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/Tyler Stewart

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

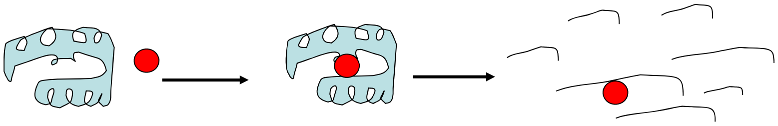
- **Enzymes often undergo posttranslational modifications in order to be active under the conditions in a cell**
  - **for example, many enzymes in the signal transduction pathways are activated by phosphorylation on serine, threonine and tyrosine residues**
    - **EGF receptor (tyrosine kinase), TGF 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 (this is coming up in Peter Prevelige's lectures)**
- **simulation of phosphorylation may be necessary by mutating serine and threonine groups to aspartate and glutamate, respectively**

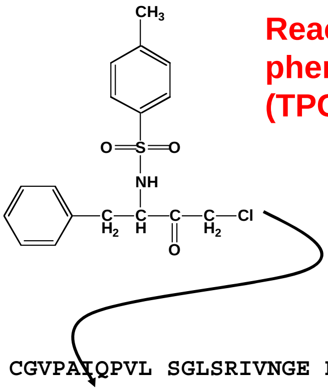
MS of enzymes



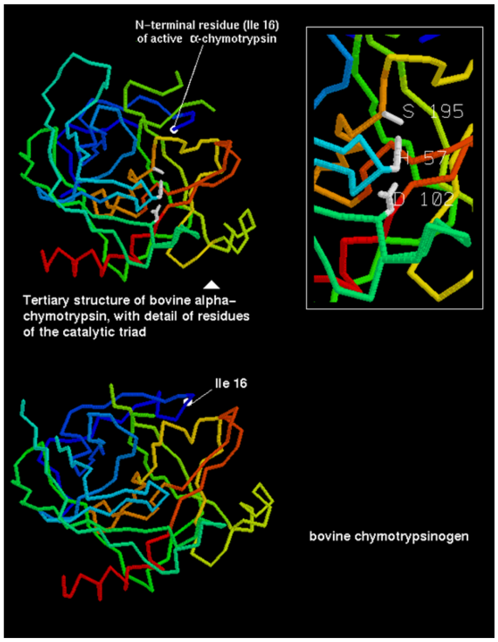
Enzymes can be inactivated by suicide substrates - these come into the active site and undergo a covalent reaction, thereby blocking the approach of other substrate molecules

- **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
```

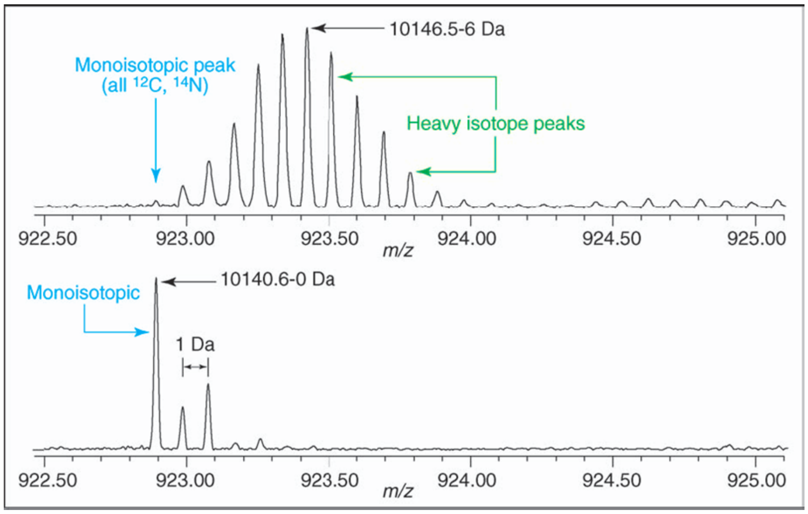
Glu-C

```
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

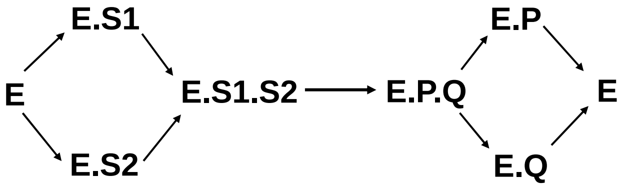
Mass spectrometry and enzyme-catalyzed reactions

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



$$\begin{tikzcd}
 & E.S1 \arrow[dr] & & & E.P \arrow[dr] & \\
E \arrow[ur] \arrow[dr] & & E.S1.S2 \arrow[r] & E.P.Q \arrow[ur] \arrow[dr] & & E \\
 & E.S2 \arrow[ur] & & & E.Q \arrow[ur] &
\end{tikzcd}$$

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

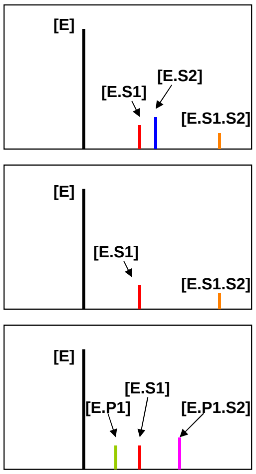
Mass spectrometry and enzyme-catalyzed reactions

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

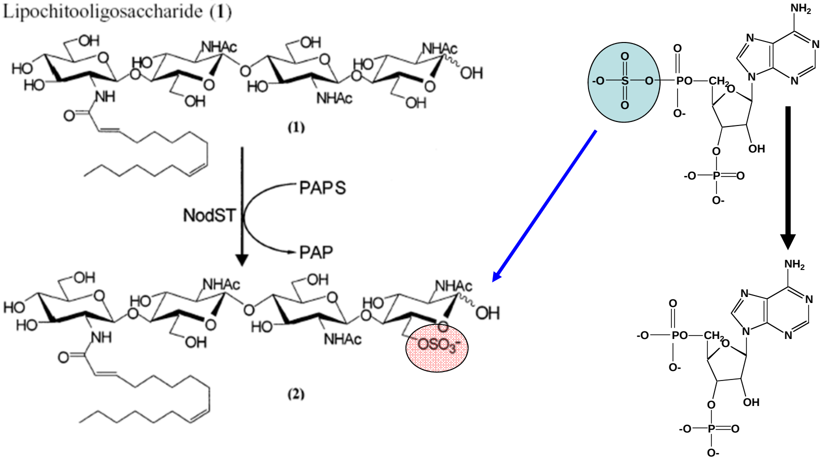


Mass spectrometry and substrates and products of enzyme reactions

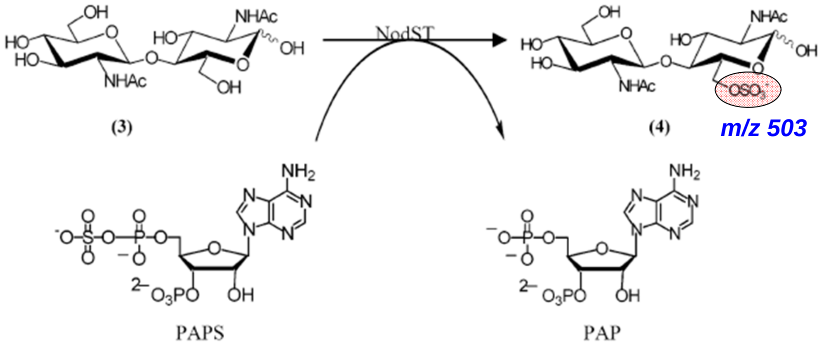
- Most enzyme reactions are studied by measuring the appearance of a product or (more rarely) the disappearance of a substrate
- If the substrate or product has a unique absorbance or fluorescence, the reaction can be followed in real time
- Some substrates have no usable absorbance or fluorescence -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



m/z 503

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

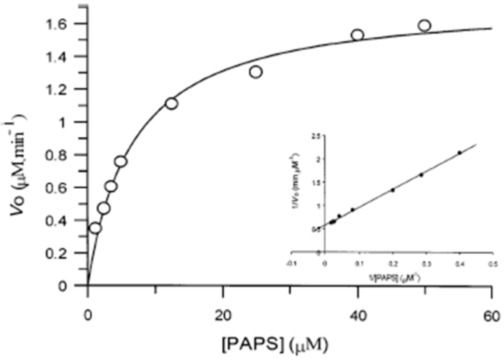


FIGURE 3: Saturation plot of V_0 vs [PAPS]. The inset is a double-reciprocal plot of $1/V_0$ vs 1/[PAPS] ([PAPS] = 1.25, 2.5, 3.5, 5, 12.5, 25, 40, and 50 μM, [chistobiose] = 1 mM, [NodST] = 90 nM, and pH 8.0).

Inhibition of ST by PAP using ESI-MS

Pi et al., Biochemistry 41:13283

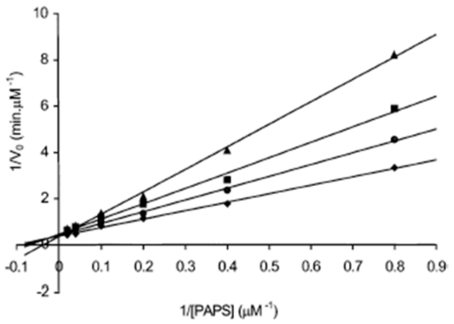
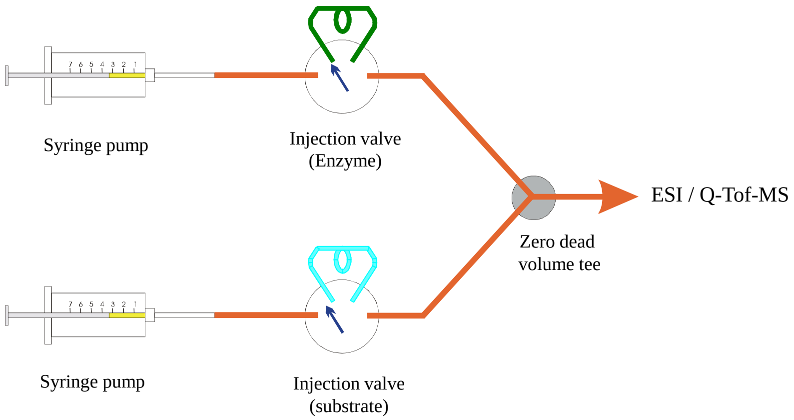


FIGURE 5: Double-reciprocal plot of $1/V_0$ vs 1/[PAPS] at different PAP concentrations: 0 µM PAP (▲), 0.75 µM PAP (■), 1.5 µM PAP (●), and 3.0 µM PAP (◆) ([PAPS] = 1.25, 2.5, 5, 10, 25, and 50 µM, [chitobiose] = 1 mM, [NodST] = 90 nM, and pH 8.0).

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


Syringe pump

Injection valve
(Enzyme)

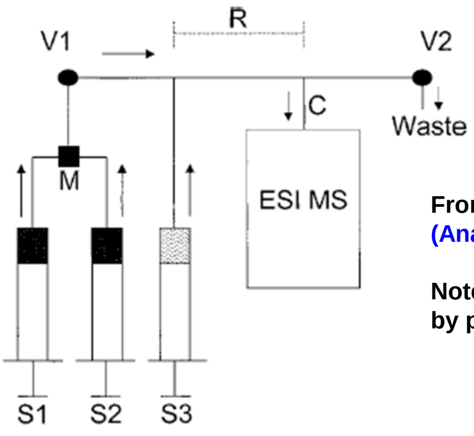
ESI / Q-Tof-MS

Zero dead
volume tee

Syringe pump

Injection valve
(substrate)

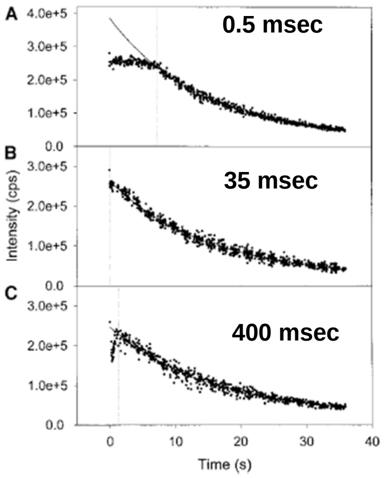
Stopped flow set up



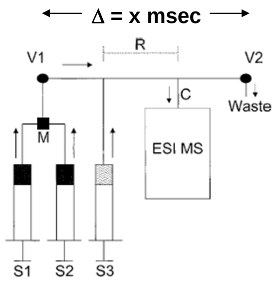
From **Kolakowski and Konermann (Anal Biochem 292:107)**

Note the additional flow introduced by pump S3

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

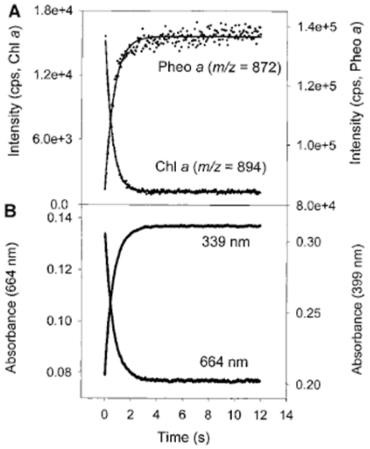


In this reaction, hydrolysis of acetylcholine in an alkaline buffer is monitored by the ion at *m/z* 146



Kolakowski and Konermann (Anal Biochem 292:107)

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

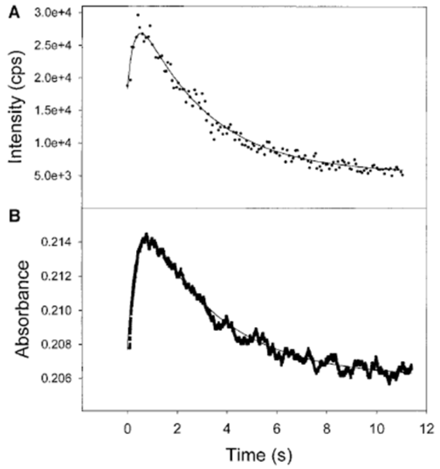


These data are from the conversion of chlorophyll A to pheophytin A (loss of Mg and gain of two protons).

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**

Studying multiple enzymes simultaneously

- Old style of research
  - Measure one purified enzyme at a time
- New style of research
  - Measure all the enzymes at the same time
  - Equivalent to study of a signaling pathway (many are phosphorylation steps)

Drug analysis in industry

- The reality is that drug targeting and drug development eventually encounter the realities of metabolism
- A major activity in the post discovery phase is to determine the metabolic reactivity of the drug, particularly the cytochrome P450 system

Multiplexed analysis of the drug metabolizing enzymes

Approach	Advantages	Disadvantages
Cocktail strategy	Several activities in a single experiment Reduced number of samples Less time and expense Enhanced throughput Rapid microsomal characterization Rapid phenotype of tissues	Probe-probe interaction Ion suppression Metabolism overlapping
Individual strategy	Selective CYP activity Avoids overlapping metabolism Avoids probe-probe interaction	Large number of strategies Time consuming analysis

Lahoz et al., 2008

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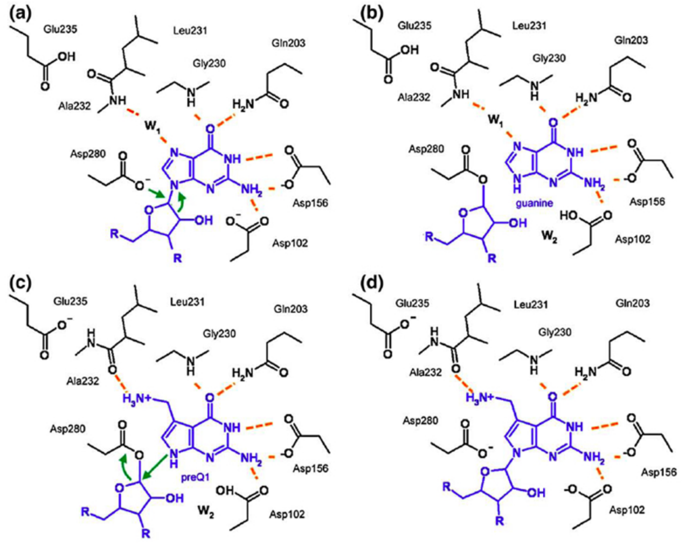
tRNA-guanine transglycosylase

- **This enzyme (Tgt) modifies tRNAs and may be a target for antibiotics**
- **The enzyme forms a dimer with tRNA**
  - **Can we observe the dimer using mass spectrometry?**
  - **Can we explore the dimer interface by carrying out mutations of the Tgt part of the interface?**

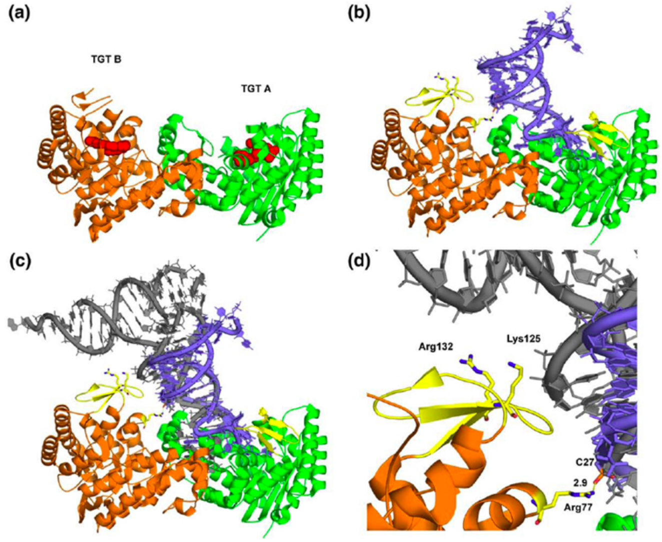
Ritschel et al. J Mol Biol (393:833-847, 2009)

Tgt enzyme reaction



Stephen Barnes BMG 744 02-13-13

Structures of Tgt complex



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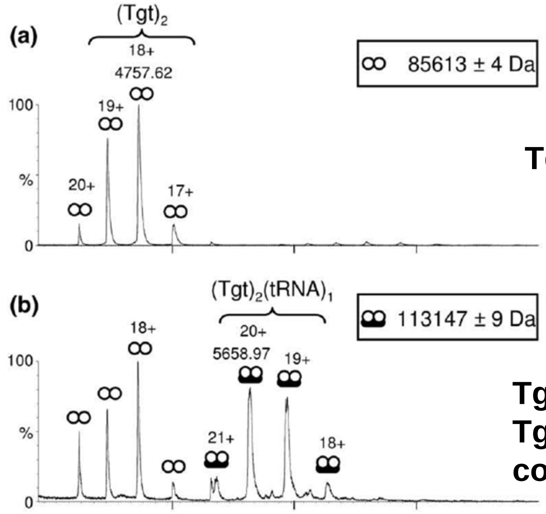
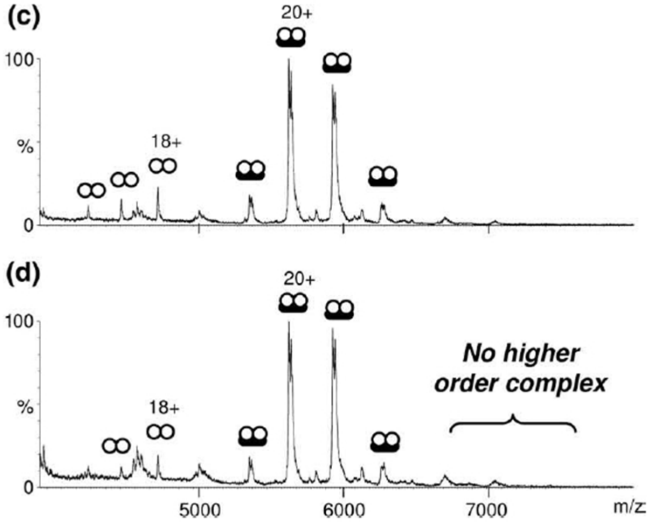
Non-covalent ESI-MS of Tgt



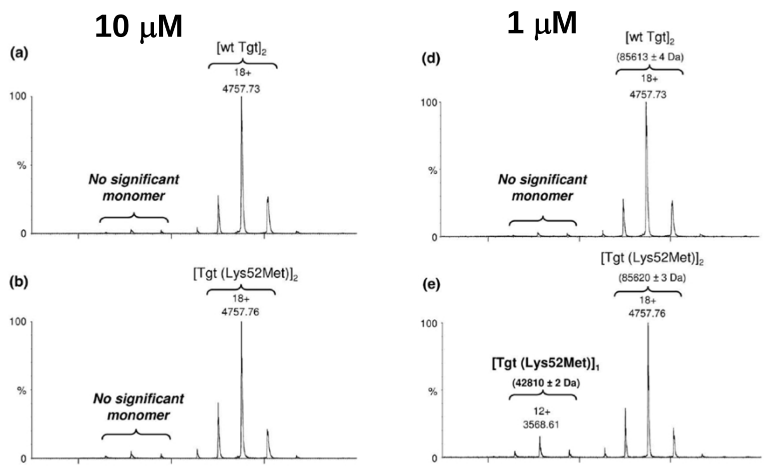
Tgt dimer

Tgt dimer and Tgt-tRNA complex

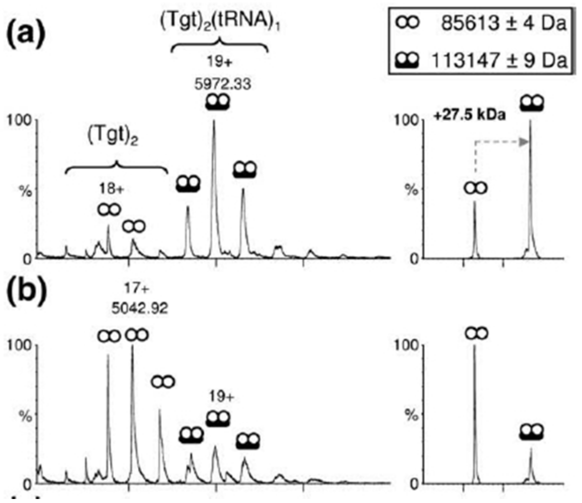
Adding more tRNA does not lead to higher order complexes



Evidence for Tgt dimer dissociation in Lys52Met mutant



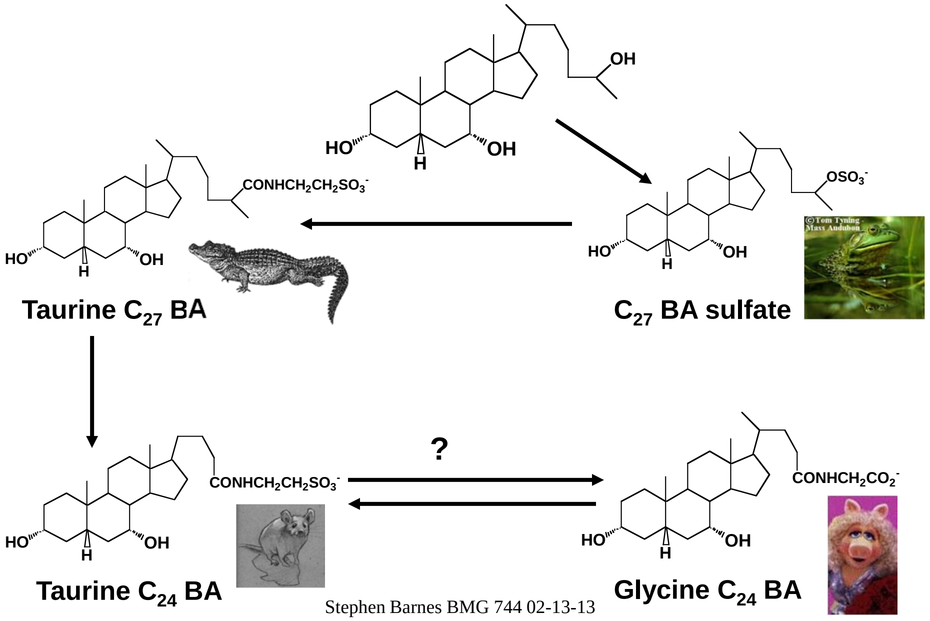
Effect of an inhibitor on formation of Tgt dimer-tRNA complex



Conclusions about non-covalent complexes by ESI-MS

- The physical state of the enzyme with its substrates and inihibitors can be readily observed and quantified by ESI-MS
- Requires sufficient protein (in the Ritschel study they used 1-10 μM – for a 25 kDa protein, this would be 25-250 mg/L or 25-250 μg/ml)
- The spraying medium is important – in this case 500 mM ammonium acetate, pH 8

Evolution of bile acid conjugation



Taurine C_{27} BA

C_{27} BA sulfate

Taurine C_{24} BA

Glycine C_{24} BA

Bile acid N-acylamidate formation (in hepatocytes)

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

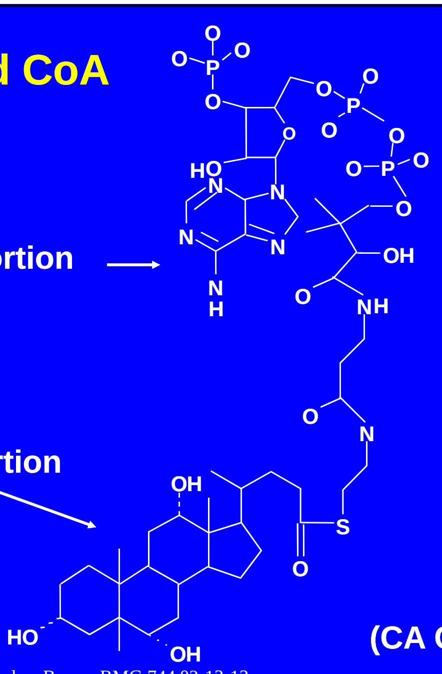
$$\begin{tikzcd}\text{Bile acid-SCoA + amino acids} \arrow[r, "\text{BAT}"] & \text{Bile acid amidate} \\ & \text{CoASH} \arrow[from=1-1, to=2-2, phantom] \end{tikzcd}$$

$\downarrow$ CoASH

Bile acid CoA

Hydrophilic portion (CoA)

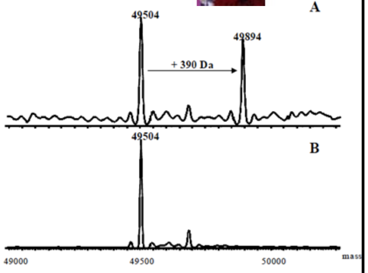
Hydrophobic portion (Bile acid)

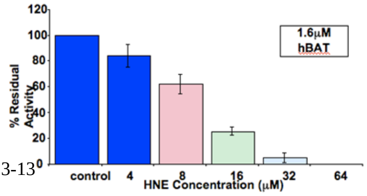


(CA CoA)

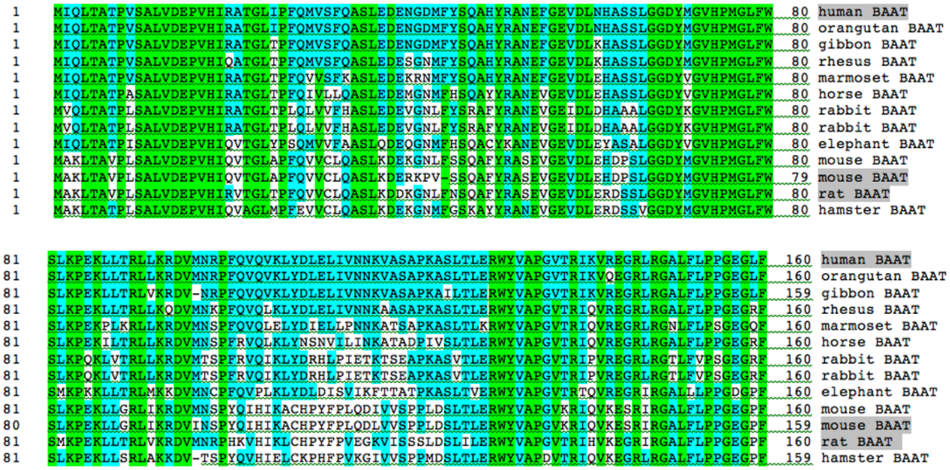
Background

- Bile acid CoA:amino acid N-acyltransferase (hBAT) catalyzes the conjugation of bile acid thioesters with glycine and taurine
- It has a Ping-Pong reaction mechanism
  - This involves the formation of a covalent intermediate between the bile acid and hBAT
  - The covalent intermediate is sufficiently stable that it can be observed by electrospray ionization mass spectrometry
  - It has a Cys-Asp-His catalytic triad
- hBAT is inactivated by 4-hydroxynonenal in a dose-dependent manner (Shonsey et al. 2007)
  - Both the active site Cys and His residues form Michael adducts
  - Several other His and Lys residues are altered by 4HNE





BAAT homologies



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1    MIQLTATPVSALVDEPVHIRATGLIPFQMVSFQASLEDENGDMFYSQAHYRANEFGEVDLNHASSLGGDYMGVHPMGLFW  80 human BAAT
1    MIQLTATPVSALVDEPVHIRATGLIPFQMVSFQASLEDENGDMFYSQAHYRANEFGEVDLNHASSLGGDYMGVHPMGLFW  80 orangutan BAAT
1    MIQLTATPVSALVDEPVHIRATGLTPFQMVSFQASLEDENGDMFYSQAHYRANEFGEVDLKHASSLGGDYMGVHPMGLFW  80 gibbon BAAT
1    MIQLTATPVSALVDEPVHIQATGLTPFQMVSFQASLEDESGNMFYSQAHYRANEFGEVDLKHASSLGGDYMGVHPMGLFW  80 rhesus BAAT
1    MIQLTATPVSALVDEPVHIRATGLTPFQVVSFKASLEDEKRNMFYSQAHYRANEFGEVDLEHASSLGGDYVGVHPMGLFW  80 marmoset BAAT
1    MIQLTATPASALVDEPVHIRATGLTPFQIVLLQASLEDEMGNMFHSQAYYRANEVGEVDLEHASSLGGDYVGVHPMGLFW  80 horse BAAT
1    MVQLTATPLSALVDEPVHIRATGLTPLQLVVFHASLEDEVGNLFYSRAFYRANEVGEIDLDHAAALGGDYKGVHPMGLFW  80 rabbit BAAT
1    MVQLTATPLSALVDEPVHIRATGLTPLQLVVFHASLEDEVGNLFYSRAFYRANEVGEIDLDHAAALGGDYKGVHPMGLFW  80 rabbit BAAT
1    MIQLTATPISALVDEPVHIQVTGLYPSQMVVFAASLQDEQGNMFHSQACYKANEVGEVDLEYASALGGDYVGVHPMGLFW  80 elephant BAAT
1    MAKLTAVPLSALVDEPVHIQVTGLAPFQVVCLQASLKDEKGNLFSSQAFYRASEVGEVDLEHDPSLGGDYMGVHPMGLFW  80 mouse BAAT
1    MAKLTAVPLSALVDEPVHIQVTGLAPFQVVCLQASLKDERKPV-SSQAFYRASEVGEVDLEHDPSLGGDYMGVHPMGLFW  79 mouse BAAT
1    MAKLTAVPLSALVDEPVHIRVTGLTPFQVVCLQASLKDDKGNLFNSQAFYRASEVGEVDLERDSSLGGDYMGVHPMGLFW  80 rat BAAT
1    MAKLTATPLSALVDEPVHIQVAGLMPFEVVCLQASLKDEKGNMFGSKAYYRANEVGEVDLERDSSVGGDYMGVHPMGLFW  80 hamster BAAT

81  SLKPEKLLTRLLKRDVMNRPFQVQVKLYDLELIVNNKVASAPKASLTLERWYVAPGVTRIKVREGRLRGALFLPPGEGLF  160 human BAAT
81  SLKPEKLLTRLLKRDVMNRPFQVQVKLYDLELIVNNKVASAPKASLTLERWYVAPGVTRIKVQEGRLRGALFLPPGEGLF  160 orangutan BAAT
81  SLKPEKLLTRLVKRDV-NRPFQVQVKLYDLELIVNNKVASAPKAILTLERWYVAPGVTRIKVREGRLRGALFLPPGEGLF  159 gibbon BAAT
81  SLKPEKLLTRLLKQDVMNKPFQVQLKLYDLELIVNNKAASAPKASLTLERWYVAPGVTRIQVREGRLRGALFLPPGEGRF  160 rhesus BAAT
81  SLKPEKPLKRLLKRDVMNSPFQVQLELYDIELLPNNKATSAPKASLTLKRWYVAPGVTRIQVREGRLRGNLFLPSGEGQF  160 marmoset BAAT
81  SLKPEKILTRLLKRDVMNSPFRVQLKLYNSNVILINKATADPIVSLTLERWYVAPGVTRIQVREGRLRGALFLPPGEGRF  160 horse BAAT
81  SLKPQKLVTRLLKRDVMTSPFRVQIKLYDRHLPIETKTSEAPKASVTLERWYVAPGVTRIPVREGRLRGTLFVPSGEGRF  160 rabbit BAAT
81  SLKPQKLVTRLLKRDVMTSPFRVQIKLYDRHLPIETKTSEAPKASVTLERWYVAPGVTRIPVREGRLRGTLFVPSGEGRF  160 rabbit BAAT
81  SMKPKKLLTRLMKKDVMNCPFQVPLKLYDLDISVIKFTTATPKASLTVERWYVAPGVTRTQVREGRIRGALLLPPGDGPF  160 elephant BAAT
81  SLKPEKLLGRLIKRDVMNSPYQIHIKACHPYFPLQDIVVSPPLDSLTLERWYVAPGVKRIQVKESRIRGALFLPPGEGPF  160 mouse BAAT
80  SLKPEKLLGRLIKRDVINSPYQIHIKACHPYFPLQDLVVSPPLDSLTLERWYVAPGVKRIQVKESRIRGALFLPPGEGPF  159 mouse BAAT
81  SMKPEKLLTRLVKRDVMNRPHKVHIKLCHPYFPVEGKVISSSLDSLILERWYVAPGVTRIHVKEGRIRGALFLPPGEGPF  160 rat BAAT
81  SLKPEKLLSRLAKKDV-TSPYQVHIELCKPHFPVKGIVVSPPMDSLTLERWYVAPDVTRIQVKEGRLRGALFLPPGEGPF  159 hamster BAAT
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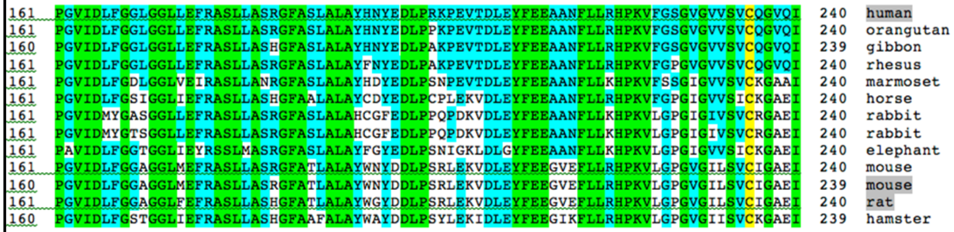
BAAT homologies



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161  PGVIDLFGGLGGLLEFRASLLASRGFASLALAYHNYEDLPRKPEVTDLEYFEEAANFLLRHPKVFGSGVGVVSVCQGVQI  240  human
161  PGVIDLFGGLGGLLEFRASLLASRGFASLALAYHNYEDLPPKPEVTDLEYFEEAANFLLRHPKVFGSGVGVVSVCQGVQI  240  orangutan
160  PGVIDLFGGLGGLLEFRASLLASHGFASLALAYHNYEDLPAKPEVTDLEYFEEAANFLLRHPKVFGSGVGVVSVCQGVQI  239  gibbon
161  PGVIDLFGGLGGLLEFRASLLASRGFASLALAYFNYEDLPAKPEVTDLEYFEEAANFLLRHPKVFGPGVGVVSVCQGVQI  240  rhesus
161  PGVIDLFGDLGGLVEIRASLLANRGFASLALAYHDYEDLPSNPEVTDLEYFEEAANFLLKHPKVFSSGIGVVSVCKGAAI  240  marmoset
161  PGVIDLFGSIGGLIEFRASLLASHGFAALALAYCDYEDLPCPLEKVDLEYFEEAANFLLRHPKVFGPGIGVVSICKGAEI  240  horse
161  PGVIDMYGASGGLLEFRASLLASRGFASLALAHCGFEDLPPQPDKVDLEYFEEAANFLLKHPKVLGPGIGIVSVCRGAEI  240  rabbit
161  PGVIDMYGTSGGLLEFRASLLASRGFASLALAHCGFEDLPPQPDKVDLEYFEEAANFLLKHPKVLGPGIGIVSVCRGAEI  240  rabbit
161  PAVIDLFGGTGGLIEYRSSLMASRGFASLALAYFGYEDLPSNIGKLDLGYFEEAANFLLKHPKVLGPGIGVVSICKGAEI  240  elephant
161  PGVIDLFGGAGGLMEFRASLLASRGFATLALAYWNYDDLPSRLEKVDLEYFEEGVEFLLRHPKVLGPGVGILSVCIGAEI  240  mouse
160  PGVIDLFGGAGGLMEFRASLLASRGFATLALAYWNYDDLPSRLEKVDLEYFEEGVEFLLRHPKVLGPGVGILSVCIGAEI  239  mouse
161  PGVIDLFGGAGGLFEFRASLLASHGFATLALAYWGYDDLPSRLEKVDLEYFEEGVEFLLRHPKVLGPGVGILSVCIGAEI  240  rat
160  PGVIDLFGSTGGLIEFRASLLASHGFAAFALAYWAYDDLPSYLEKIDLEYFEEGIKFLLRHPKVLGPGVGIISVCKGAEI  239  hamster
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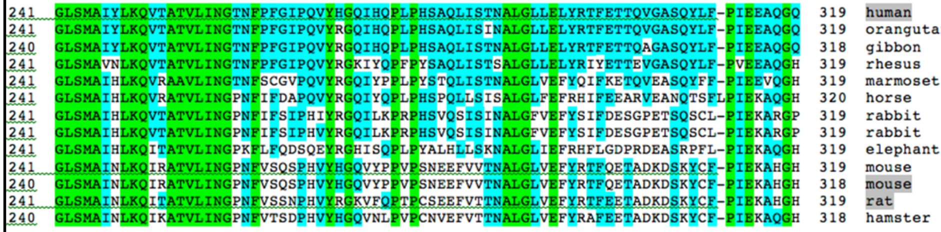

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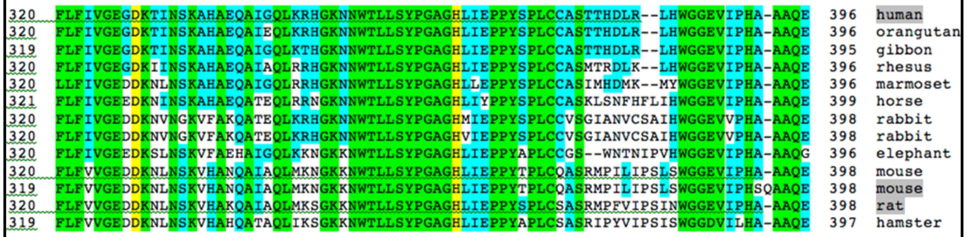
241  GLSMAIYLKQVTATVLINGTNFPFGIPQVYHGQIHQPLPHSAQLISTNALGLLELYRTFETTQVGASQYLF-PIEEAQGQ  319  human
241  GLSMAIYLKQVTATVLINGTNFPFGIPQVYRGQIHQPLPHSAQLISINALGLLELYRTFETTQVGASQYLF-PIEEAQGQ  319  orangutan
240  GLSMAIYLKQVTATVLINGTNFPFGIPQVYRGQIHQPLPHSAQLISTNALGLLELYRTFETTQAGASQYLF-PIEEAQGQ  318  gibbon
241  GLSMAVNLKQVTATVLINGTNFPFGIPQVYRGKIYQPFPYSAQLISTSALGLLELYRIYETTEVGASQYLF-PVEEAQGH  319  rhesus
241  GLSMAIHLKQVRAAVLINGTNFSCGVPQVYRGQIYPPLPYSTQLISTNALGLVEFYQIFKETQVEASQYFF-PIEEVQGH  319  marmoset
241  GLSMAIHLKQVRATVLINGPNFIFDAPQVYRGQIYQPLPHSPQLLSISALGLFEFRHIFEEARVEANQTSFLPIEKAQGH  320  horse
241  GLSMAIHLKQVTATVLINGPNFIFSIPHIYRGQILKPRPHSVQSISINALGFVEFYSIFDESGPETSQSCL-PIEKARGP  319  rabbit
241  GLSMAIHLKQVTATVLINGPNFIFSIPHVYRGQILKPRPHSVQSISINALGFVEFYSIFDESGPETSQSCL-PIEKARGP  319  rabbit
241  GLSMAIHLKQITATVLINGPKFLFQDSQEYRGHISQPLPYALHLLSKNALGLIEFRHFLGDPRDEASRPFL-PIEKAQGH  319  elephant
241  GLSMAINLKQIRATVLINGPNFVSQSPHVYHGQVYPPVPSNEEFVVTNALGLVEFYRTFQETADKDSKYCF-PIEKAHGH  319  mouse
240  GLSMAINLKQIRATVLINGPNFVSQSPHVYHGQVYPPVPSNEEFVVTNALGLVEFYRTFQETADKDSKYCF-PIEKAHGH  318  mouse
241  GLSMAINLKQITATVLINGPNFVSSNPHVYRGKVFQPTPCSEEFVTTNALGLVEFYRTFEETADKDSKYCF-PIEKAHGH  319  rat
240  GLSMAINLKQIKATVLINGPNFVTSDPHVYHGQVNLPVPCNVEFVTTNALGLVEFYRAFEETADKDSKYCF-PIEKAQGH  318  hamster
```

BAAT homologies



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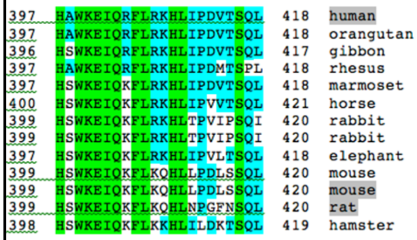
319  FLFIVGEGDKTINSKAHAEQAIGQLKTHGKNNWTLLSYPGAGHLIEPPYSPLCCASTTHDLR--LHWGGEVIPHA-AAQE  395  gibbon
320  FLFIVGEGDKIINSKAHAEQAIAQLRRHGKNNWTLLSYPGAGHLIEPPYSPLCCASMTRDLK--LHWGGEVIPHA-AAQE  396  rhesus
320  LLFIVGEDDKNLNSKAHAEQAIGQLRRHGKNNWTLLSYPGAGHLLEPPYSPLCCASIMHDMK--MYWGGEVIPHA-AAQE  396  marmoset
321  FLFIVGEEDKNINSKAHAEQATEQLRRNGKNNWTLLSYPGAGHLIYPPYSPLCCASKLSNFHFLIHWGGEVIPHA-AAQE  399  horse
320  FLFIVGEDDKNVNGKVFAKQATEQLKRHGKNNWTLLSYPGAGHMIEPPYSPLCCVSGIANVCSAIHWGGEVVPHA-AAQE  398  rabbit
320  FLFIVGEDDKNVNGKVFAKQATEQLKRHGKNNWTLLSYPGAGHVIEPPYSPLCCVSGIANVCSAIHWGGEVVPHA-AAQE  398  rabbit
320  FLFIVGEEDKSLNSKVFAEHAIGQLKKNGKKNWTLLSYPGAGHLIEPPYAPLCCGS--WNTNIPVHWGGEVIPHA-AAQG  396  elephant
320  FLFVVGEDDKNLNSKVHANQAIAQLMKNGKKNWTLLSYPGAGHLIEPPYTPLCQASRMPILIPSLSWGGEVIPHA-AAQE  398  mouse
319  FLFVVGEDDKNLNSKVHANQAIAQLMKNGKKNWTLLSYPGAGHLIEPPYTPLCQASRMPILIPSLSWGGEVIPHSQAAQE  398  mouse
320  FLFVVGEDDKNLNSKVHAKQAIAQLMKSGKKNWTLLSYPGAGHLIEPPYSPLCSASRMPFVIPSINWGGEVIPHA-AAQE  398  rat
319  FLFVVGEDDKNLNSKVHAHQATAQLIKSGKKNWTLLSYPGAGHLIEPPYAPLCSASRIPYVIPSISWGGDVILHA-AAQE  397  hamster
```



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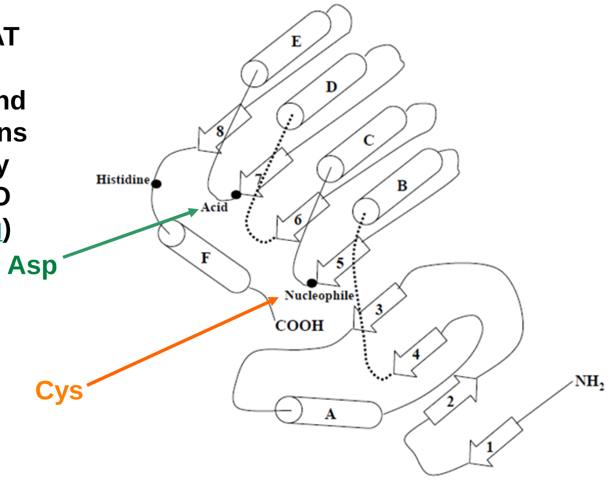
397  HAWKEIQRFLRKHLIPDVTSQL  418  human
397  HAWKEIQRFLRKHLIPDVTSQL  418  orangutan
396  HSWKEIQRFLRKHLIPDVTSQL  417  gibbon
397  HAWKEIQRFLRKHLIPDMTSPL  418  rhesus
397  HSWKEIQKFLRKHLIPDVTSQL  418  marmoset
400  HSWKEIQKFLRKHLIPVVTSQL  421  horse
399  HSWKEIQKFLRKHLTPVIPSQI  420  rabbit
399  HSWKEIQKFLRKHLTPVIPSQI  420  rabbit
397  HSWKEIQKFLRKHLIPVLTSQL  418  elephant
399  HSWKEIQKFLKQHLLPDLSSQL  420  mouse
399  HSWKEIQKFLKQHLLPDLSSQL  420  mouse
399  HSWKEIQKFLKQHLNPGFNSQL  420  rat
398  HSWKEIQKFLKKHLILDKTSQL  419  hamster
```

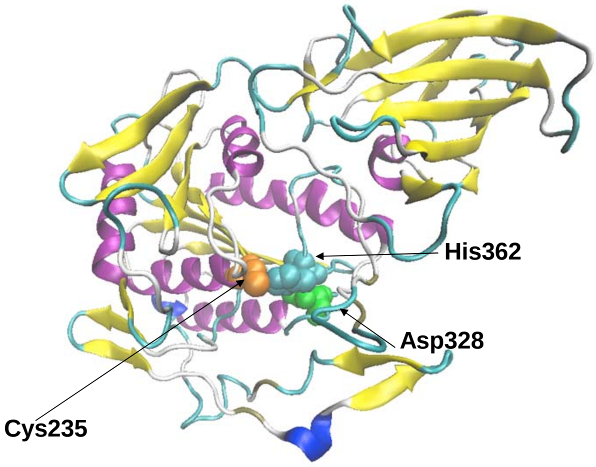
hBAT is a member of a class of proteins with an α/β hydrolase fold

The regions of hBAT that were part of α-helices, β-sheets and unstructured regions were determined by the use of the JUFO (www.Meilerlab.org)



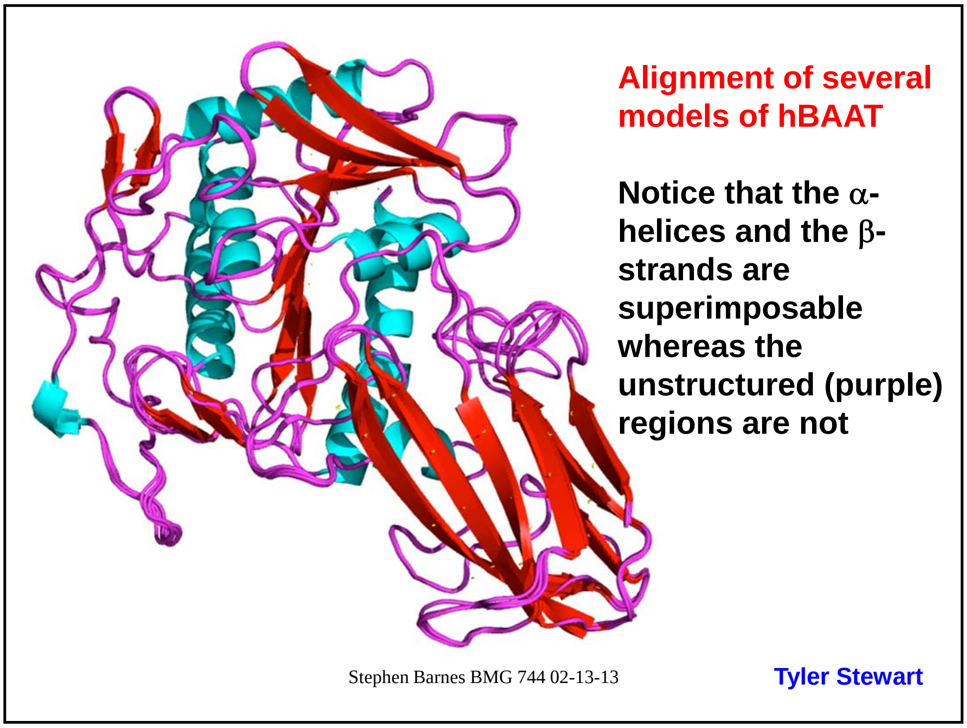
Modeled structure of hBAT

In the absence of hBAT crystals, structural homology experiments using FUGUE were performed. These identified two previously crystallized proteins, *ACOT2* and *ACOT4* as structural homologs of hBAT. These two structures were used to create a threaded structure for hBAT which was refined using Modeller 9v10



Tyler Stewart

http://tardis.nibio.go.jp/fugue/prfsearch.html

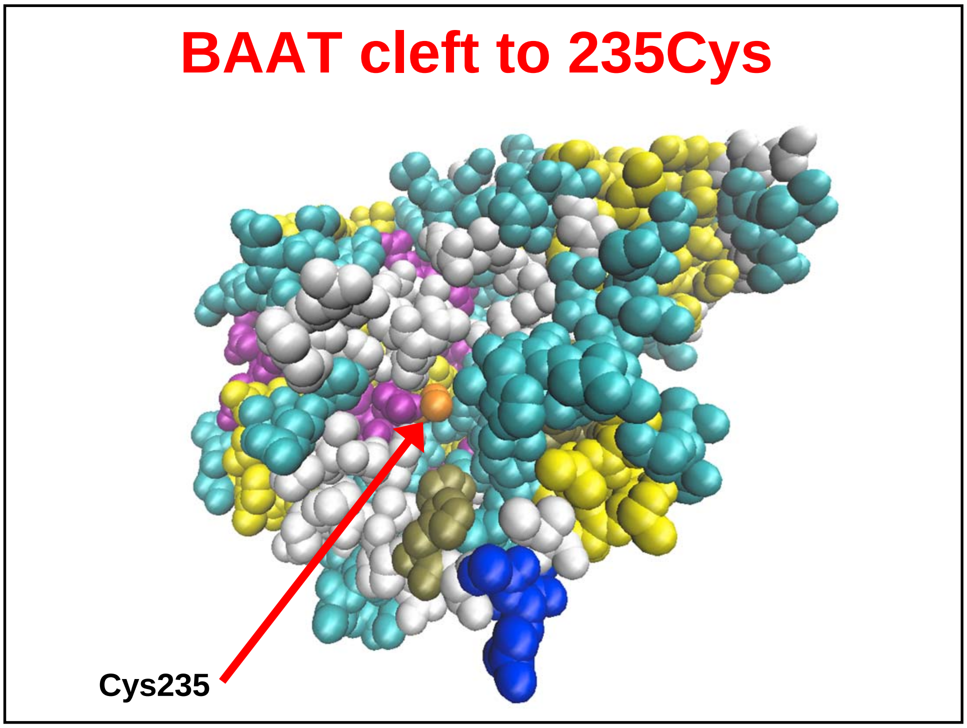


Alignment of several models of hBAAT

Notice that the α-helices and the β-strands are superimposable whereas the unstructured (purple) regions are not

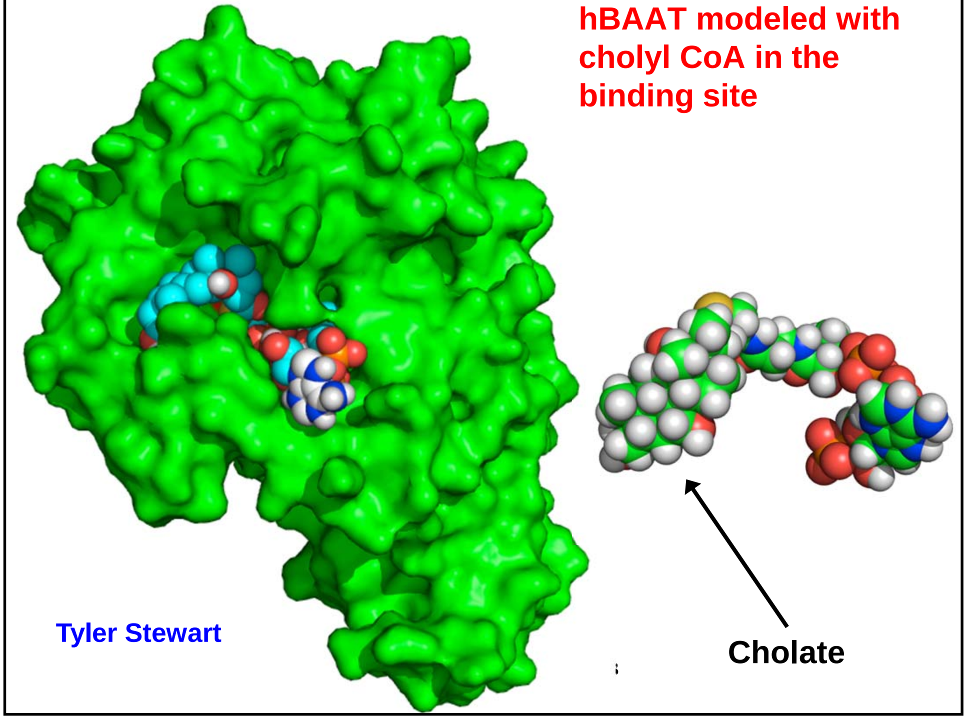
Stephen Barnes BMG 744 02-13-13

Tyler Stewart



BAAT cleft to 235Cys

hBAAT modeled with cholyl CoA in the binding site



Cholate

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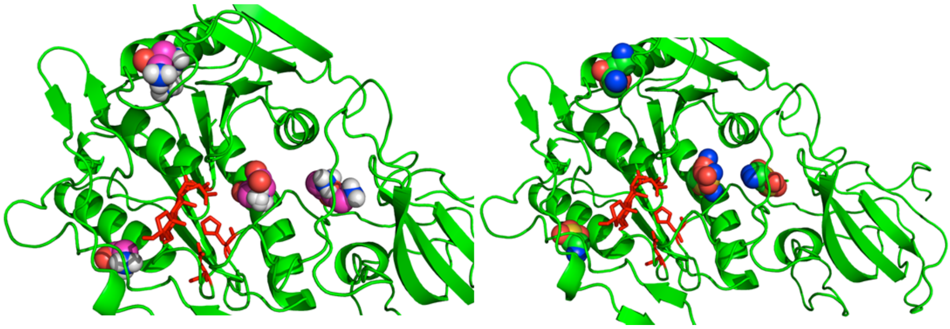
Where do glycine and taurine bind to hBAAT?



Glycine

Taurine

Seem to have binding sites in common

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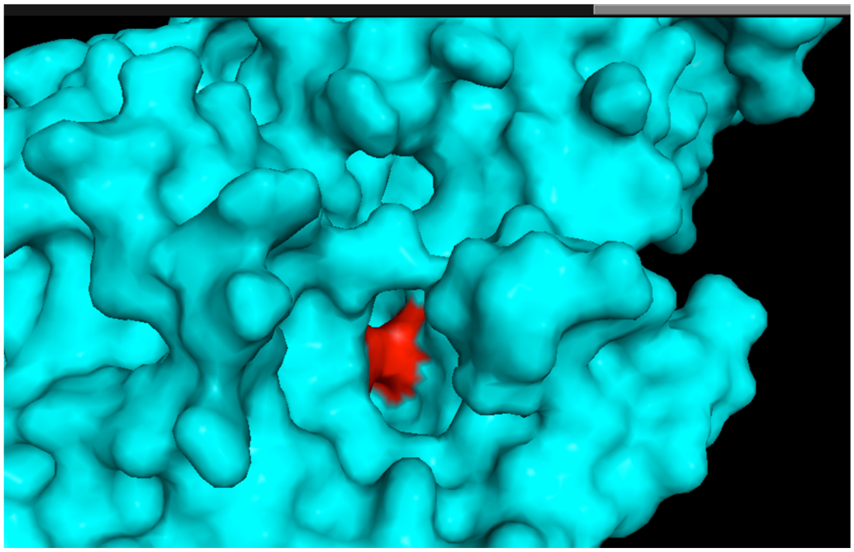
Visualization of the taurine “tunnel” on hBAAT



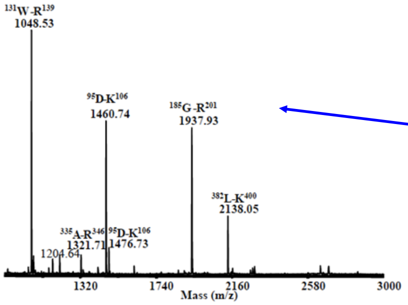
Tyler Stewart

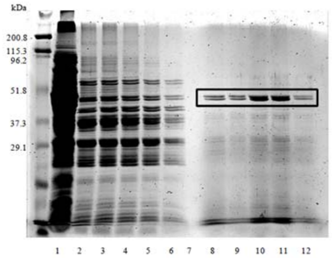
Hypothesis

- **The ability of 4HNE to react with Cys, His and Lys residues is dependent on their accessibility to solvent containing 4HNE.**
- **Therefore, in the presence of cholyl CoA, and hence covalently linked cholate to Cys235, the accessibility of 4HNE to the active site will be blocked, thereby revealing the residues that are involved**

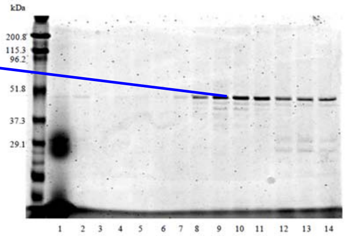
Purification of wt-hBAT

Untagged wt-hBAT was recombinantly expressed in *E. coli* using a pKK233.2 vector and purified using chromatofocusing and anion exchange chromatography





Chromatofocusing

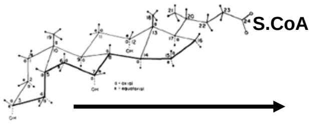


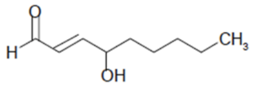
DEAE-Sephacryl

Erin Shonsey

Experimental plan

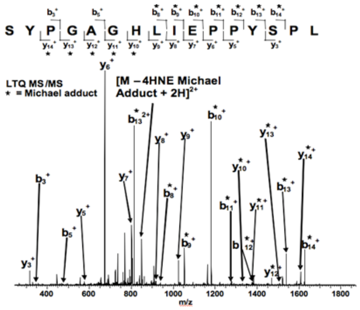
hBAT -NH$_2$
-SH
-imidazole



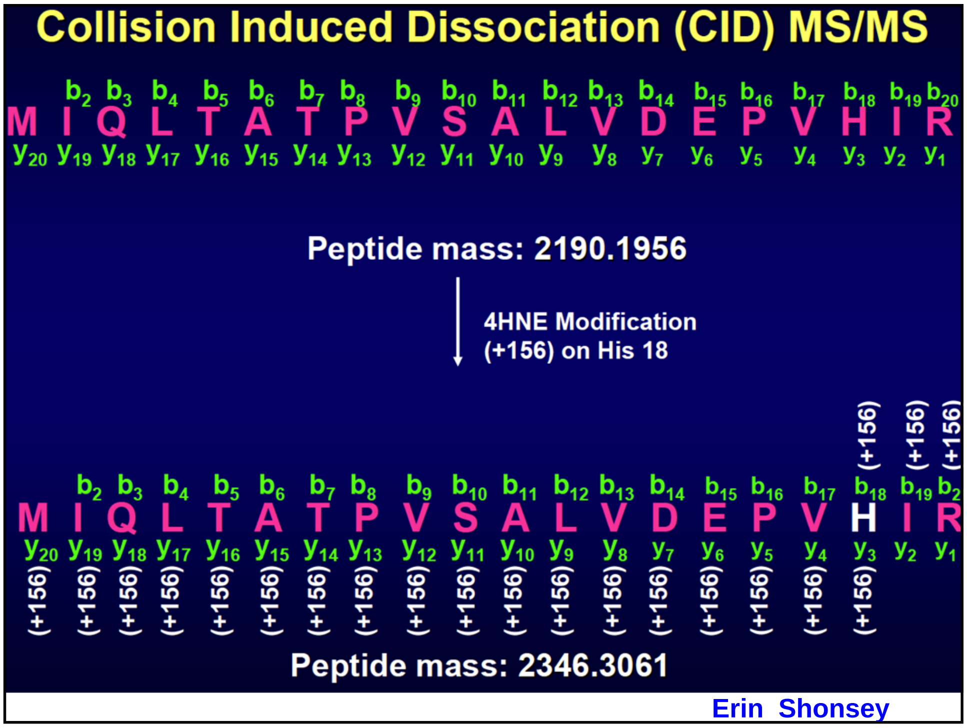


hBAT adducts

Digestion with trypsin and chymotrypsin



Erin Shonsey



Collision Induced Dissociation (CID) MS/MS

b_2 b_3 b_4 b_5 b_6 b_7 b_8 b_9 b_{10} b_{11} b_{12} b_{13} b_{14} b_{15} b_{16} b_{17} b_{18} b_{19} b_{20}
M I Q L T A T P V S A L V D E P V H I R
y_{20} y_{19} y_{18} y_{17} y_{16} y_{15} y_{14} y_{13} y_{12} y_{11} y_{10} y_9 y_8 y_7 y_6 y_5 y_4 y_3 y_2 y_1

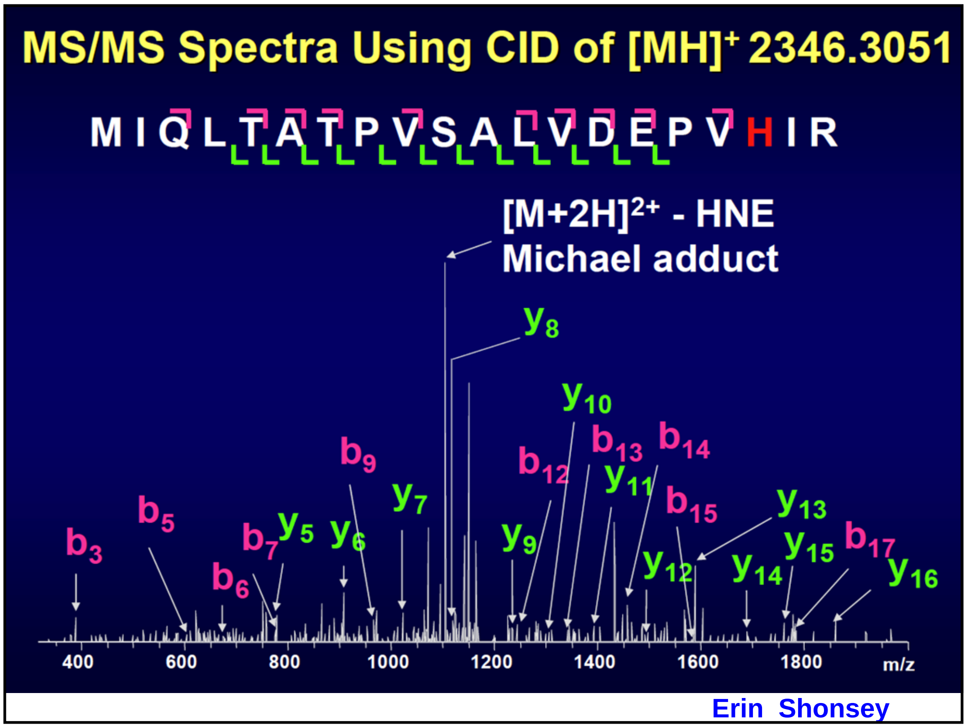
Peptide mass: 2190.1956

4HNE Modification
(+156) on His 18

(+156) (+156) (+156)
b_2 b_3 b_4 b_5 b_6 b_7 b_8 b_9 b_{10} b_{11} b_{12} b_{13} b_{14} b_{15} b_{16} b_{17} b_{18} b_{19} b_2
M I Q L T A T P V S A L V D E P V H I R
y_{20} y_{19} y_{18} y_{17} y_{16} y_{15} y_{14} y_{13} y_{12} y_{11} y_{10} y_9 y_8 y_7 y_6 y_5 y_4 y_3 y_2 y_1
(+156) (+156) (+156) (+156) (+156) (+156) (+156) (+156) (+156) (+156) (+156) (+156) (+156) (+156) (+156) (+156) (+156) (+156)

Peptide mass: 2346.3061

Erin Shonsey



MS/MS Spectra Using CID of $[MH]^+$ 2346.3051

M I Q L T A T P V S A L V D E P V H I R

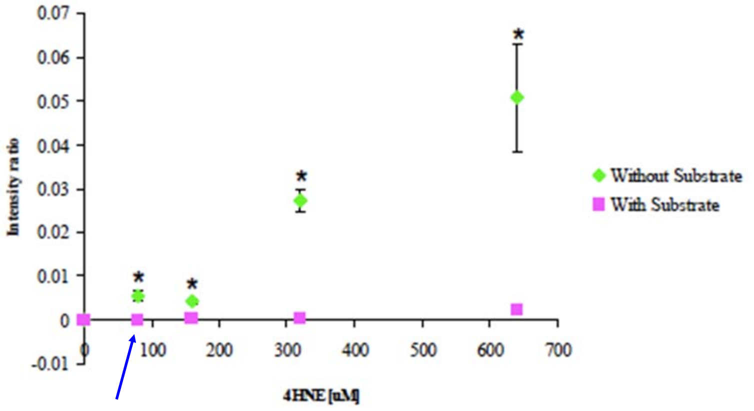
Erin Shonsey

Concentration-dependent sites of 4HNE modification on hBAT

	Modified Amino Acid				
Peptide	128 µM HNE	64 µM HNE	32 µM HNE	16 µM HNE	8 µM HNE
AHAEQAIGQLKR	H336	H336	H336	H336	H336
RLHWGGEVIPHAAAQEHAWK	H397	H397	H397	H397	
AQGQFLFIVGEGDKTINSK	K329, K334	K329, K334	K329, K334	K329, K334	K329, K334
MIQLTATPVSALVDEPVHIR	H18	H18			
RANEFGEVDLNHASSLGGDYMGV HPMGLFWSLKPEK	H62, H74	H62, H74	H62, H74	H62, H74	H62
HGQIHQPLPHSAQL	H271, H274, H279	H271, H274, H279	H271, H274, H279	H271, H279	H271, H279
NNWTLLSYPGAGHLIEPPYSPLCCA STTHDLR	H362, C372, C373, H378	H362	H362	H362	H362

Shonsey et al., 2007

Cholyl CoA blocks 4HNE adducts on the His383 residue of hBAT



Concentration used for comparative analyses

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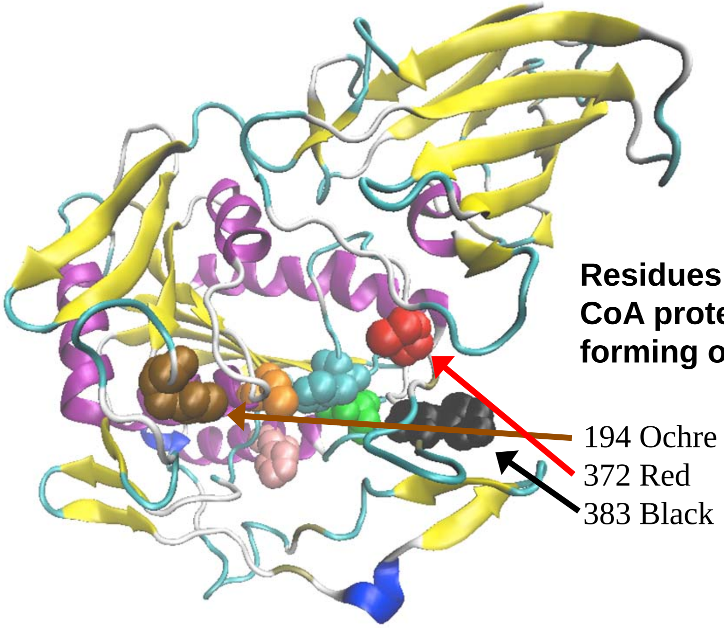

Residues that cholyl CoA protects from forming of 4HNE

194 Ochre
372 Red
383 Black

Identifying protease activity

- **Many proteins are proproteins. They can:**
  - **Modify a C-terminal glycine to produce a C-terminal amide (many brain peptides)**
  - **Cleave a head group (e.g., transmembrane region) to make an active form of the protein**
  - **Cleave “centrally” to release a polypeptide that enters the nucleus to bind to a nuclear transcription factor**