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

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**

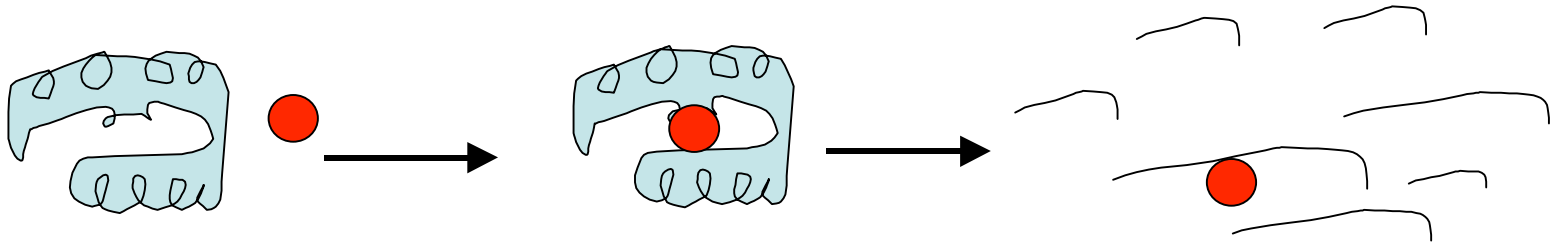
Enzymes and Mass Spec

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**

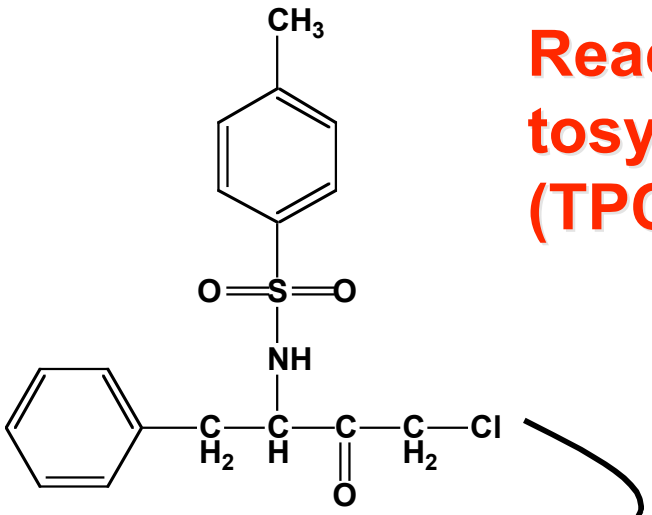
MS of enzymes



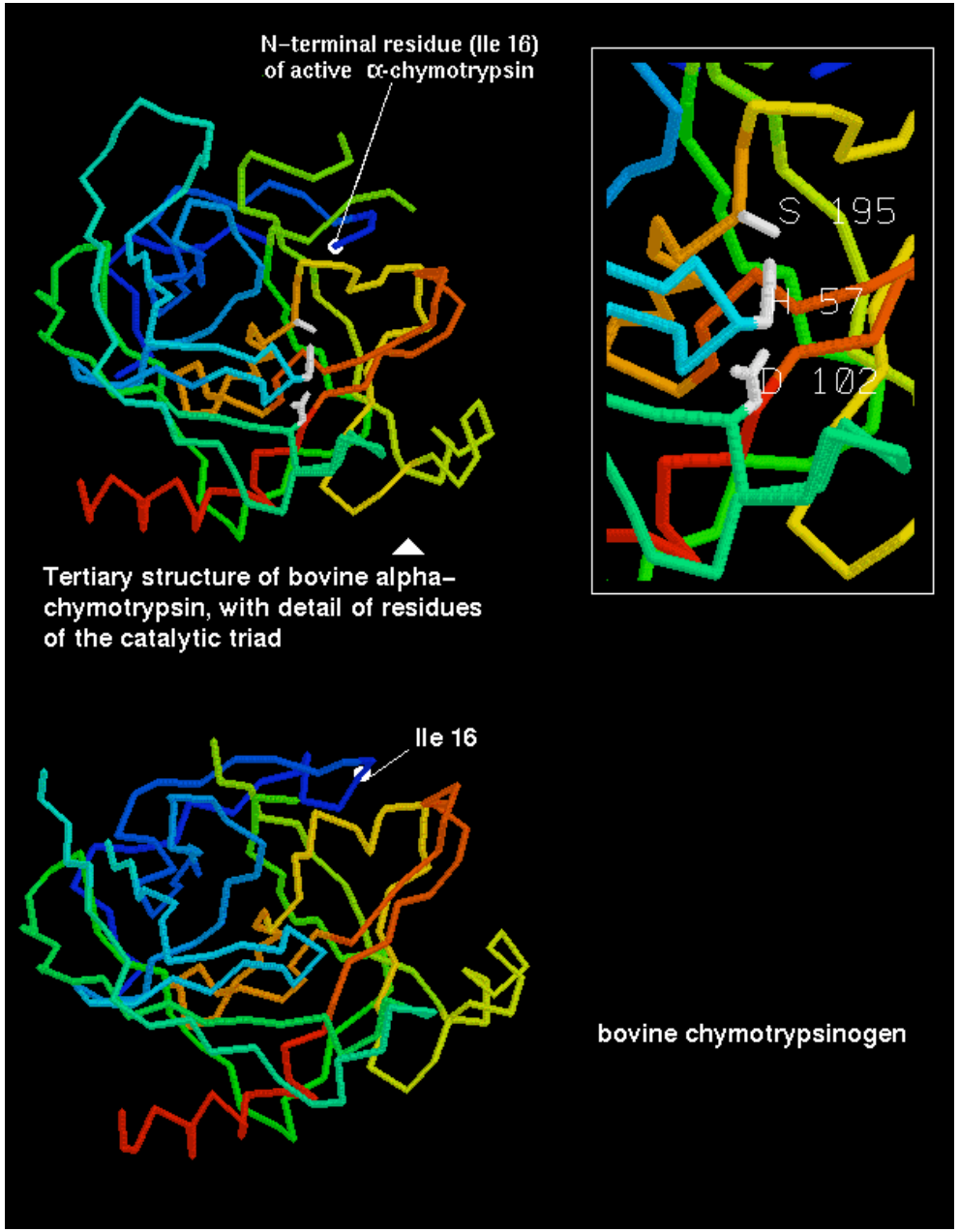
Enzymes can be inactivated by suicide substrates - these come into the active site and undergo a covalent reaction, thereby blocking the approach of other substrate molecules

- **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 tosylphenylalanylchloromethylketone (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
```

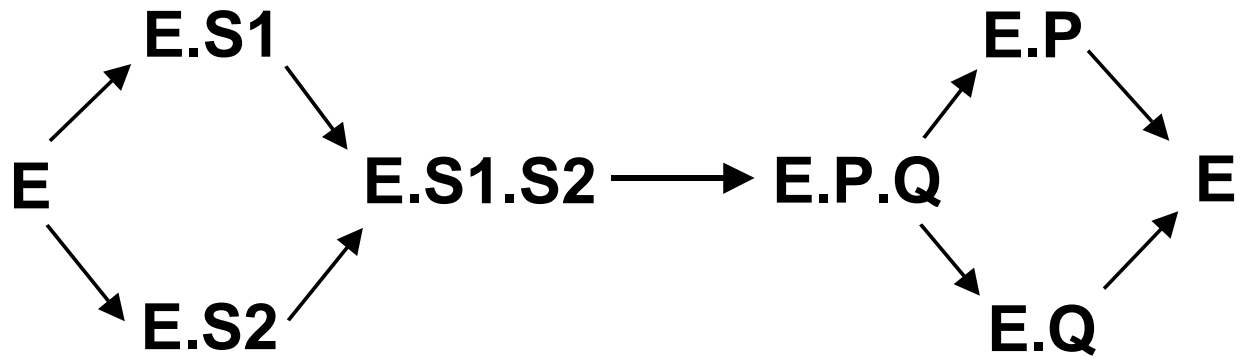
Mass spectrometry and enzyme-catalyzed reactions

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

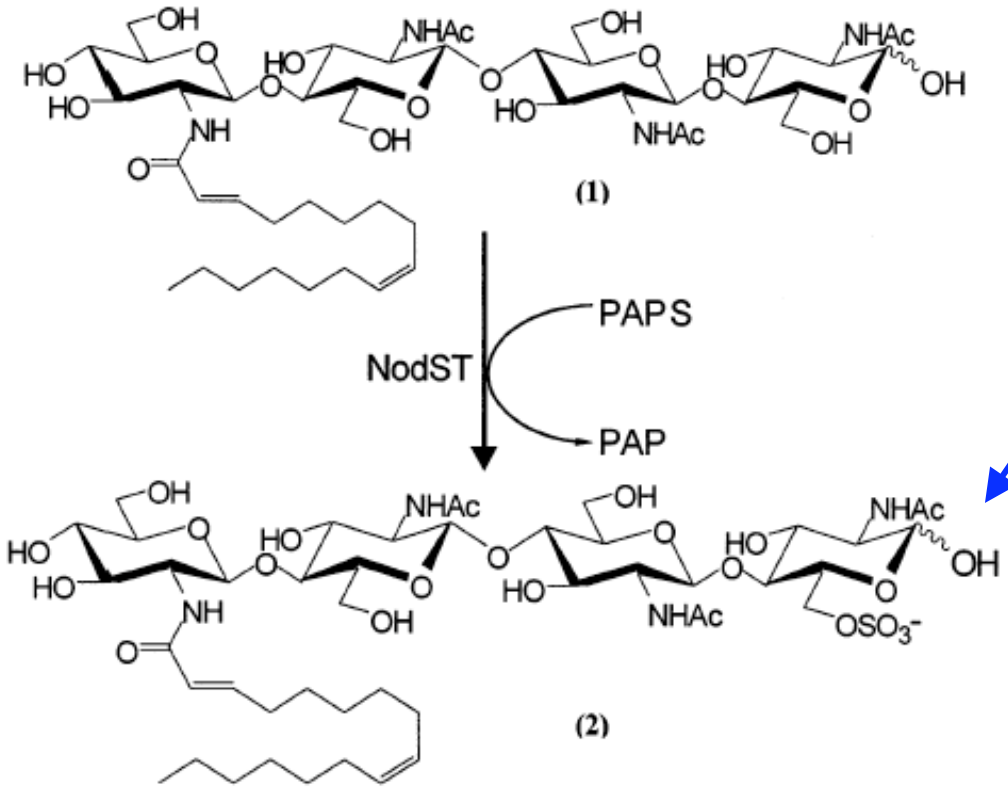
- **is it a random mechanism? If so, both E.S1 and E.S2 exist**
- **is it an ordered mechanism? In this case, S1 has to bind first. So, there will be E.S1 and E.S1.S2, but no E.S2**
- **is it a Ping-Pong mechanism? In this case, E.S1 → E.P1 before S2 binds to form E.P1.S2**

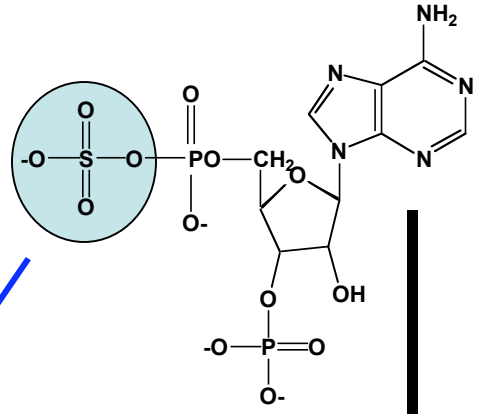
Mass spectrometry and substrates and products of enzyme reactions

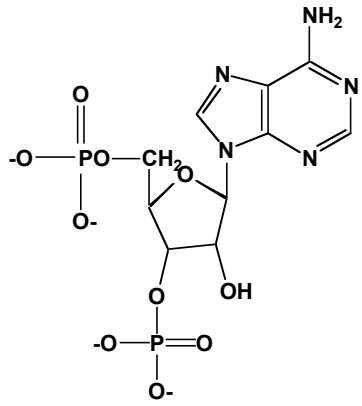
- Most enzyme reactions are studied by measuring the appearance of a product or (more rarely) the disappearance of a substrate
- If the substrate or product has a unique absorbance or fluorescence, the reaction can be followed in real time
- Some substrates have no usable absorbance or fluorescence - 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

Sulfotransferase - a reaction with no absorbance or fluorescence to follow

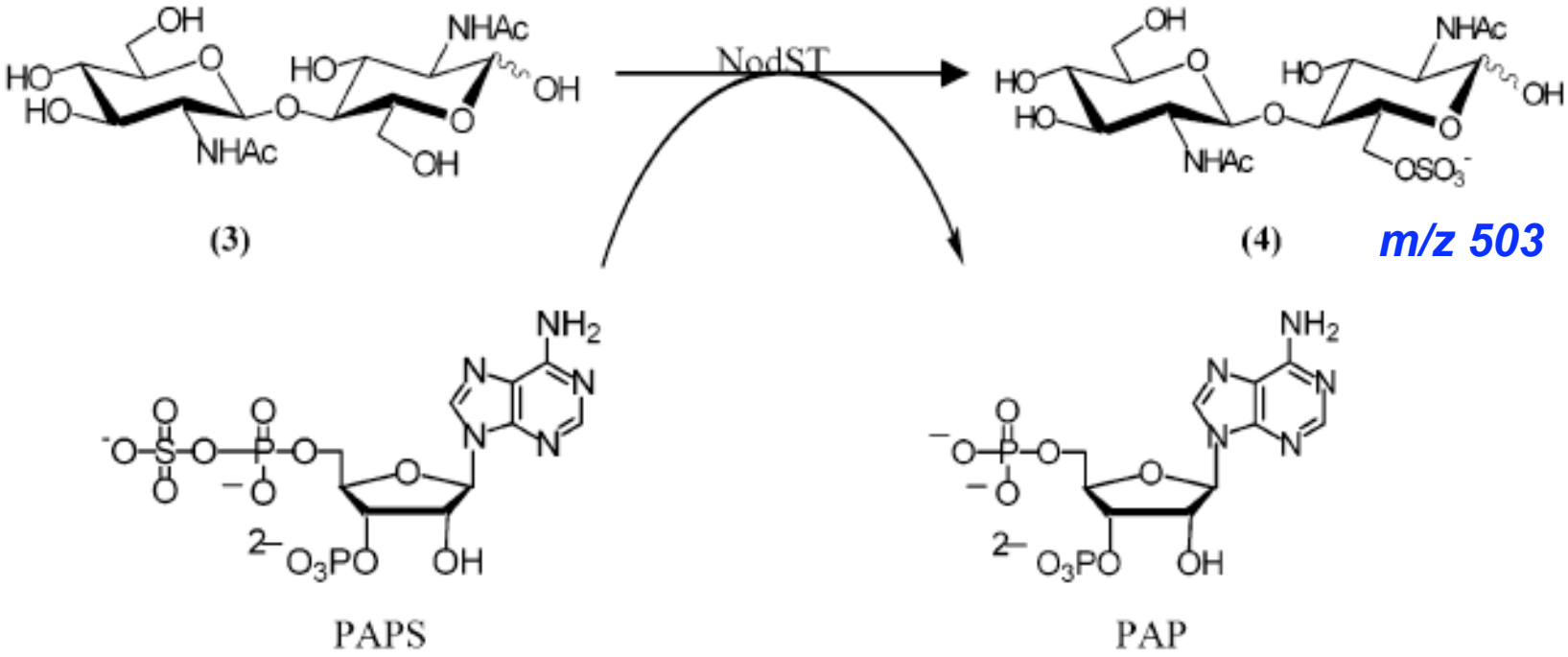
Scheme 1: NodST Catalyzes the Sulfation of a Lipochitooligosaccharide (**1**)







Sulfation of chitobiose



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

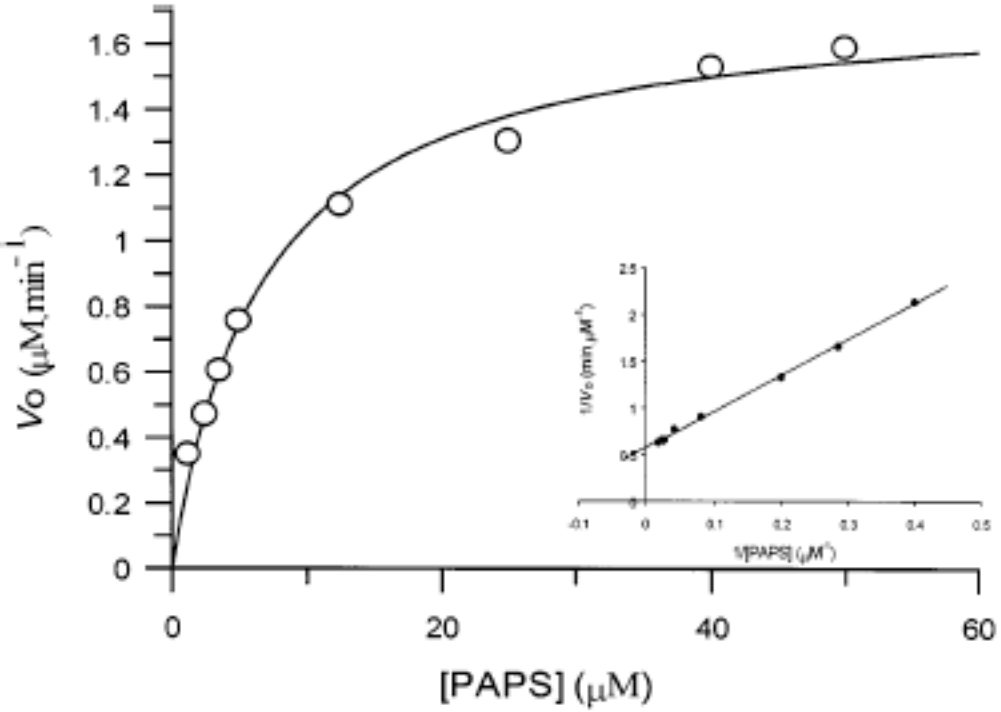


FIGURE 3: Saturation plot of V_0 vs [PAPS]. The inset is a double-reciprocal plot of $1/V_0$ vs 1/[PAPS] ([PAPS] = 1.25, 2.5, 3.5, 5, 12.5, 25, 40, and 50 μM, [chistobiose] = 1 mM, [NodST] = 90 nM, and pH 8.0).

Inhibition of ST by PAP using ESI-MS

Pi et al., Biochemistry 41:13283

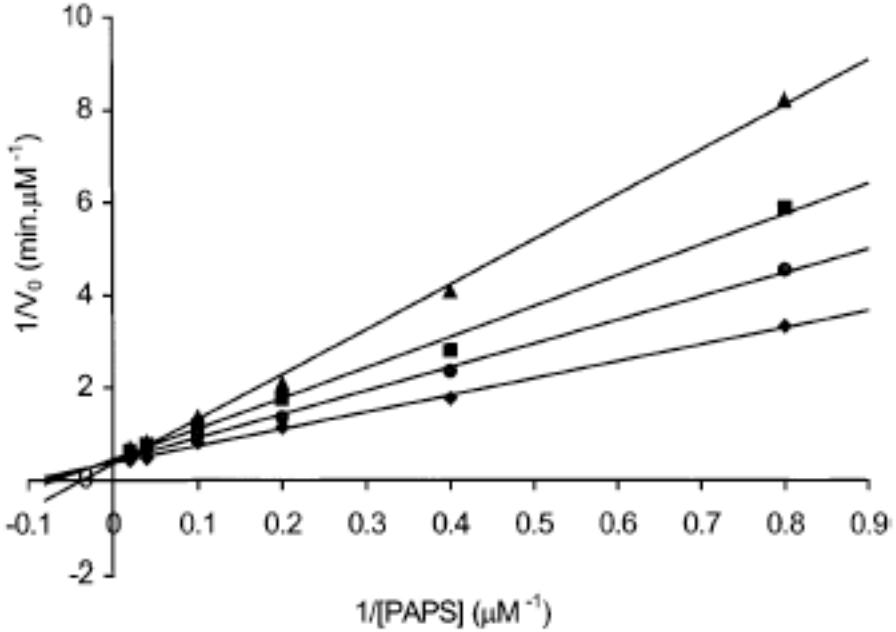
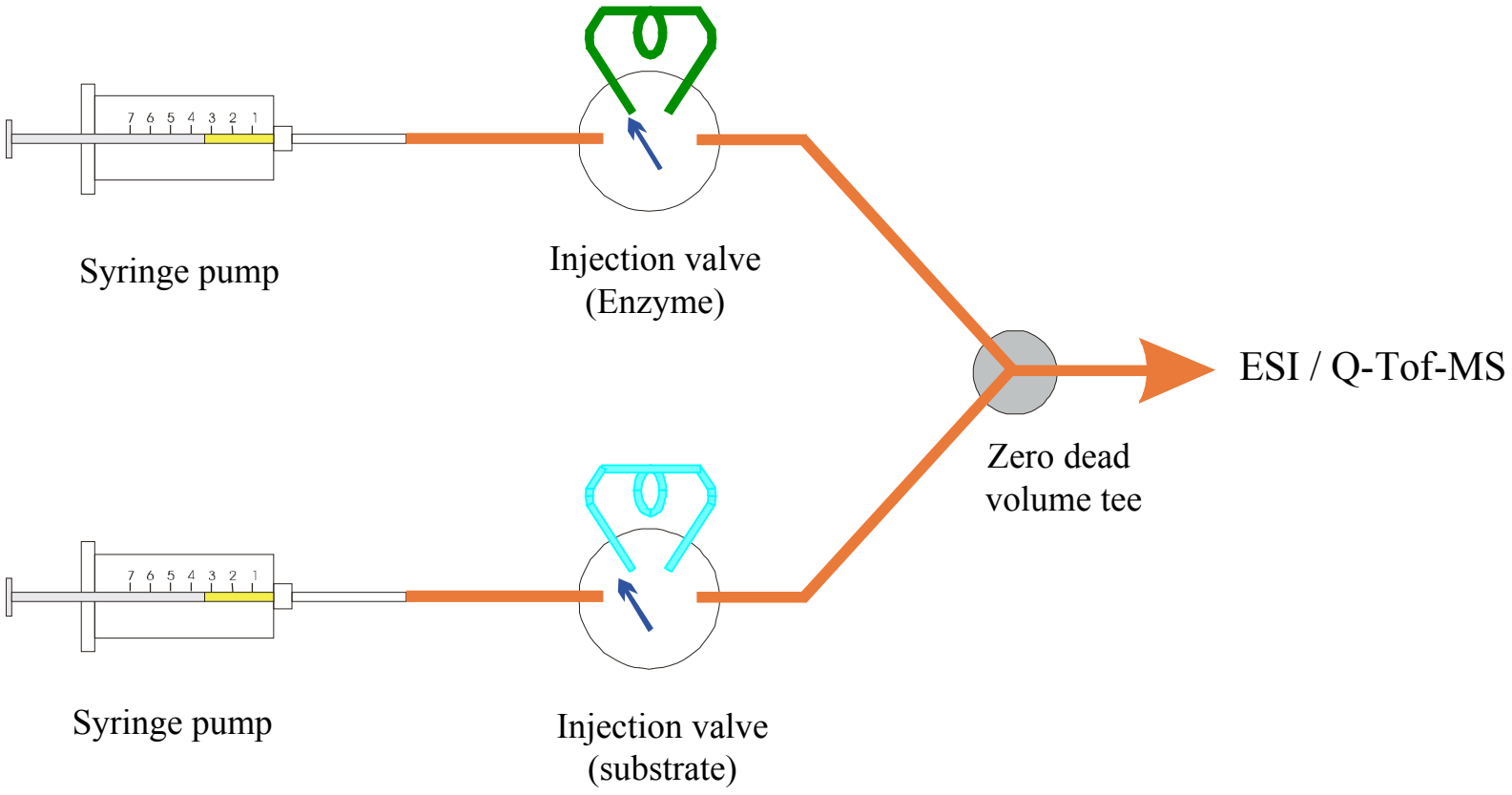


FIGURE 5: Double-reciprocal plot of $1/V_0$ vs 1/[PAPS] at different PAP concentrations: 0 μM PAP (▲), 0.75 μM PAP (■), 1.5 μM PAP (●), and 3.0 μM PAP (◆) ([PAPS] = 1.25, 2.5, 5, 10, 25, and 50 μM, [chitobiose] = 1 mM, [NodST] = 90 nM, and pH 8.0).

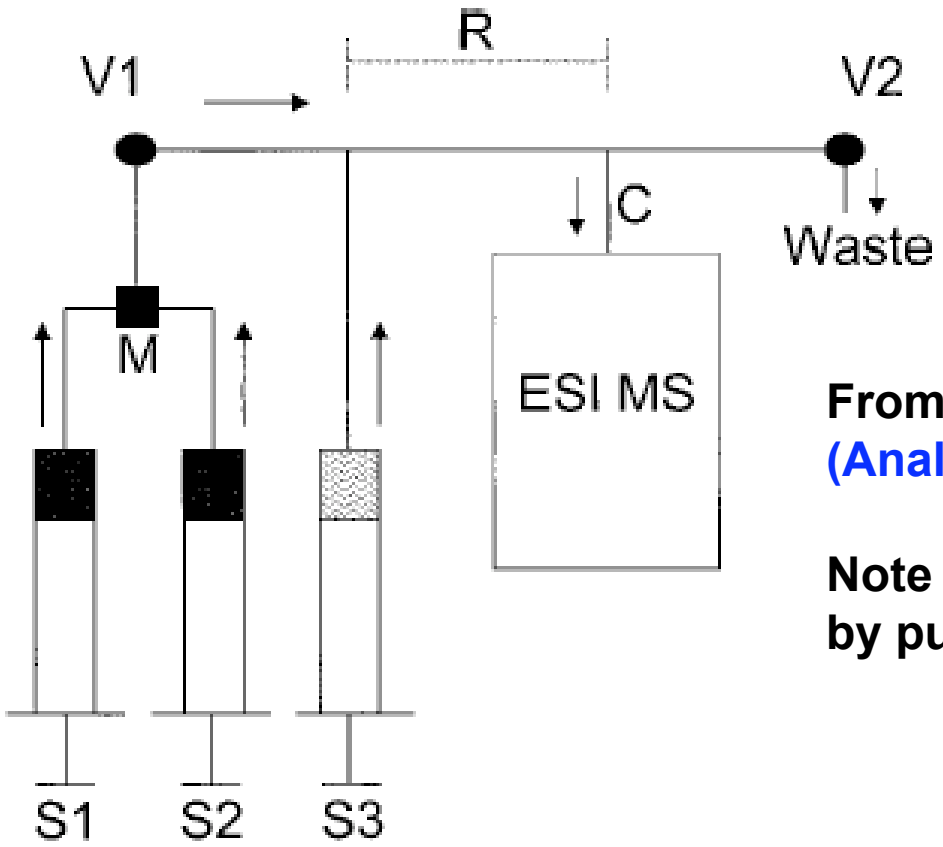
Non-covalent enzyme:substrate complexes

- **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



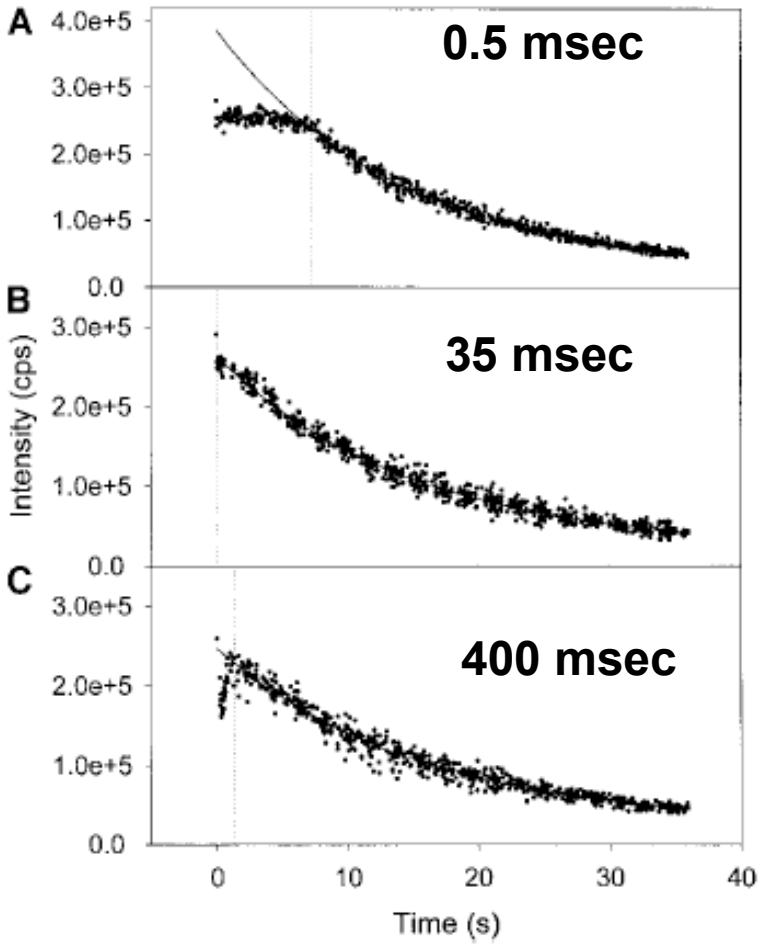
Stopped flow set up



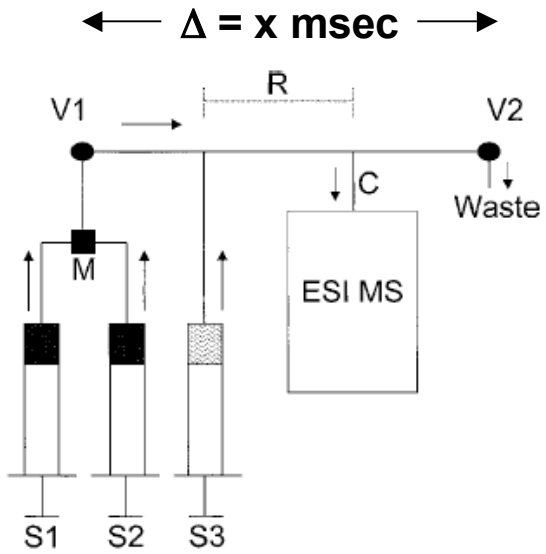
From Kolakowski and Konermann (Anal Biochem 292:107)

Note the additional flow introduced by pump S3

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

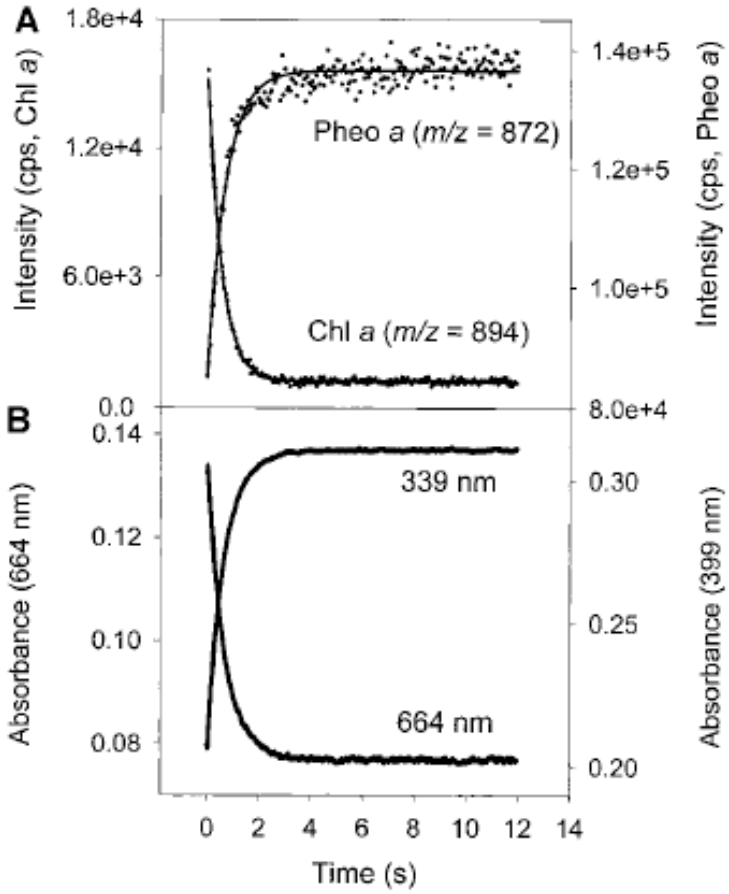


In this reaction, hydrolysis of acetylcholine in an alkaline buffer is monitored by the ion at *m/z* 146



Kolakowski and Konermann (Anal Biochem 292:107)

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

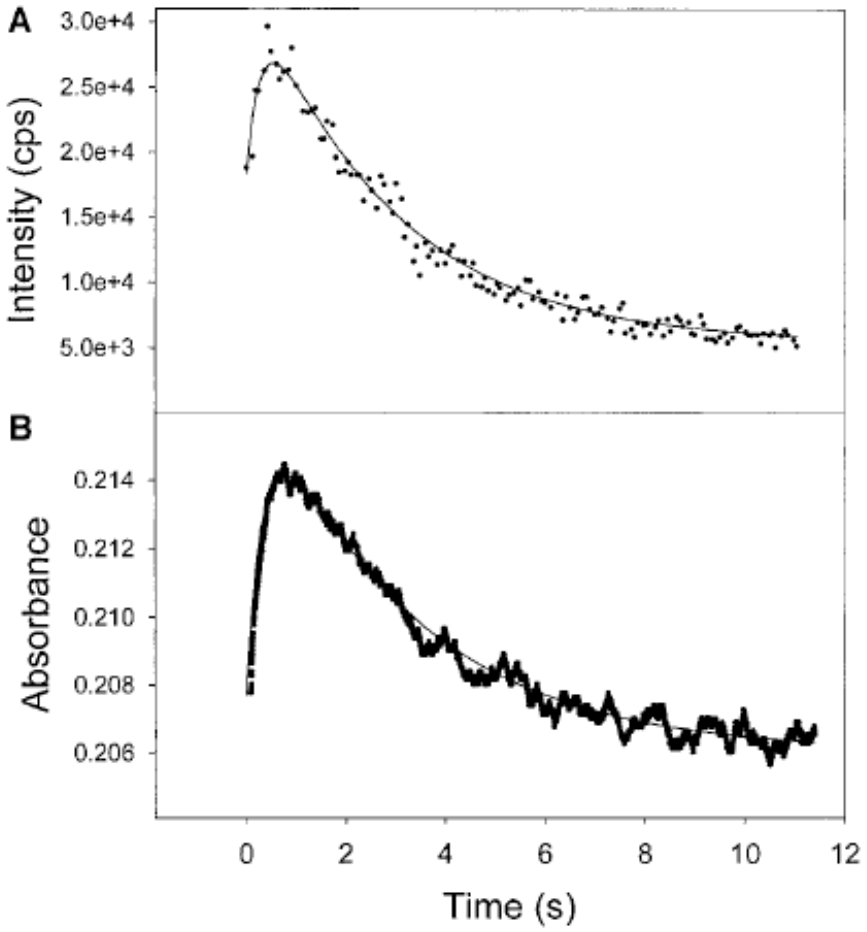


These data are from the conversion of chlorophyll A to pheophytin A (loss of Mg and gain of two protons).

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

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

Kolakowski and Konermann (Anal Biochem 292:107)

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

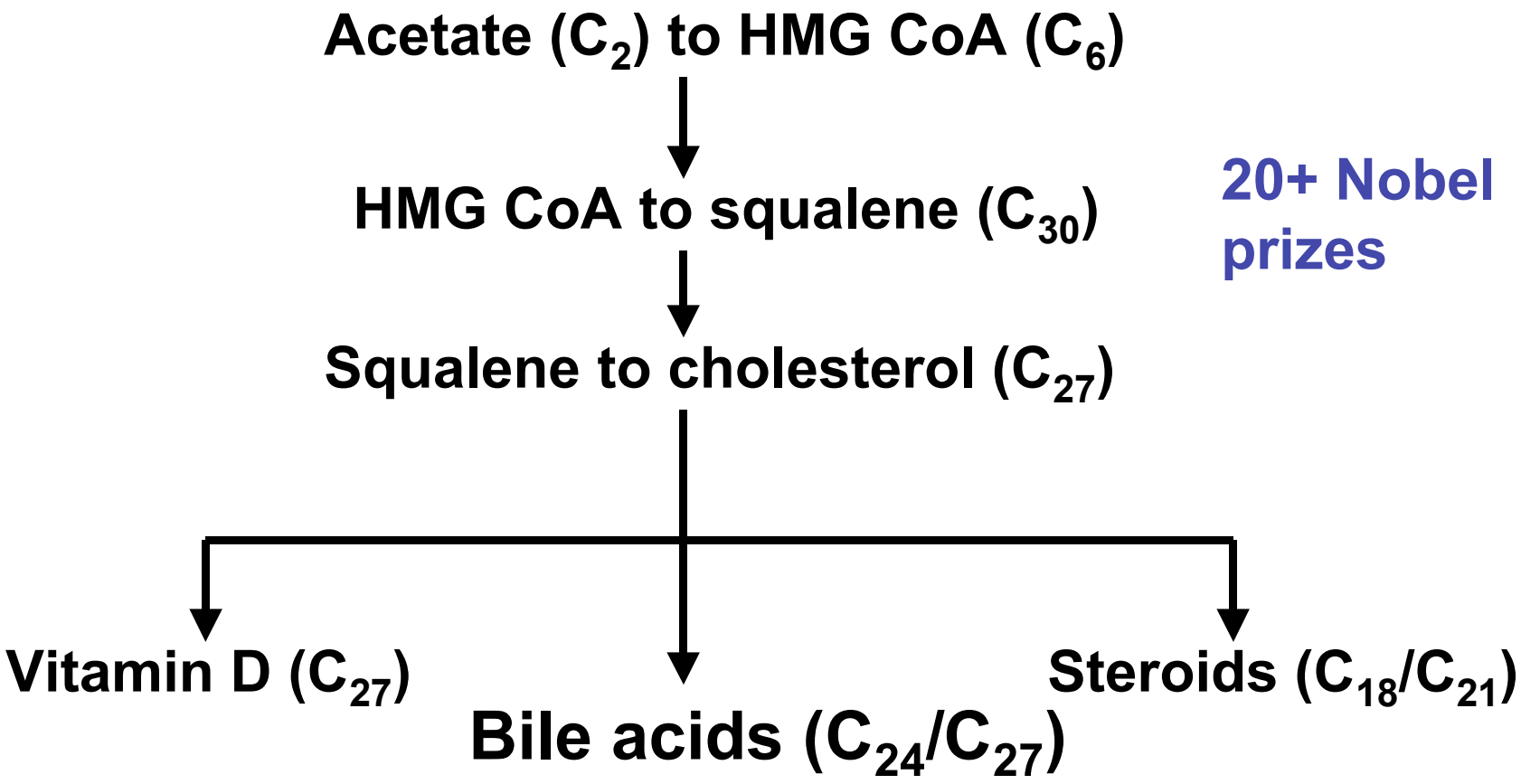
- **The pros:**
  - **All the substrates and products (as well as the enzyme itself) can be studied simultaneously**
  - **It's applicable to compounds with no absorbance or fluorescence**

- **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**

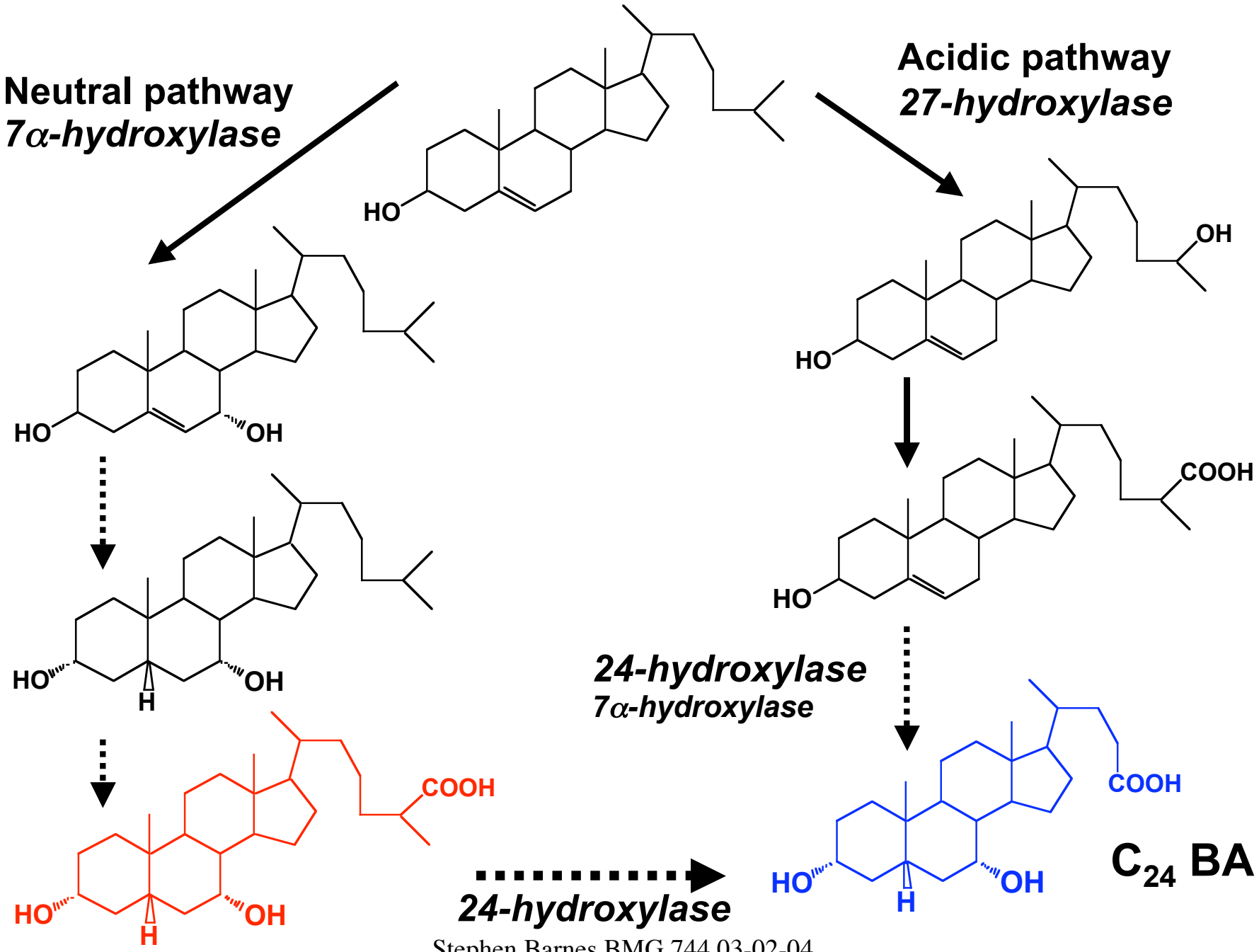
A practical example of use of MS in enzymology - the enzyme BAT

- 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)

First let's remind ourselves of some basic biochemistry

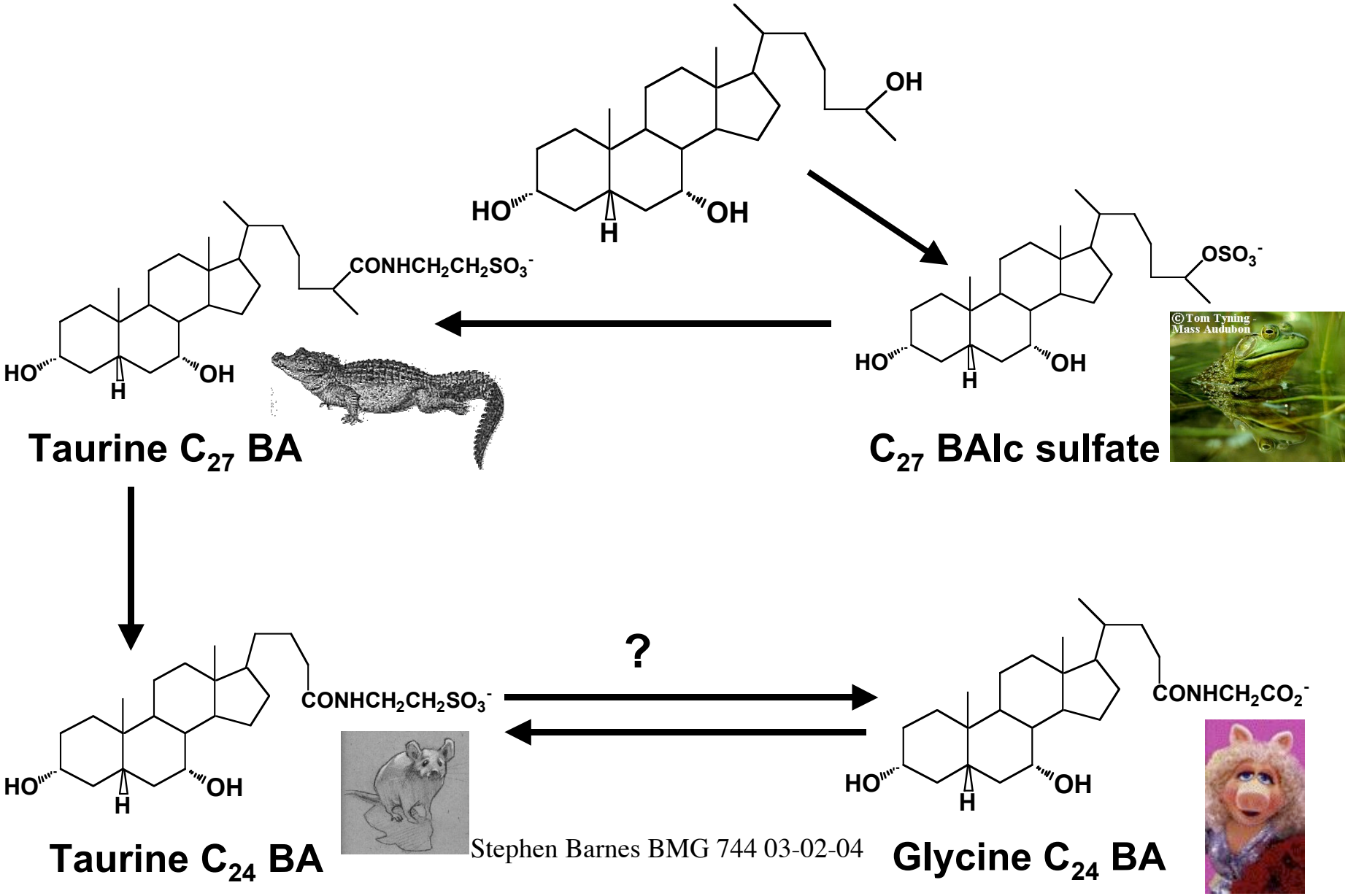


20+ Nobel prizes

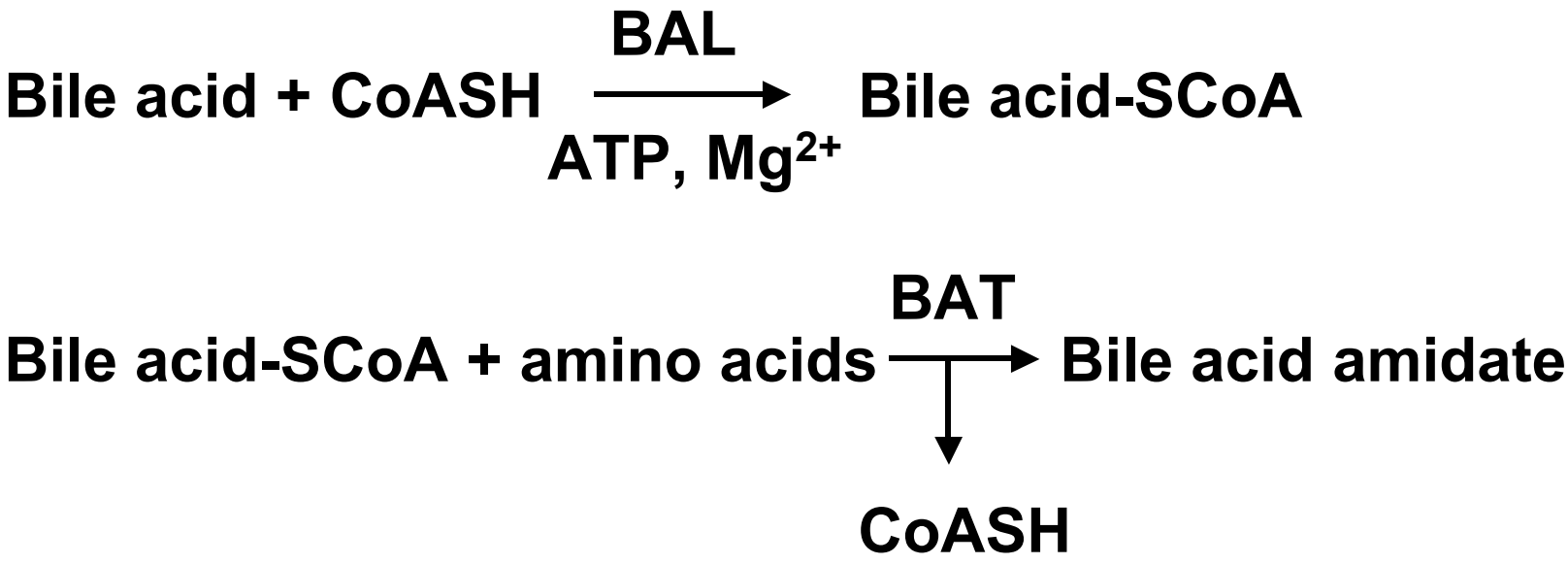


Stephen Barnes BMG 744 03-02-04

Evolution of bile acid conjugation



Bile acid N-acylamidate formation (in hepatocytes)

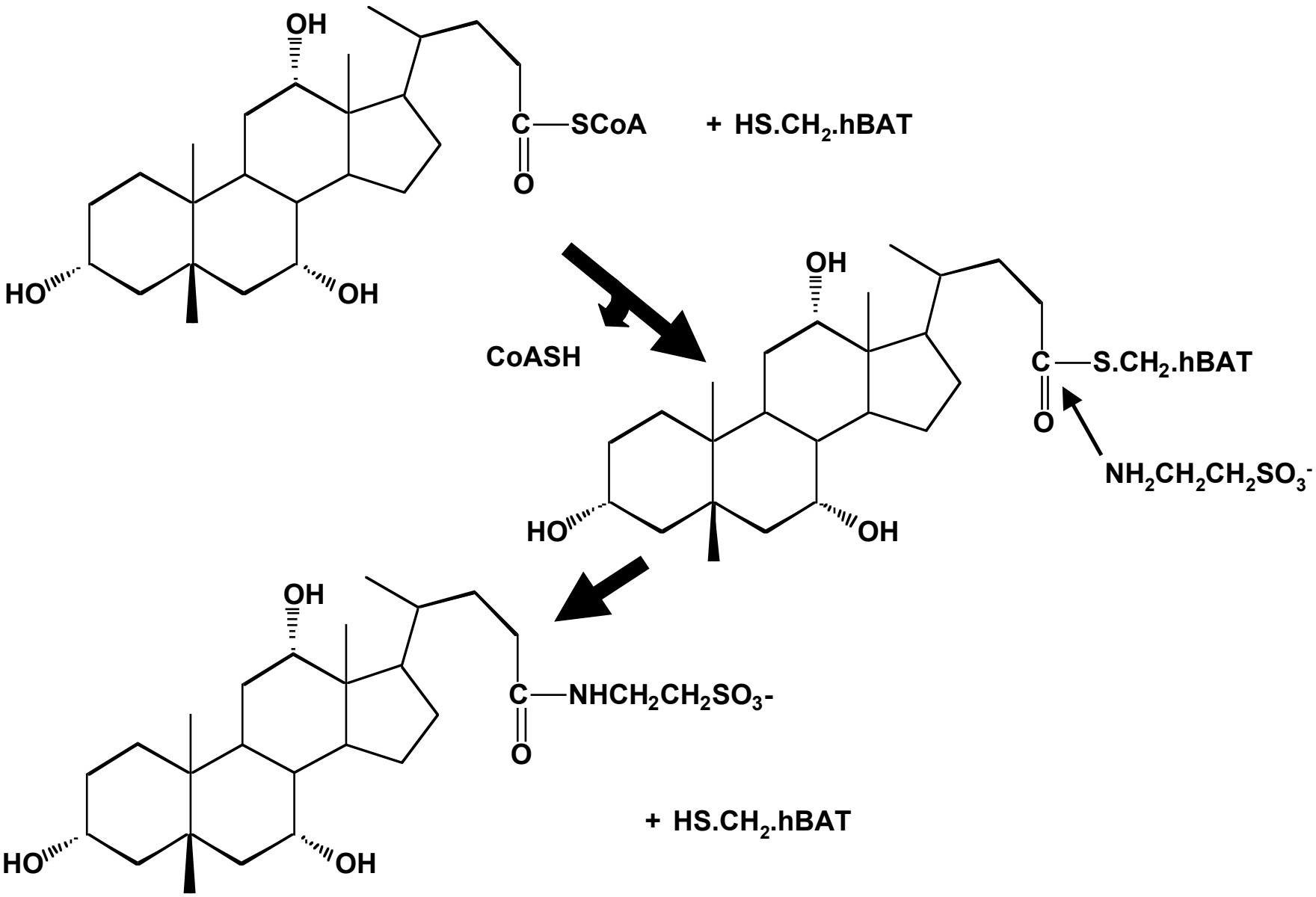


Characterization of BAT

- **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 λgt11 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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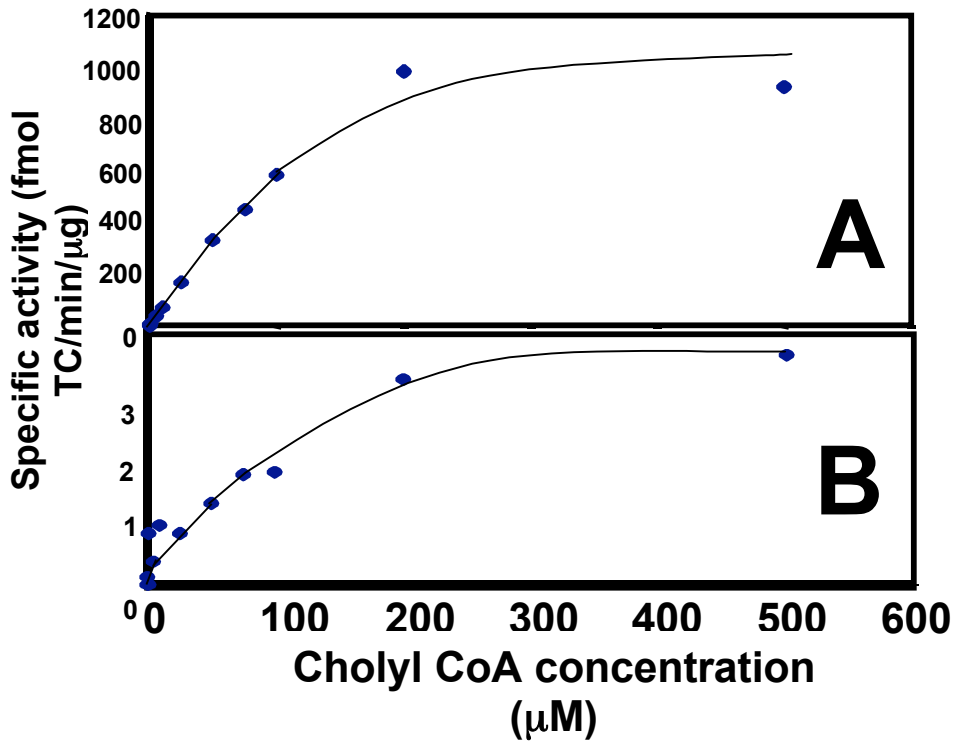
Sequence comparisons of mouse, rat and human BATs

```
                                                                  *235
r   65%  SLLASHGFATLALAYWGYDDLPSRLEKVDLEYFEEGVEFLLRHPKVLGPGVGILSVCIGA 238
m   63%  SLLASRGFATLALAYWNYDDLPSRLEKVDLEYFEEGVEFLLRHPKVLGPGVGILSVCIGA 237
h  100%  SLLASRGFASLALAYHNYEDLPRKPEVTDLEYFEEAANFLLRHPKVFGSGVGVVSVCQGV 238

r        EIGLSMAINLKQITATVLINGPNFVSSNPHVYRGKVFQPTPCSEEFVTTNALGLVEFYRT 298
m        EIGLSMAINLKQIRATVLINGPNFVSQSPHVYHGQVYPPVPSNEEFVVTNALGLVEFYRT 297
h        QIGLSMAIYLKQVTATVLINGTNFPFGIPQVYHGQIHQPLPHSAQLISTNALGLLELYRT 288
                                      *328
r        FEETADKDSKYCFPIEKAHGHFLFVVGEDDKNLNSKVHAKQAIAQLMKSGKKNWTLLSYP 358
m        FQETADKDSKYCFPIEKAHGHFLFVVGEDDKNLNSKVHANQAIAQLMKNGKKNWTLLSYP 357
h        FETTQVGASQYLFPIEEAQGQFLFIVGEGDKTINSKAHAEQAIGQLKRHGKNNWTLLSYP 358
           *362       *372
r       GAGHLIEPPYSPLCSASRMPFVIPSINWGGEVIPH-AA 395
m       GAGHLIEPPYTPLCQASRMPILIPSLSWGGEVIPHSQA 395
h       GAGHLIEPPYSPLCCASTTHDLR--LHWGGEVIPH-AA 393
```

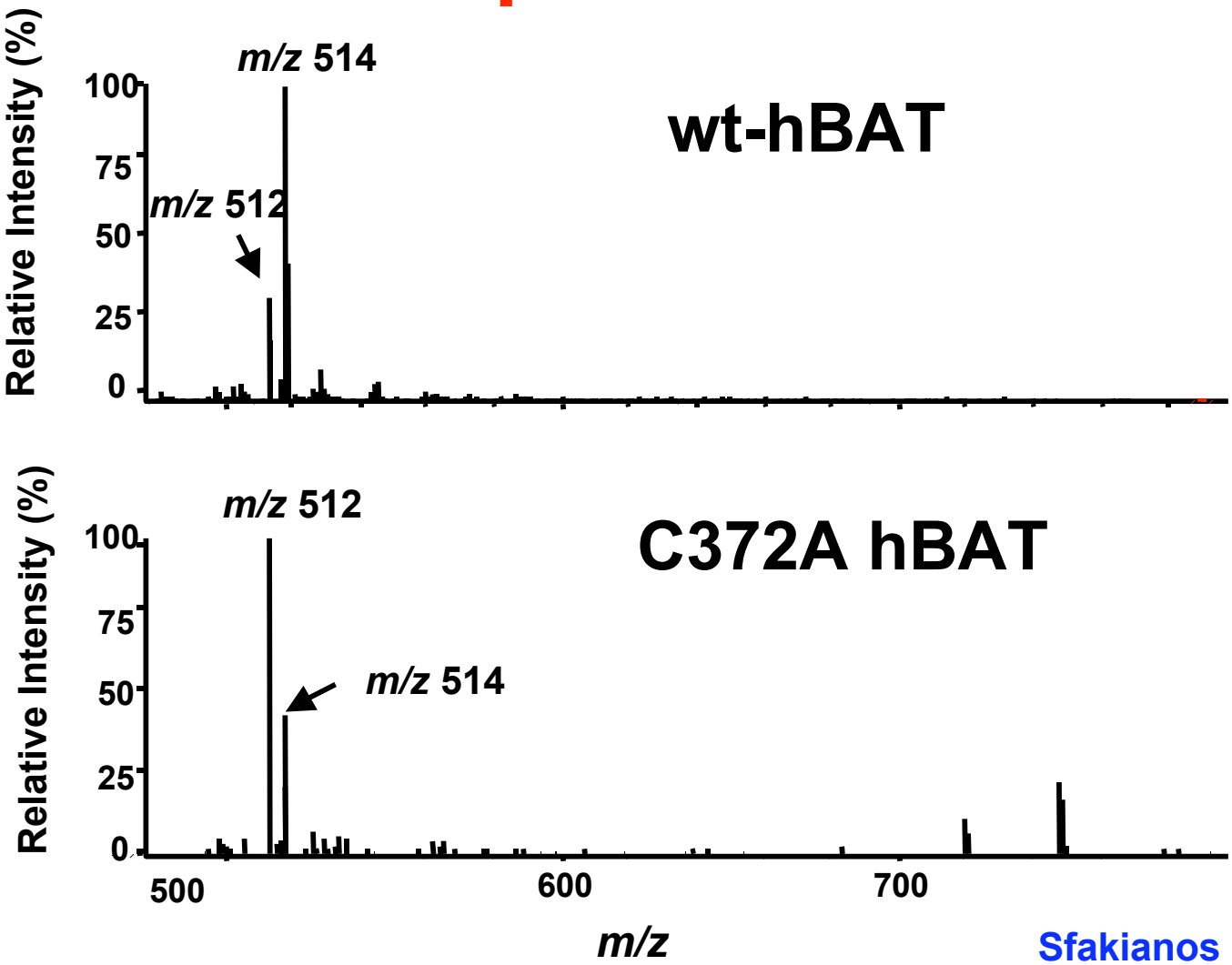
Site-specific Cys mutations

- **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**



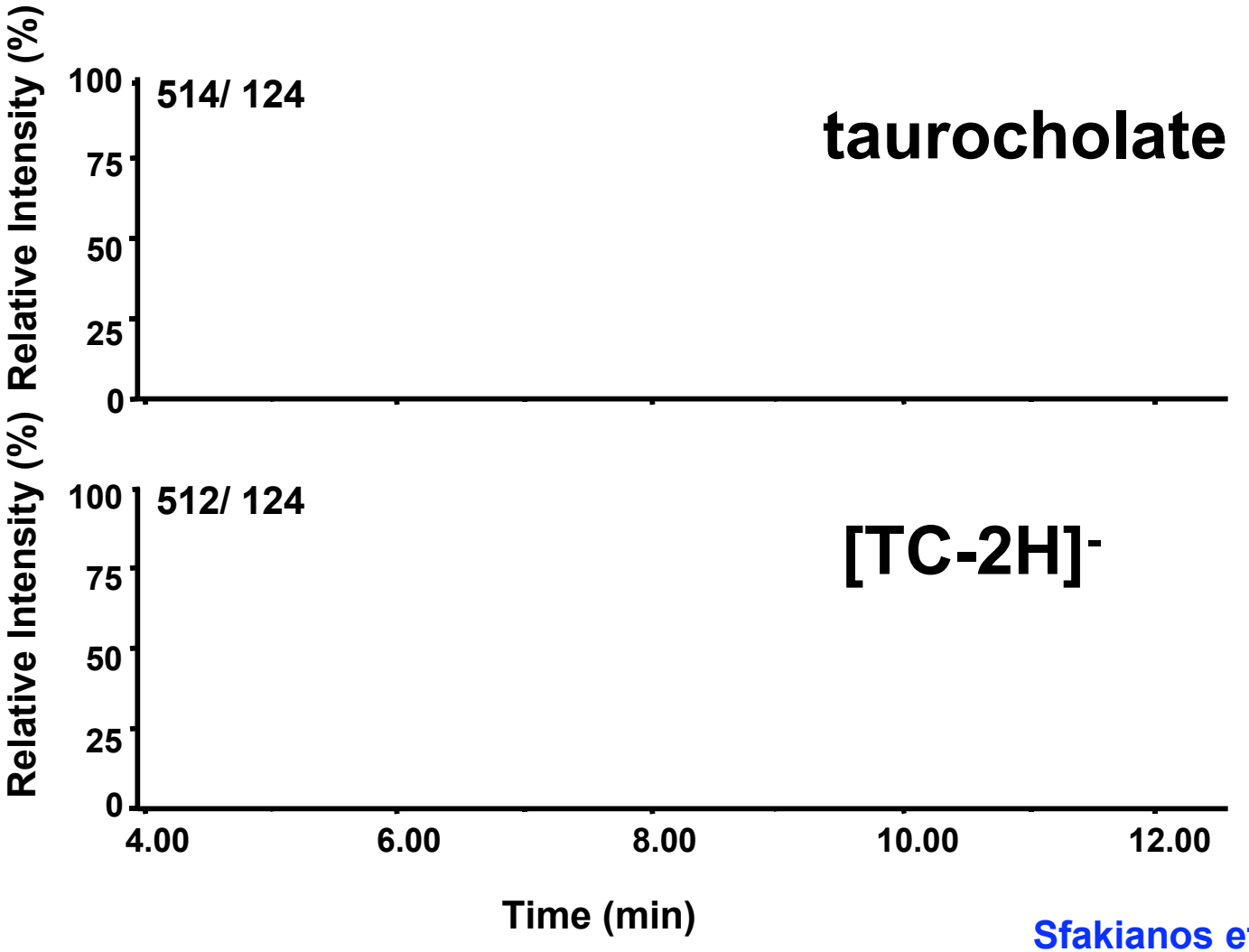
Sfakianos et al.

ESI-mass spectrum of hBAT products



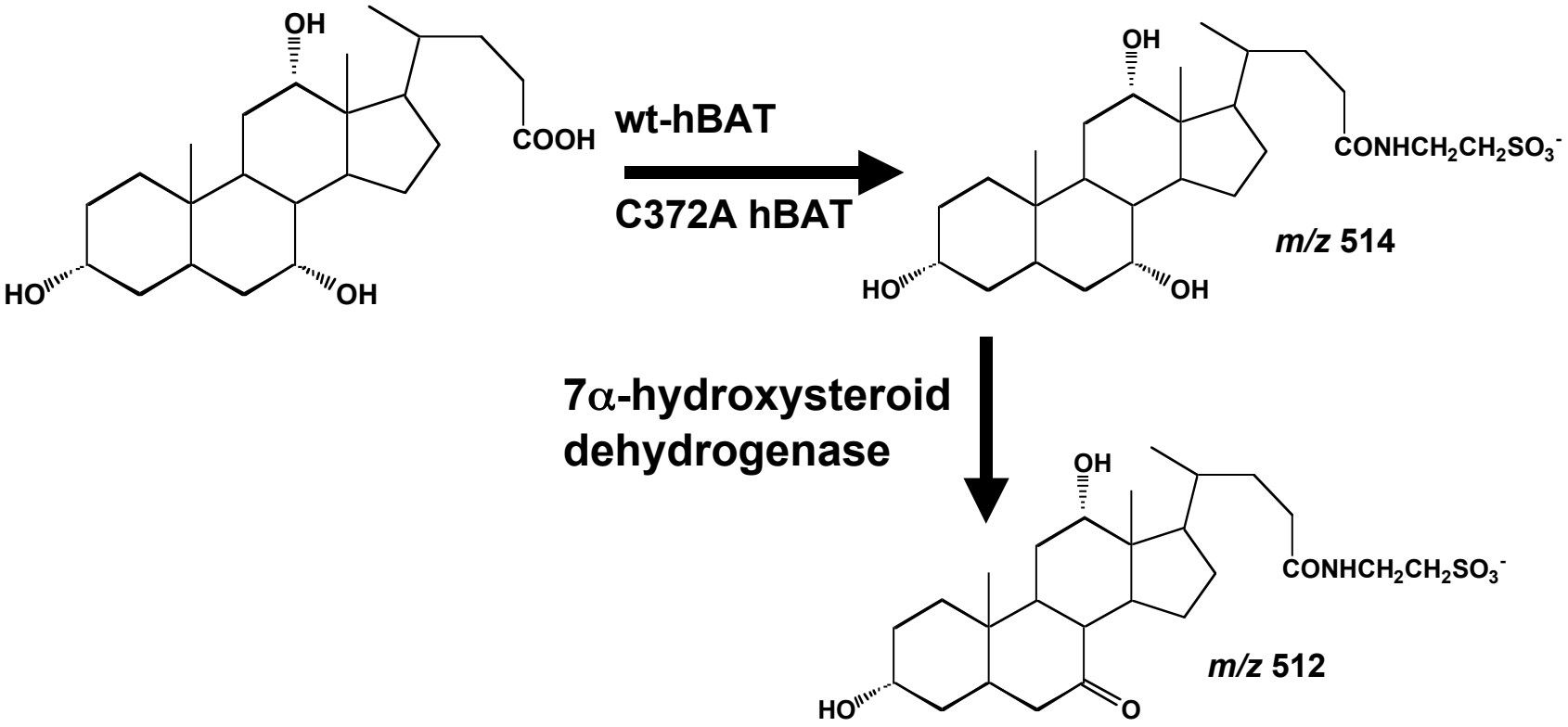
Sfakianos et al.

LC-MS of C372A hBAT product



Sfakianos et al.

Metabolism in *E. coli* expression system



Sfakianos et al.

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

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                                                                  235
kan-1   SLLASHGFATLALAYWGYDDLPSRLEKVDLEYFEEGVEFLLRHPKVLGPGVGILSVCIGA 238
mBAT    SLLASRGFATLALAYWNYDDLPSRLEKVDLEYFEEGVEFLLRHPKVLGPGVGILSVCIGA 237
hBAT    SLLASRGFASLALAYHNYEDLPRKPEVTDLEYFEEAANFLLRHPKVFGSGVGVVSVCQGV 238
MTE-I   SLLAGKGFAVMALAYYNYDDLPKTMETMRIEYFEEAVNYLRGHPEVKGPGIGLLGISKGG 276
CTE-I   SLLAGKGFAVMALAYYNYDDLPKTMETMRIEYFEEAVNYLRGHPEVKGPGIGLLGISKGG 235
CLCTE   SLLAGKGFAVMALAYYNYDDLPKTMETMRIEYFEEAVNYLRGHPEVKGPGIGLLGISKGG 235
PLCTE   SLLAGKGFAVMALAYYNYEDLPKTMETLHLEYFEEAMNYLLSHPEVKGPGVGLLGISKGG 235
PTE-Ia  SLLAGKGFAVMALAYNNYEDLPKDMDIIHLEYFEEAVTYLLSHPQVTGSGVGVLGISKGG 246
DLHp    KPFAEQGYAVLALSYFAAPGLPATAEELPLEYFDRAVAWLAAQPSVDPKAIGVYGVSKGA 138
                                                  328
kan-1   ALGLVEFYR--TFEETAD-KDSKYCFPIEKAHGHFLFVVGEDDKNLNSKVHAKQAIAQLM 345
mBAT    ALGLVEFYR--TFQETAD-KDSKYCFPIEKAHGHFLFVVGEDDKNLNSKVHANQAIAQLM 344
hBAT    ALGLLELYR--TFETTQV-GASQYLFPIEEAQGQFLFIVGEGDKTINSKAHAEQAIGQLK 345
MTE-I   KDGLLDVVE--ALQSPL--VDKKSFIPVERSDTTFLFLVGQDDHNWKSEFYAREASKRLQ 382
CTE-I   KDGLKDVVD--ALQSPL--VEQKSFIPVERSDTTFLFLVGQDDHNWKSEFYANEISKRLQ 341
CLCTE   KDGLKDVVD--ALQSPL--VEQKSFIPVERSDTTFLFLVGQDDHNWKSEFYANEISKRLQ 341
PLCTE   KDGYADIVD--VLNSPLEGPDQKSFIPVERAESTFLFLVGQDDHNWKSEFYANEACKRLQ 343
PTE-Ia  KDGLKDIVD--LLNNPLEGPDQKSLIPVERSDTAFLFLVGQDDHNWKSEFYAREASKRLQ 354
DLHp    SNYMAFIYGLYDTGLKAADAHPQAAIPVEKIHGPVMLISGRADAMWSSSAMSDAVVARLK 258
                        362
kan-1   KSGKK-NWTLLSYPGAGHLIEPPYSPLCSASRMPFVIPSINWGGEVIPH-AA 395
mBAT    KNGKK-NWTLLSYPGAGHLIEPPYTPLCQASRMPILIPSLSWGGEVIPHSQA 395
hBAT    RHGKN-NWTLLSYPGAGHLIEPPYSPLCCASTTHDLR--LHWGGEVIPH-AA 393
MTE-I   AHGKE-KPQIICYPEAGHYIEPPYFPLCSAGMHLLVGANITFGGEPKPH-SV 432
CTE-I   AHGKE-KPQIICYPEAGHYIEPPYFPLCSAGMHLLVGANITFGGEPKPH-SV 391
CLCTE   AHGKE-KPQIICYPEAGHYIEPPYFPLCSAGMHLLVGANITFGGEPKPH-SV 391
PLCTE   AHGRR-KPQIICYPETGHYIEPPYFPLCRASLHALVGSPIIWGGEPRAH-AM 393
PTE-Ia  AHGKE-KPQIICYPETGHHIEPPYFPLCKASLNSLVGGPVIWGGEPRAH-AM 404
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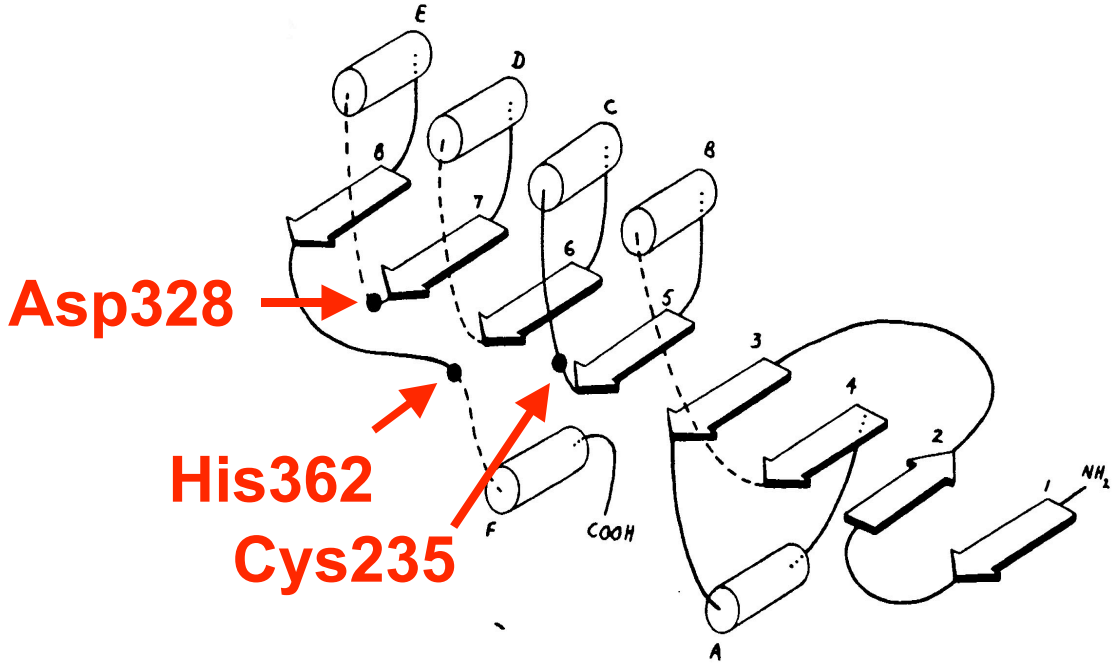
The Protein Structure Modeling of hBAT



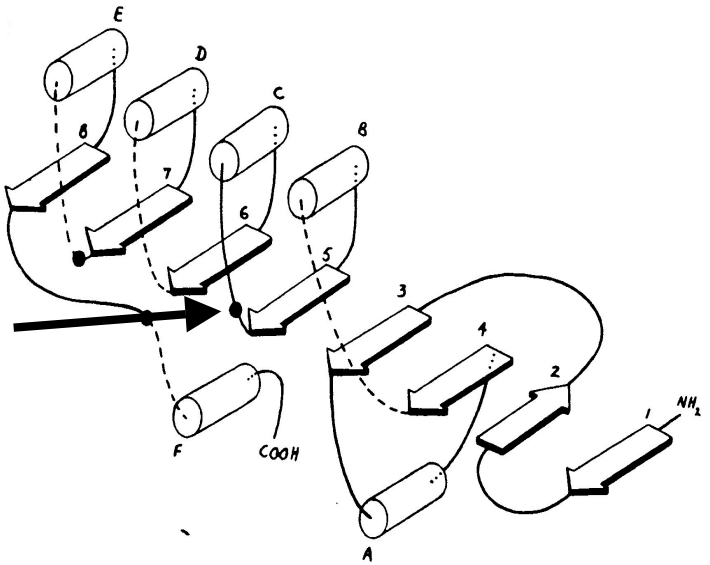
Ollis D.L., Protein Engineering 5(3): pp.197-221.

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

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

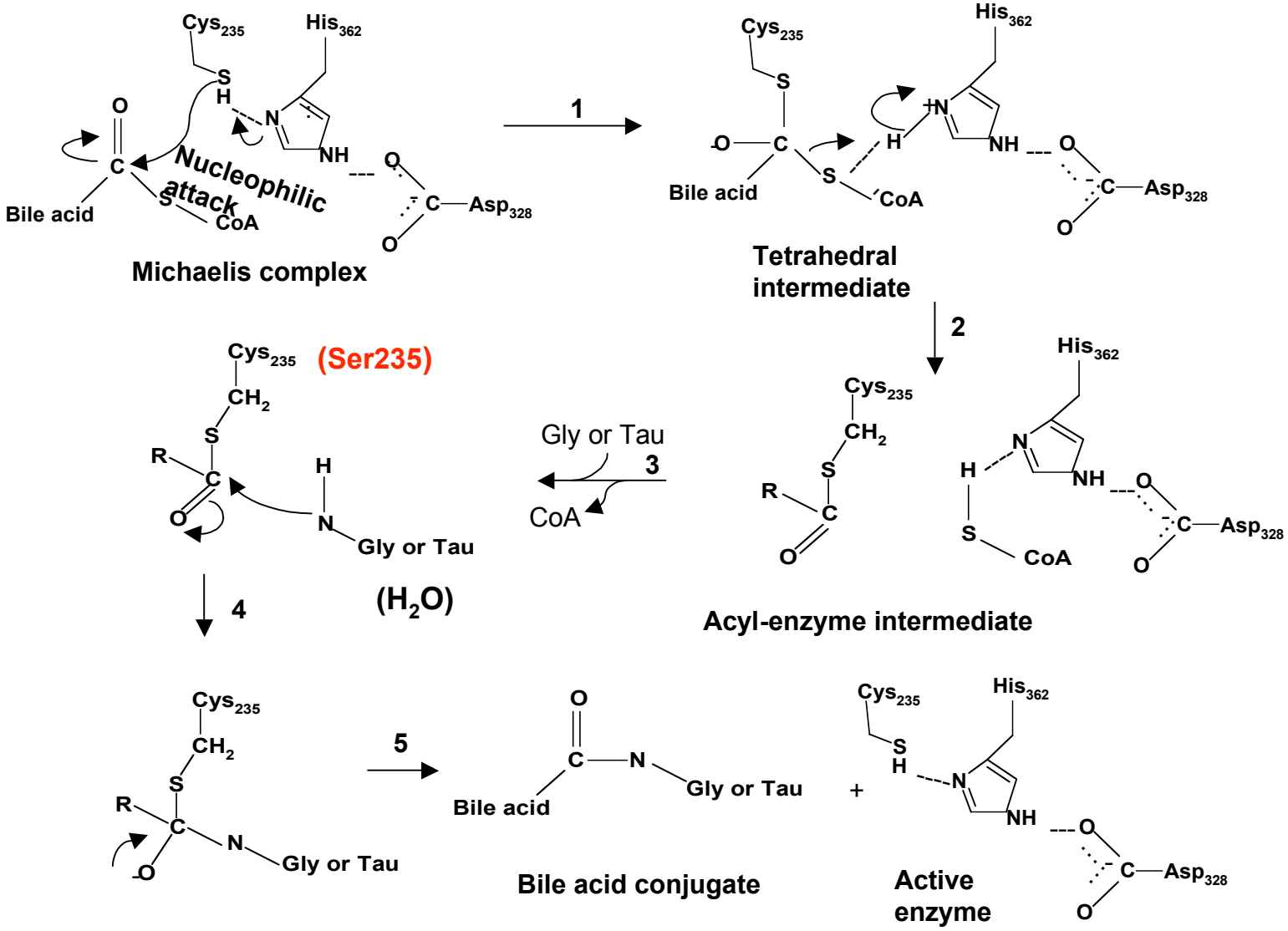
```
                                                                  235
kan-1   SLLASHGFATLALAYWGYDDLPSRLEKVDLEYFEEGVEFLLRHPKVLGPGVGILSVCIGA 238
mBAT    SLLASRGFATLALAYWNYDDLPSRLEKVDLEYFEEGVEFLLRHPKVLGPGVGILSVCIGA 237
hBAT    SLLASRGFASLALAYHNYEDLPRKPEVTDLEYFEEAANFLLRHPKVFGSGVGVVSVCQGV 238
MTE-I   SLLAGKGFAVMALAYYNYDDLPKTMETMRIEYFEEAVNYLRGHPEVKGPGIGLLGISKGG 276
CTE-I   SLLAGKGFAVMALAYYNYDDLPKTMETMRIEYFEEAVNYLRGHPEVKGPGIGLLGISKGG 235
CLCTE   SLLAGKGFAVMALAYYNYDDLPKTMETMRIEYFEEAVNYLRGHPEVKGPGIGLLGISKGG 235
PLCTE   SLLAGKGFAVMALAYYNYEDLPKTMETLHLEYFEEAMNYLLSHPEVKGPGVGLLGISKGG 235
PTE-Ia  SLLAGKGFAVMALAYNNYEDLPKDMDIIHLEYFEEAVTYLLSHPQVTGSGVGVLGISKGG 246
DLHp    KPFAEQGYAVLALSYFAAPGLPATAEELPLEYFDRAVAWLAAQPSVDPKAIGVYGVSKGA 138
```

? Nucleophile
Cys235→Ser

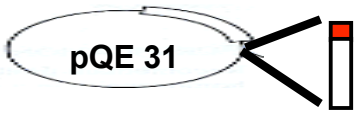


Stepher

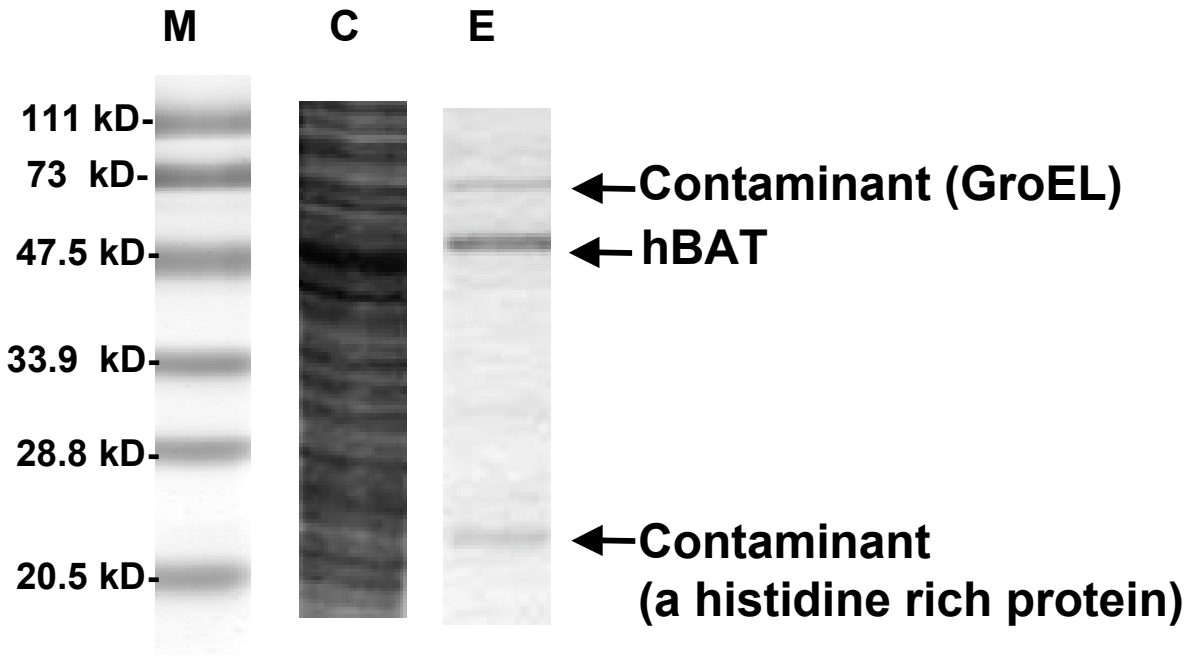
Charge Relay Mechanism shared by hBAT, thioesterases, and a large group of hydrolases



Sfakianos, JBC 277:47270

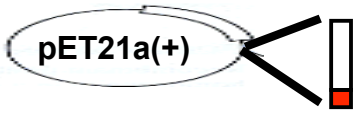


Purification of hBAT with 6x His-tag

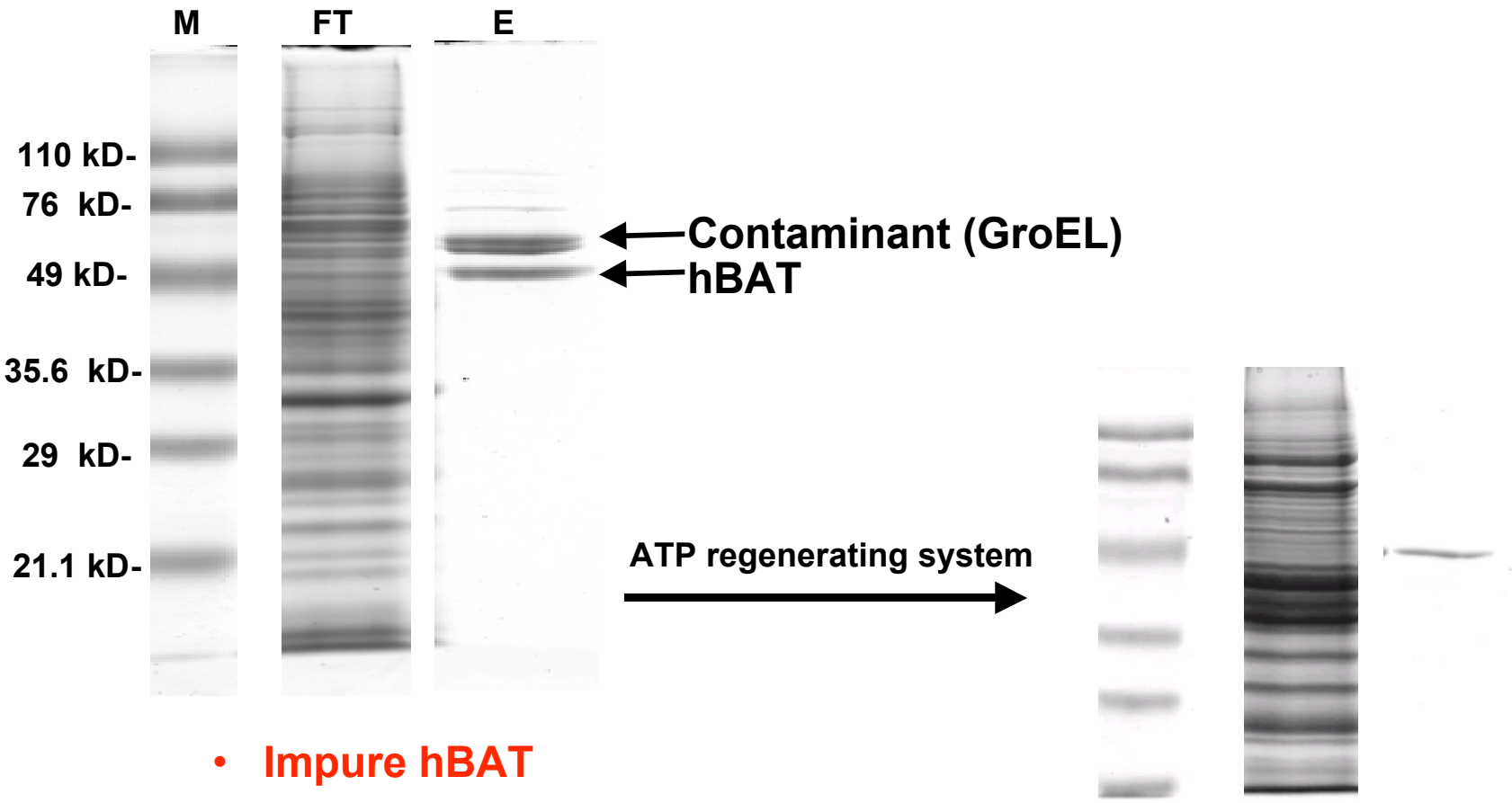


- Impure hBAT
- Inactivated by imidazole in elution buffer

Sfakianos et al.



Purification of hBAT with Avi-tag



- **Impure hBAT**

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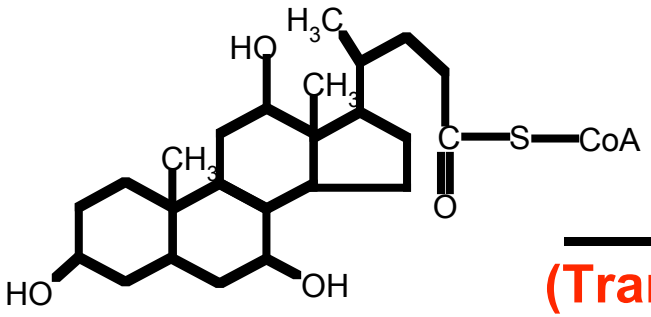
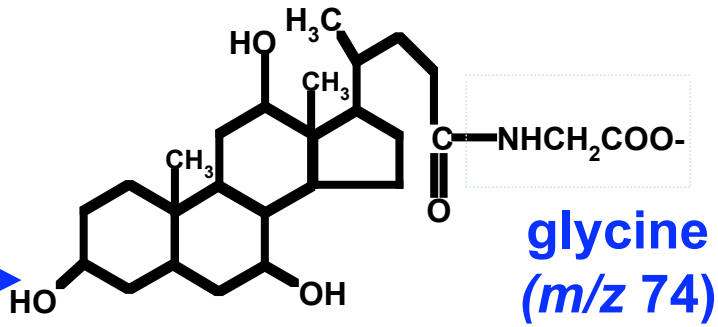


cholyl CoA

hBAT

(Transferase)

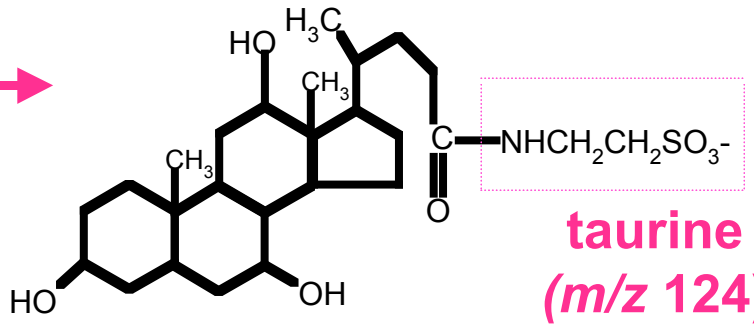
glycine



glycocholate
m/z 464

glycine
(m/z 74)

taurine

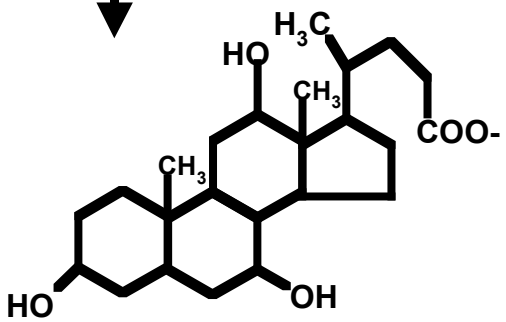


taurocholate
m/z 514

taurine
(m/z 124)

hBAT

(Thioesterase)



cholic acid
m/z 407

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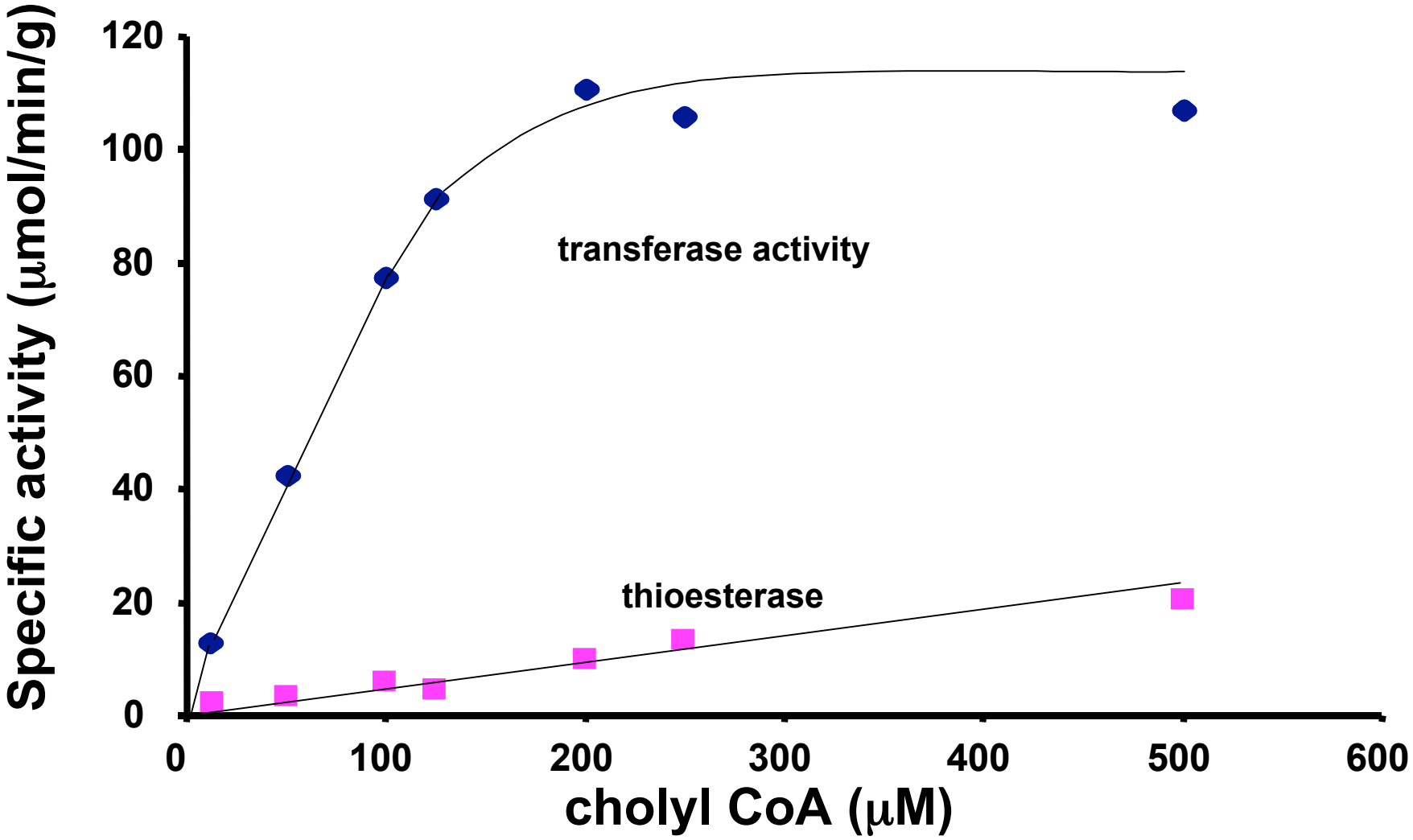
Kinetics of transferase and thioesterase activities of wild-type hBAT with glycine



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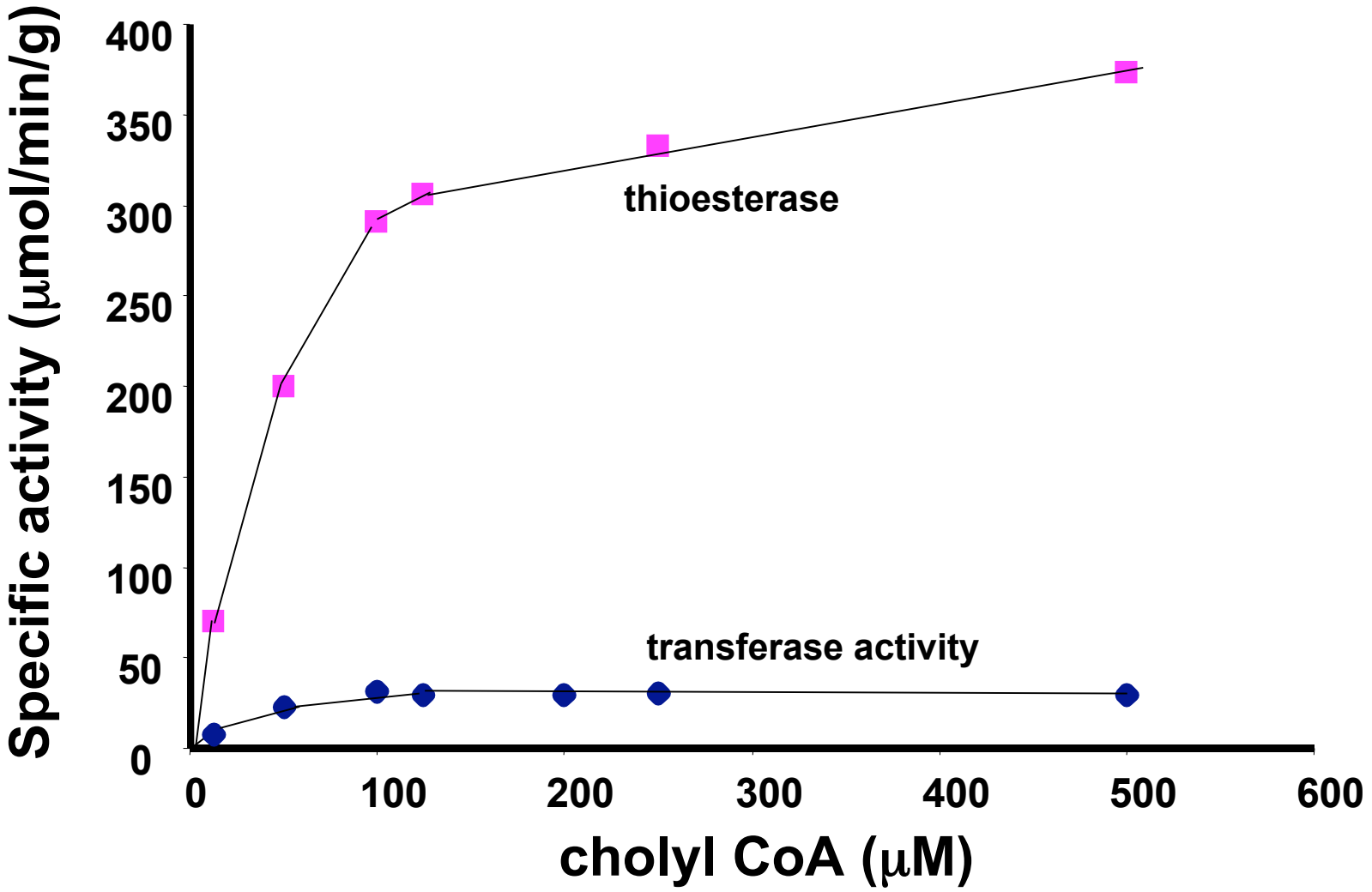
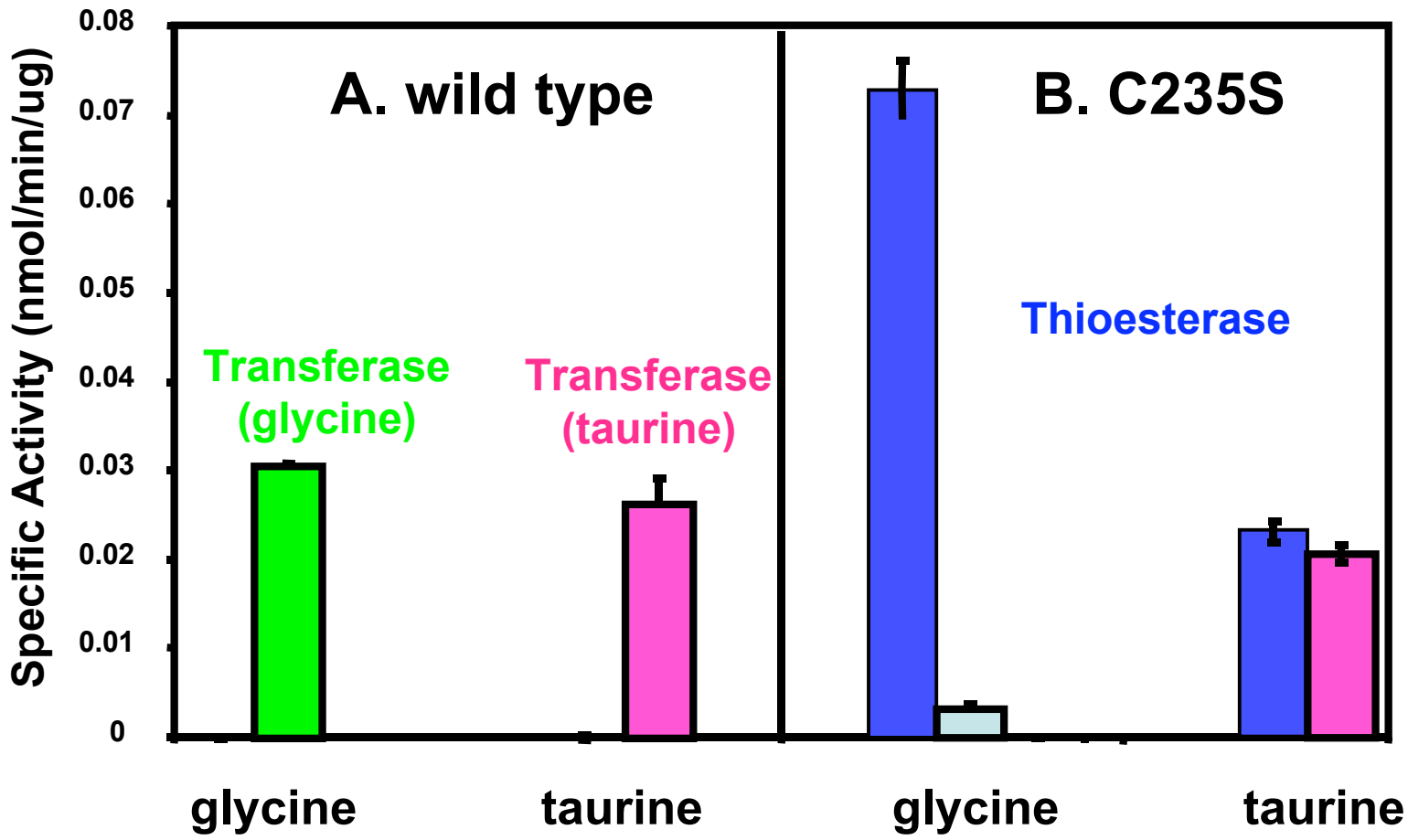
Kinetics of transferase and thioesterase activities of C235S hBAT variant with glycine



Sfakianos et al.

LC-ESI-MS-MRM Analysis of Reaction Products



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**