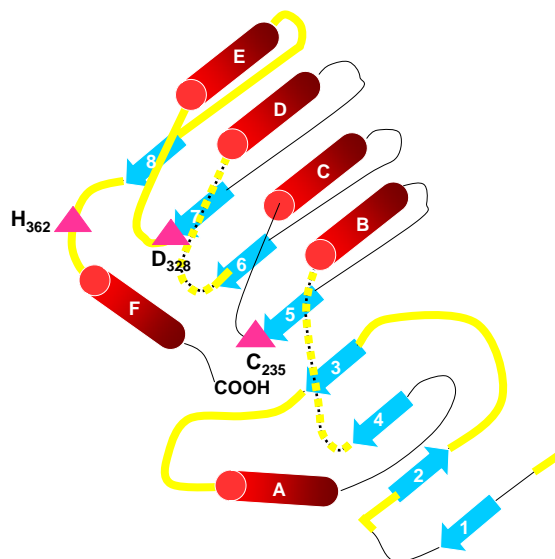


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## The Protein Structure Modeling of hBAT



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## A practical example of use of MS in enzymology - the enzyme BAT

- Johnson et al., J Biol Chem, 266: 10227-10233, 1991 (human BAT enzyme - purification)
- Falany et al., J Biol Chem, 269: 19375-19379, 1994 (human cDNA cloning and expression)
- 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)
- Sfakianos et al., J Biol Chem, 277: 47270-47275, 2002 (mechanism of human BAT)
- Shonsey et al., *Methods in Enzymology*, Vol. 400, Chapter 21, pp 360-373, 2005.
- Shonsey et al., *Methods in Enzymology*, Vol. 400, Chapter 22, pp 374-394, 2005.

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