

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**

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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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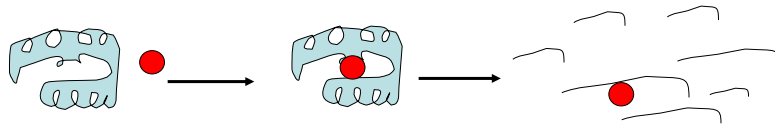
Enzymes and Mass Spec

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 (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

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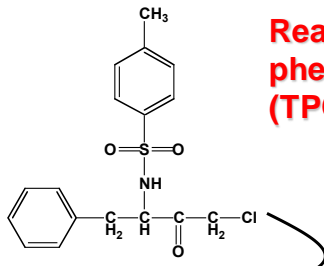
MS of enzymes



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)

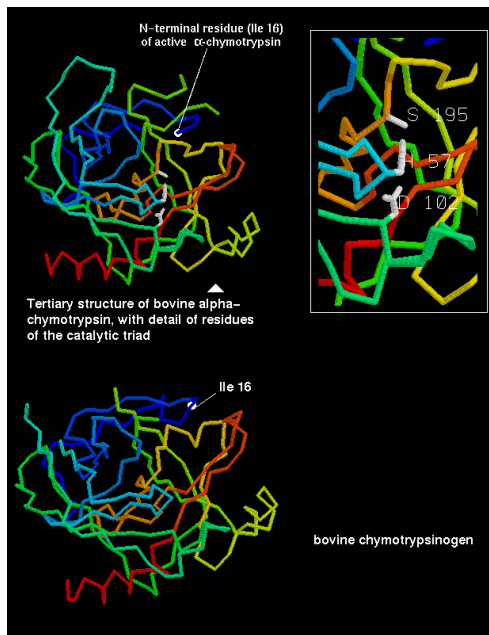
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Reaction of chymotrypsin with tosyl phenylalanyl chloromethylketone (TPCK) in His57

CGVPAIQPVL SGLSRIVNGE EAVPGSWPWQ VSLQDKTGFH FCGGSLINEN
 50
 WVVTAAH**CGV** TTSDVVVAGE FDQGSSEKI QKLKIAKVKF NSKYNSLTIN
 100
 NDITLLKLST AASFSQTVSA 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 TTSDDVVAGE FDQGSSEKI QKLKIAKVKF NSKYNSLTIN
100

NDITLLKLST AASFSQTVSA VCLPSASDDF AAGTTCVTTG WGLTRYTNAN
150

TPDRLQQASL PLLSNTNCKK YWGTKIKDAM ICAGASGVSS
150
CGVPAIQPVL SGLSRIVNGE EAVPGSWPWQ VSLQDKTGFH FCGGSLINEN
50
CMGDSGGPLY 200

CKKNGAWTLV GIVSWGSSTC STSTPGVYAR VTALVNWVQQ TLAAN
50
WVVTAAHCGV TTSDDVVAGE FDQGSSEKI QKLKIAKVKF NSKYNSLTIN
100

NDITLLKLST AASFSQTVSA VCLPSASDDF AAGTTCVTTG WGLTRYTNAN
150

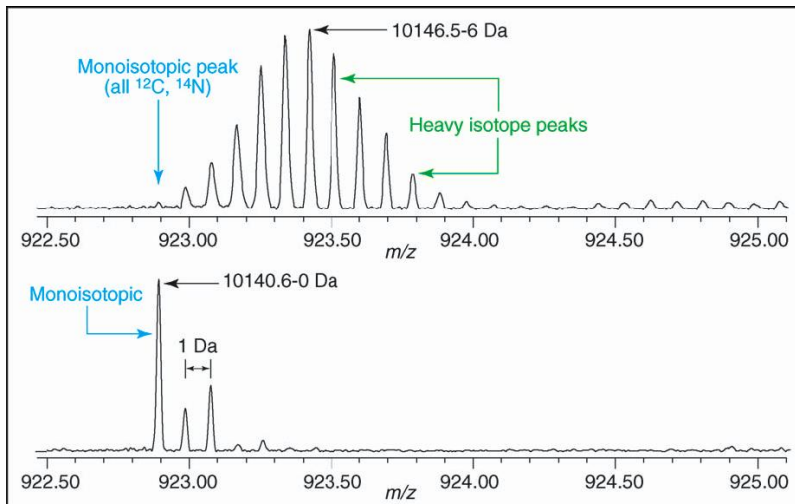
TPDRLQQASL PLLSNTNCKK YWGTKIKDAM ICAGASGVSS
150
CGVPAIQPVL SGLSRIVNGE EAVPGSWPWQ VSLQDKTGFH FCGGSLINEN
50
CMGDSGGPLY 200

WVVTAAHCGV TTSDDVVAGE FDQGSSEKI QKLKIAKVKF
50
NSKYNSLTIN 100 NDITLLKLST AASFSQTVSA VCLPSASDDF
100

AAGTTCVTTG WGLTRYTNAN 150 TPDRLQQASL PLLSNTNCKK
150
YWGTKIKDAM ICAGASGVSS CMGDSGGPLY 200 CKKNGAWTLV
200
GIVSWGSSTC STSTPGVYAR VTALVNWVQQ TLAAN
250

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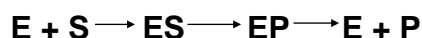
Advantage of growing recombinant bacteria on ¹²C/¹⁴N labeled substrates - protein is close to monoisotopic mass



Stephen Barnes BMG 744 02-13-12 Kelleher & Hicks, 2005

Mass spectrometry and enzyme-catalyzed reactions

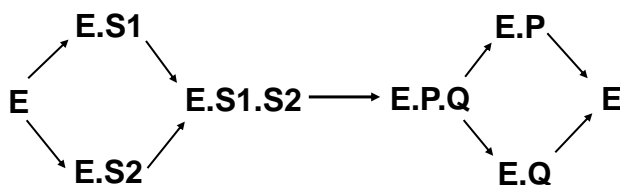
In the simplest case, an enzyme (E) reacts with a substrate (S) - an intermediate complex is formed (ES) and it is converted to an enzyme: product complex (E:P) before the product dissociates.



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



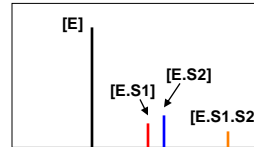
By measuring the molecular weights of the forms of the enzyme:substrate (product) complexes, mass spectrometry can throw enormous light on the mechanism

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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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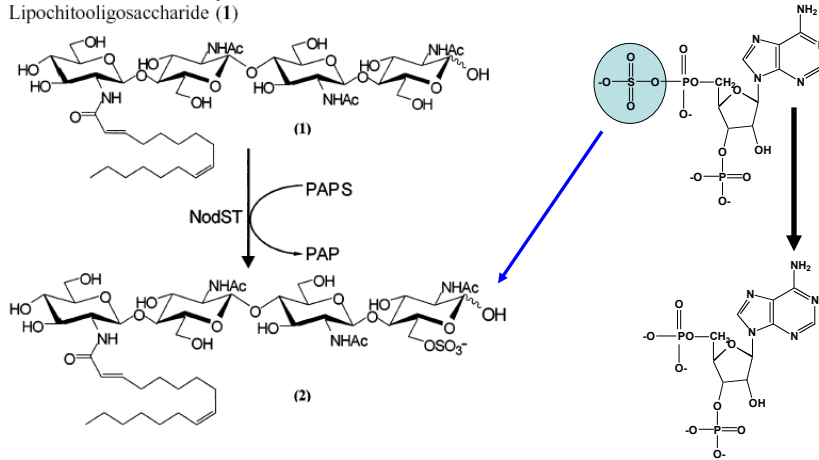
Mass spectrometry and substrates and products of enzyme reactions

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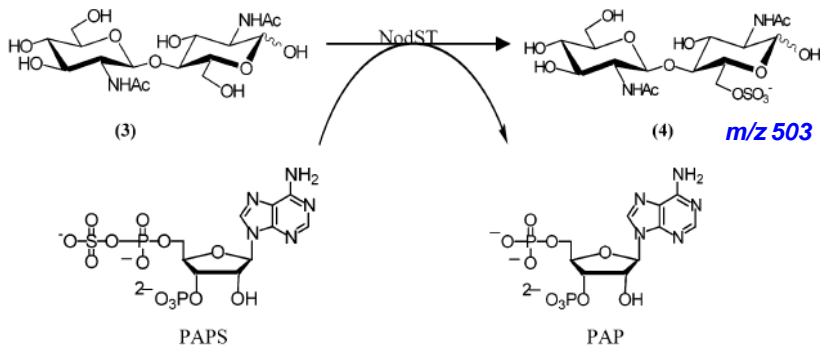
Sulfotransferase - a reaction with no absorbance or fluorescence to follow

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



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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 $\mu\text{l}/\text{min}$
- 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

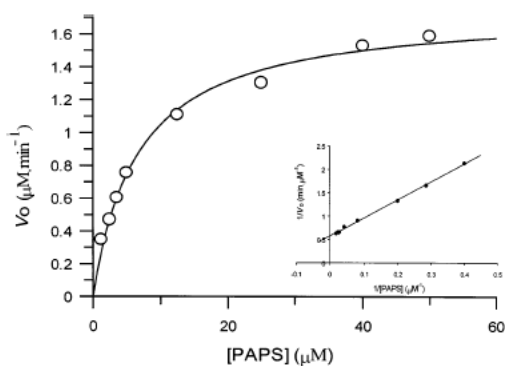


FIGURE 3: Saturation plot of V_0 vs [PAPS]. The inset is a double-reciprocal plot of $1/V_0$ vs $1/[PAPS]$ ([PAPS] = 1.25, 2.5, 3.5, 5, 12.5, 25, 40, and 50 μM , [chitobiose] = 1 mM, [NodST] = 90 nM, and pH 8.0).

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Inhibition of ST by PAP using ESI-MS

Pi et al., Biochemistry 41:13283

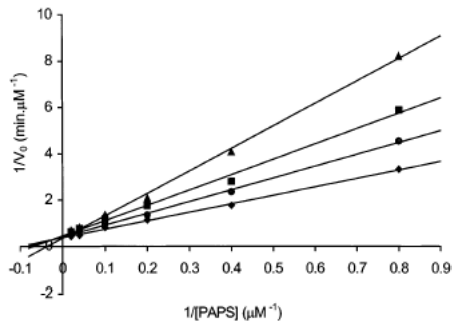


FIGURE 5: Double-reciprocal plot of $1/V_0$ vs $1/[PAPS]$ at different PAP concentrations: 0 μ M PAP (▲), 0.75 μ M PAP (■), 1.5 μ M PAP (●), and 3.0 μ M PAP (◆) ([PAPS] = 1.25, 2.5, 5, 10, 25, and 50 μ M, [chitobiose] = 1 mM, [NodST] = 90 nM, and pH 8.0).

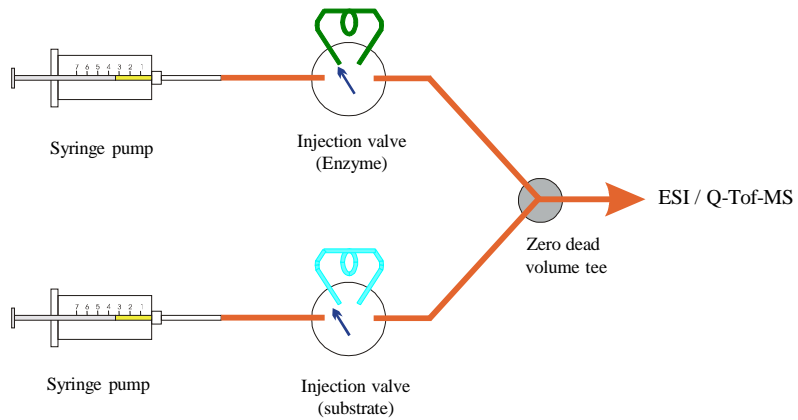
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Non-covalent enzyme:substrate complexes

- 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

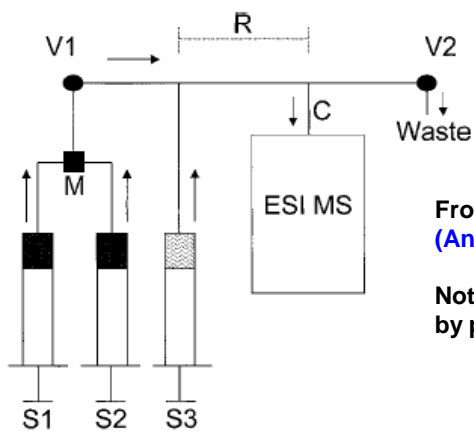
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Schematic diagram of a stop-flow system



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Stopped flow set up

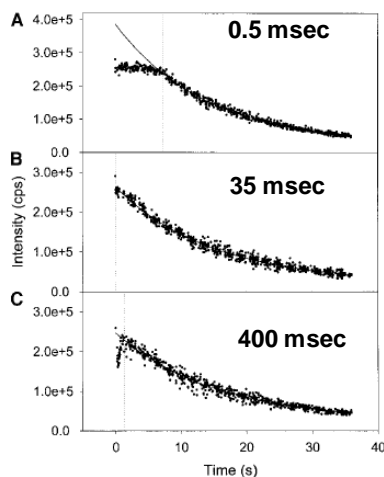


From Kolakowski and Konermann
(Anal Biochem 292:107)

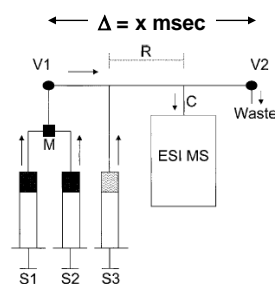
Note the additional flow introduced
by pump S3

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



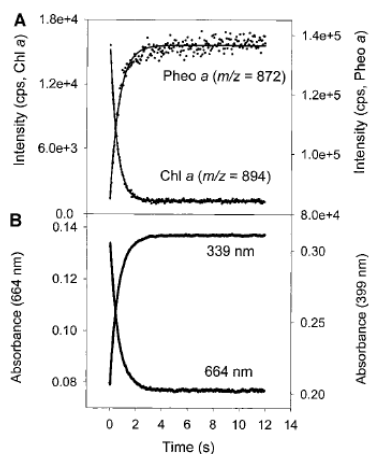
In this reaction, hydrolysis of acetylcholine in an alkaline buffer is monitored by the ion at m/z 146



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Kolakowski and Konermann
(Anal Biochem 292:107)

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



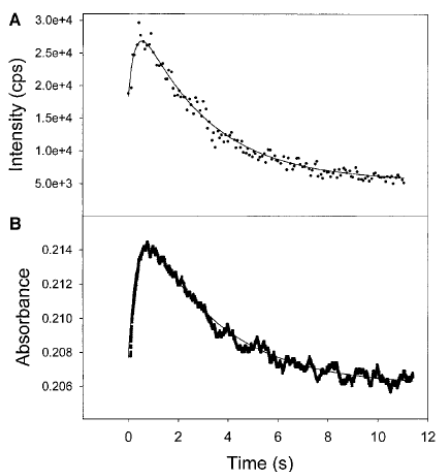
These data are from the conversion of chlorophyll A to pheophytin A (loss of Mg and gain of two protons).

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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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)

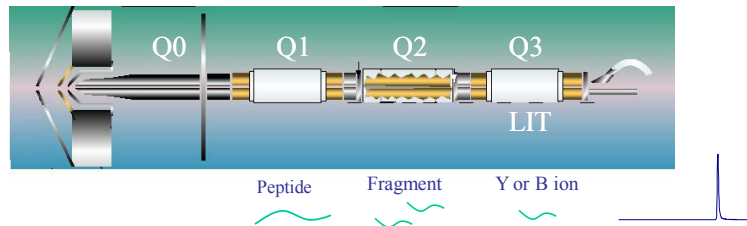
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Multiple reaction ion monitoring

- **This technique allows us to measure the proteins as well as the substrate and products**
 - Based on selection of the parent ion, fragmentation, and selection of specific fragment ions

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Multiple Reaction Monitoring



- High specificity and sensitivity for detecting components in a complex mixture
- Largest linear dynamic range for quantitation
- Well accepted as the MS technique for quantification (small molecule world)
- Triple quadrupole or Q TRAP[®] system mass spectrometers

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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

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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

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[Lahoz et al., 2008](#)

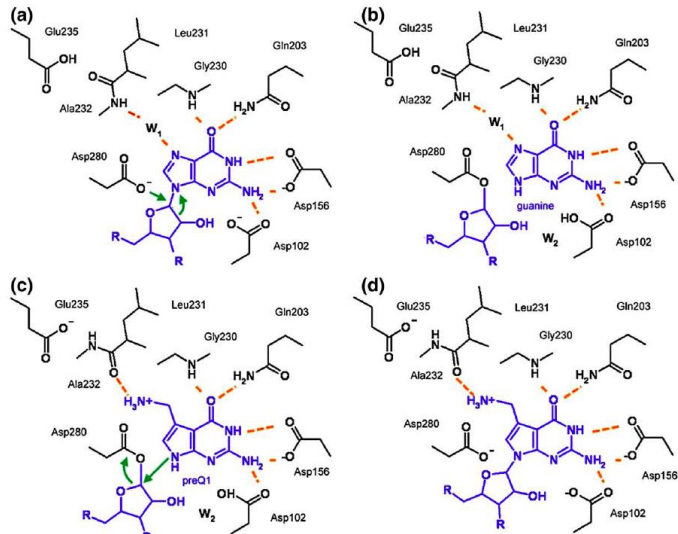
tRNA-guanine transglycosylase

- 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 Tgt part of the interface?

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

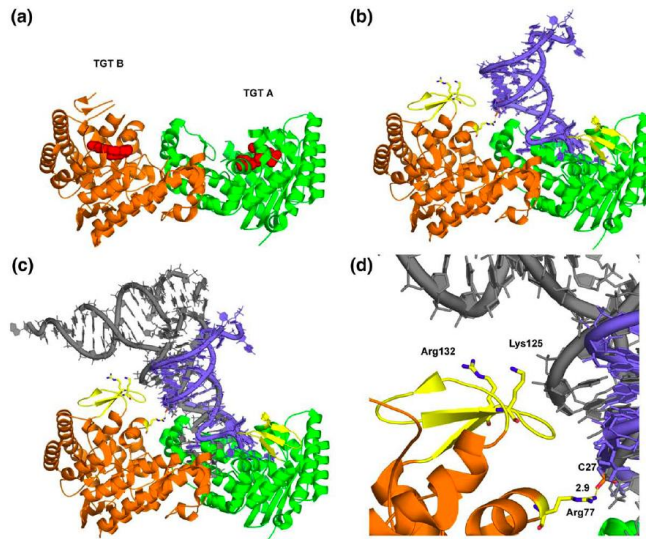
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Tgt enzyme reaction



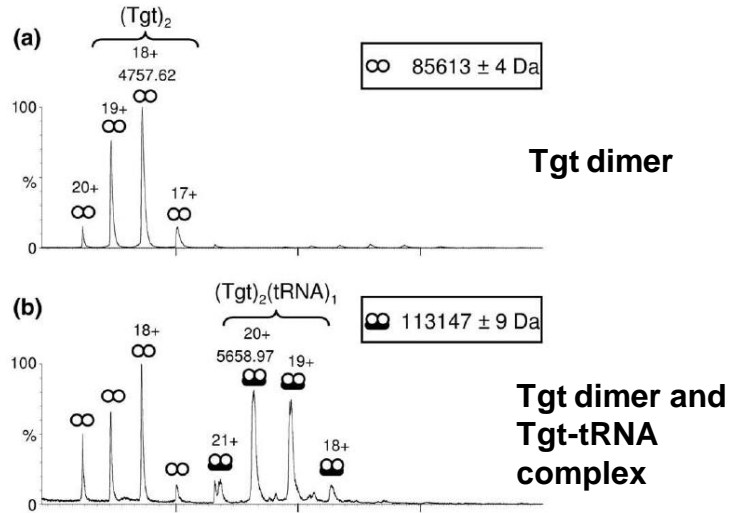
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Structures of Tgt complex



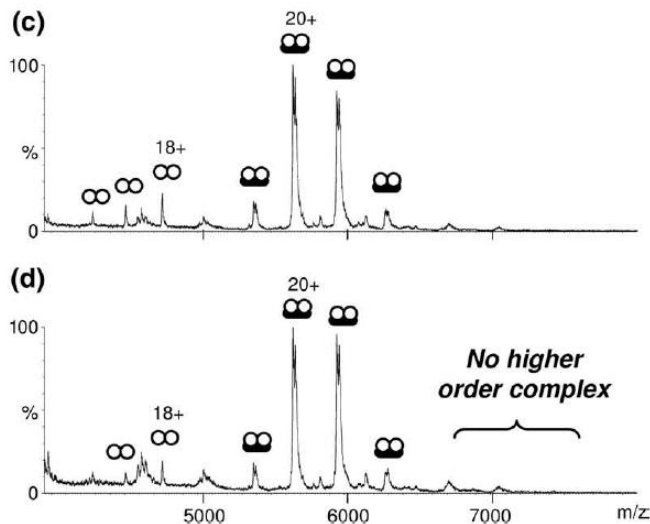
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Non-covalent ESI-MS of Tgt



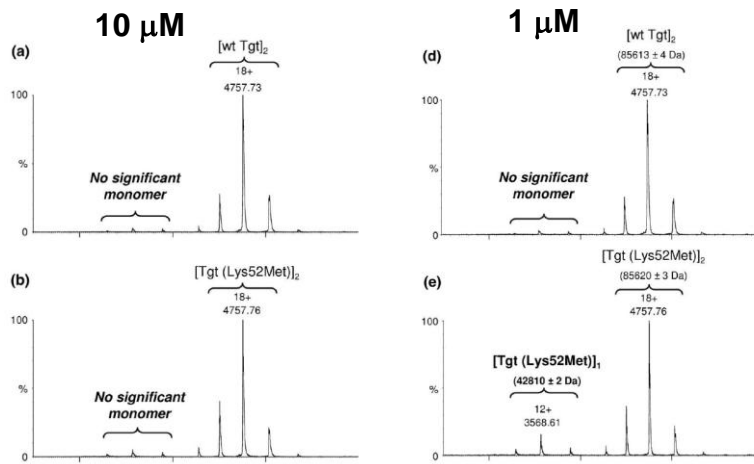
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Adding more tRNA does not lead to higher order complexes



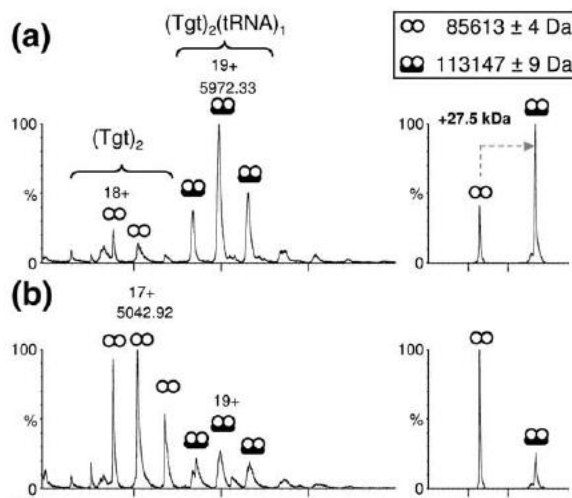
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Evidence for Tgt dimer dissociation in Lys52Met mutant



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



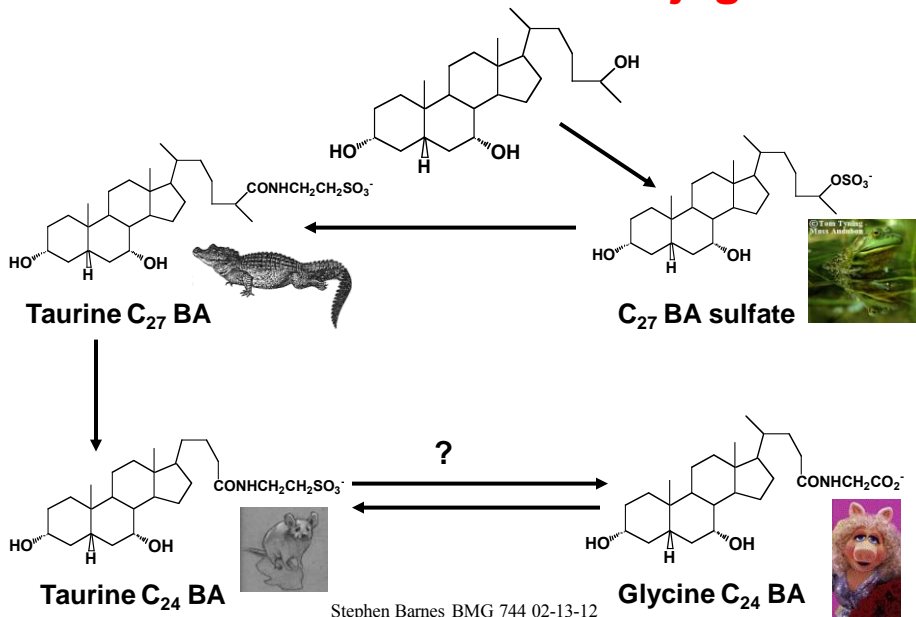
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Conclusions about non-covalent complexes by ESI-MS

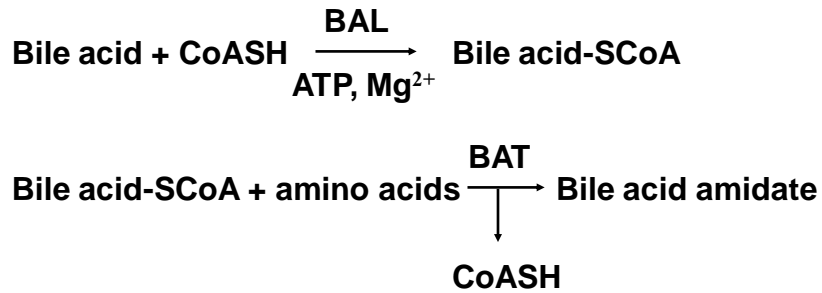
- The physical state of the enzyme with its substrates and inhibitors can be readily observed and quantified by ESI-MS
- Requires sufficient protein (in the Ritschel study they used 1-10 μM – for a 25 kDa protein, this would be 25-250 mg/L or 25-250 $\mu\text{g/ml}$)
- The spraying medium is important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

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Evolution of bile acid conjugation



Bile acid N-acylamidate formation (in hepatocytes)

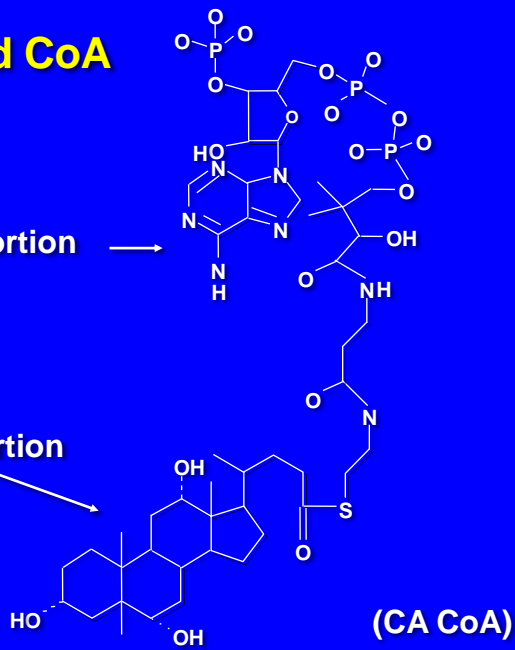


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Bile acid CoA

Hydrophilic portion
(CoA)

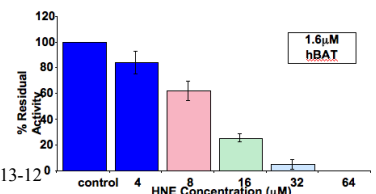
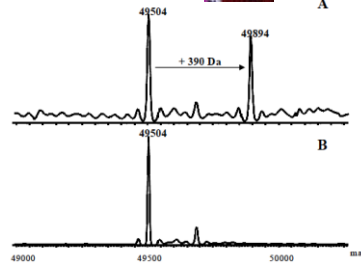
Hydrophobic portion
(Bile acid)



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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



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BAAT homologues

1	MIQIATATVTSALVDEPVHIRATGILPFQMVSFQASLEDENGDMFYSOAHYRANFGEVDLNHASSLGGDYMGVHPMGLFW	80	human BAAT
1	MIQIATATVTSALVDEPVHIRATGILPFQMVSFQASLEDENGDMFYSOAHYRANFGEVDLNHASSLGGDYMGVHPMGLFW	80	orangutan BAAT
1	MIQIATATVTSALVDEPVHIRATGILPFQMVSFQASLEDENGDMFYSOAHYRANFGEVDLNHASSLGGDYMGVHPMGLFW	80	gibbon BAAT
1	MIQIATATVTSALVDEPVHIRATGILPFQMVSFQASLEDESGNMFYSOAHYRANFGEVDLNHASSLGGDYMGVHPMGLFW	80	rhesus BAAT
1	MIQIATATVTSALVDEPVHIRATGILPFQVVSFKASLEDEKRNMFYSOAHYRANFGEVDLNHASSLGGDYMGVHPMGLFW	80	marmoset BAAT
1	MIQIATATVTSALVDEPVHIRATGILPFQILVLLQASLEDEMGNMHSOAHYRANFGEVDLNHASSLGGDYMGVHPMGLFW	80	horse BAAT
1	IVQIATATVTSALVDEPVHIRATGILVLFQVVFHASLEDEVGNLVYRRAEYRANFGEVDLDDHAALGGDYMGVHPMGLFW	80	rabbit BAAT
1	IVQIATATVTSALVDEPVHIRATGILVLFQVVFHASLEDEVGNLVYRRAEYRANFGEVDLDDHAALGGDYMGVHPMGLFW	80	rabbit BAAT
1	MIQIATATVTSALVDEPVHIRATGILVLSQVVFHASLEDEQGNMHSOAHYRANFGEVDLDEYASALGGDYMGVHPMGLFW	80	elephant BAAT
1	MAKLTAVLTSALVDEPVHIRVITGLTFFQVVCLOASLKDCKGNLNSQEFYRASVGEVDLHDPSSLGGDYMGVHPMGLFW	80	mouse BAAT
1	MAKLTAVLTSALVDEPVHIRVITGLTFFQVVCLOASLKDCKGNLNSQEFYRASVGEVDLHDPSSLGGDYMGVHPMGLFW	79	mouse BAAT
1	MAKLTAVLTSALVDEPVHIRVITGLTFFQVVCLOASLKDCKGNLNSQEFYRASVGEVDLHDPSSLGGDYMGVHPMGLFW	80	rat BAAT
1	MAKLTAVLTSALVDEPVHIRVITGLTFFQVVCLOASLKDCKGNLNSQEFYRASVGEVDLHDPSSLGGDYMGVHPMGLFW	80	hamster BAAT
81	SLKPEKLLTRLLKRDVMNRFQVOVKLYDLELIVNNKVASAPKASLTTLRWVYVAGVTRIKVREGRLRGAFLFPPGEGLE	160	human BAAT
81	SLKPEKLLTRLLKRDVMNRFQVOVKLYDLELIVNNKVASAPKASLTTLRWVYVAGVTRIKVREGRLRGAFLFPPGEGLE	160	orangutan BAAT
81	SLKPEKLLTRLLKRDVNRPFQVOVKLYDLELIVNNKVASAPKASLTTLRWVYVAGVTRIKVREGRLRGAFLFPPGEGLE	159	gibbon BAAT
81	SLKPEKLLTRLLKRDVMNRFQVOVKLYDLELIVNNKASAPKASLTTLRWVYVAGVTRIQVREGRLRGAFLFPPGEGLE	160	rhesus BAAT
81	SLKPEKLLKLLKRDVMNRFQVLELYDLELIPNNKATSPKASLTTLRWVYVAGVTRIQVREGRLRGNLFLFPPGEGLE	160	marmoset BAAT
81	SLKPEKLLTRLLKRDVMNRFVQLKLYNSVLLINKATADPIVSLTLRWVYVAGVTRIQVREGRLRGAFLFPPGEGLE	160	horse BAAT
81	SLKPEKLVTRLLKRDVMNRFVQVKLYDRHLEPIETKTSEAPKASVTLRWVYVAGVTRIPVREGRLRGTFLVHSDEGR	160	rabbit BAAT
81	SLKPEKLVTRLLKRDVMNRFVQVKLYDRHLEPIETKTSEAPKASVTLRWVYVAGVTRIPVREGRLRGTFLVHSDEGR	160	rabbit BAAT
81	SMKPKLLTRLLKRDVMNRCFQVFLKLYDLDISVLEKTTATPKASLTVERWYVAGVTRIQVREGIRGALLFPPDDEP	160	elephant BAAT
81	SLKPEKLLGLLKRDVMNRFQIHIKACHYFFLQDLYVSPPLDSLTLRWVYVAGVKKHIQKSHIRGALLFPPGEGLE	160	mouse BAAT
80	SLKPEKLLGLLKRDVMNRFQIHIKACHYFFLQDLYVSPPLDSLTLRWVYVAGVKKHIQKSHIRGALLFPPGEGLE	159	mouse BAAT
81	SMKPKLLTRLLKRDVMNRHKVHIKLPYFPVQKIVSSSLDSLTLRWVYVAGVTRIHKSHIRGALLFPPGEGLE	160	rat BAAT
81	SLKPEKLLSLLKRDVTSQVHIELCKPHFPKGIIVSPMDSLTLRWVYVAGVTRIQKSHIRGALLFPPGEGLE	159	hamster BAAT

BAAT homologies

161	FGVIDLFGGLGGLEFRASLLASRGFASLALAYHNYE	DLPRKPEVTDLEYFEEAANFLLRHPKVFGSGVGVVSV	CGQVQI	240	human
161	PGVIDLFGGLGGLEFRASLLASRGFASLALAYHNYE	DLPAKPEVTDLEYFEEAANFLLRHPKVFGSGVGVVSV	CGQVQI	240	orangutan
160	PGVIDLFGGLGGLEFRASLLASRGFASLALAYHNYE	DLPAKPEVTDLEYFEEAANFLLRHPKVFGSGVGVVSV	CGQVQI	239	gibbon
161	PGVIDLFGGLGGLEFRASLLASRGFASLALAYFNVEDL	PAKPEVTDLEYFEEAANFLLRHPKVFGSGVGVVSV	CGQVQI	240	rhesus
161	PGVIDLFGGLGGLEFRASLLASRGFASLALAYHDVEDL	PSNPEVTDLEYFEEAANFLLRHPKVFGSGVGVVSV	CGQVQI	240	marmoset
161	PGVIDLFGSICGLLEFRASLLASRGFAALALAYCDYEDL	PCPLEKVDLEYFEEAANFLLRHPKVFGSGVGVVSV	ICKGAEI	240	horse
161	PGVIDLFGAGGLLEFRASLLASRGFASLALAHCGFEDL	PPQFDKVDLEYFEEAANFLLRHPKVFGSGVGVVSV	VCRGAEI	240	rabbit
161	PGVIDMYGASGGLLEFRASLLASRGFASLALAHCGFEDL	PPQFDKVDLEYFEEAANFLLRHPKVFGSGVGVVSV	VCRGAEI	240	rabbit
161	EAVIDLFGTGGLEIHYRSSLASRGFASLALAYFGVEDL	PSNIGKLDLEYFEEAANFLLRHPKVFGSGVGVVSV	ICKGAEI	240	elephant
161	PGVIDLFGGAGGLMFRASLLASRGFATLALAYWYDLDL	SRLEKVDLEYFEEGVEFLLRHPKVFGSGVGVVSV	ICKGAEI	240	mouse
160	PGVIDLFGGAGGLMFRASLLASRGFATLALAYWYDLDL	SRLEKVDLEYFEEGVEFLLRHPKVFGSGVGVVSV	ICKGAEI	239	mouse
161	PGVIDLFGGAGGLMFRASLLASRGFATLALAYWYDLDL	SRLEKVDLEYFEEGVEFLLRHPKVFGSGVGVVSV	ICKGAEI	240	rat
160	PGVIDLFGSTGGLIFRASLLASRGFAAFLLAYWYDLDL	SYLEKIDLEYFEEGVEFLLRHPKVFGSGVGVVSV	ICKGAEI	239	hamster
241	GLSMAIYLKQVITATVVLINGTNPFFGPIQVYRQIHQ	PLPHSAQLISTNALGELLELYRTFETTVGASQYLF	-FIEEAQGG	319	human
241	GLSMAIYLKQVITATVVLINGTNPFFGPIQVYRQIHQ	PLPHSAQLISTNALGELLELYRTFETTVGASQYLF	-FIEEAQGG	319	orangutan
240	GLSMAIYLKQVITATVVLINGTNPFFGPIQVYRQIHQ	PLPHSAQLISTNALGELLELYRTFETTVGASQYLF	-FIEEAQGG	318	gibbon
241	GLSMAIYLKQVITATVVLINGTNPFFGPIQVYRQIYQ	PLPHSAQLISTNALGELLELYRTFETTVGASQYLF	-FIEEAQGG	319	rhesus
241	GLSMAIHLKQVRAAVLINGTNPFFGPIQVYRQIYQ	PLPHSAQLISTNALGELLELYRTFETTVGASQYLF	-FIEEAQGG	319	marmoset
241	GLSMAIHLKQVRAVTLINGTNPFFGPIQVYRQIYQ	PLPHSAQLISTNALGELLELYRTFETTVGASQYLF	-FIEEAQGG	320	horse
241	GLSMAIHLKQVITVVLINGTNPFFGPIQVYRQIYQ	PLPHSAQLISTNALGELLELYRTFETTVGASQYLF	-FIEEAQGG	319	rabbit
241	GLSMAIHLKQVITVVLINGTNPFFGPIQVYRQIYQ	PLPHSAQLISTNALGELLELYRTFETTVGASQYLF	-FIEEAQGG	319	rabbit
241	GLSMAIHLKQVITVVLINGTNPFFGPIQVYRQIYQ	PLPHSAQLISTNALGELLELYRTFETTVGASQYLF	-FIEEAQGG	319	elephant
241	GLSMAIHLKQVITVVLINGTNPFFGPIQVYRQIYQ	PLPHSAQLISTNALGELLELYRTFETTVGASQYLF	-FIEEAQGG	319	mouse
240	GLSMAIHLKQVITVVLINGTNPFFGPIQVYRQIYQ	PLPHSAQLISTNALGELLELYRTFETTVGASQYLF	-FIEEAQGG	318	mouse
241	GLSMAIHLKQVITVVLINGTNPFFGPIQVYRQIYQ	PLPHSAQLISTNALGELLELYRTFETTVGASQYLF	-FIEEAQGG	319	rat
240	GLSMAIHLKQVITVVLINGTNPFFGPIQVYRQIYQ	PLPHSAQLISTNALGELLELYRTFETTVGASQYLF	-FIEEAQGG	318	hamster

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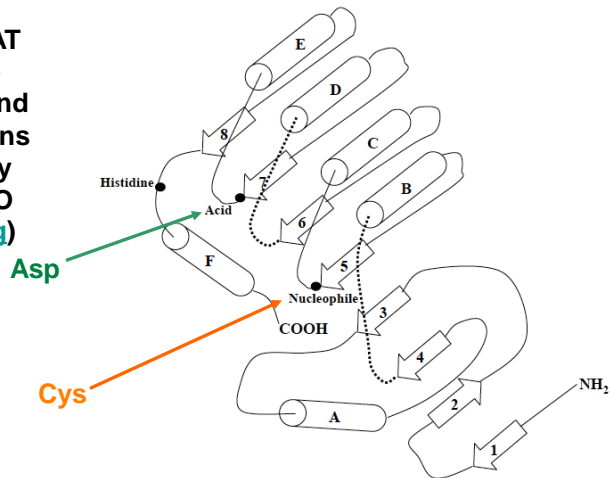
BAAT homologies

320	FLFIVGEGDKTINSKAHAEQAIQOLKRHGKNNWTL	LSYPGAGHLEIPEPYSPLCCASMTTHDLR--LHWGGEV	IPHA-AAQE	396	human
320	FLFIVGEGDKTINSKAHAEQATEOLKRHGKNNWTL	LSYPGAGHLEIPEPYSPLCCASMTTHDLR--LHWGGEV	IPHA-AAQE	396	orangutan
319	FLFIVGEGDKTINSKAHAEQAIQOLKRHGKNNWTL	LSYPGAGHLEIPEPYSPLCCASMTTHDLR--LHWGGEV	IPHA-AAQE	395	gibbon
320	FLFIVGEGDKTINSKAHAEQAIQOLKRHGKNNWTL	LSYPGAGHLEIPEPYSPLCCASMTTHDLR--LHWGGEV	IPHA-AAQE	396	rhesus
320	LLFIVGEGDDKNSKAHAEQAIQOLKRHGKNNWTL	LSYPGAGHLEIPEPYSPLCCASMTTHDLR--LHWGGEV	IPHA-AAQE	396	marmoset
321	FLFIVGEGDDKNSKAHAEQATEOLKRHGKNNWTL	LSYPGAGHLEIPEPYSPLCCASMTTHDLR--LHWGGEV	IPHA-AAQE	399	horse
320	FLFIVGEGDDKNSKAHAEQATEOLKRHGKNNWTL	LSYPGAGHLEIPEPYSPLCCASMTTHDLR--LHWGGEV	IPHA-AAQE	398	rabbit
320	FLFIVGEGDDKNSKAHAEQATEOLKRHGKNNWTL	LSYPGAGHLEIPEPYSPLCCASMTTHDLR--LHWGGEV	IPHA-AAQE	398	rabbit
320	FLFIVGEGDDKNSKAHAEQATEOLKRHGKNNWTL	LSYPGAGHLEIPEPYSPLCCASMTTHDLR--LHWGGEV	IPHA-AAQE	396	elephant
320	FLFIVGEGDDKNSKAHAEQATEOLKRHGKNNWTL	LSYPGAGHLEIPEPYSPLCCASMTTHDLR--LHWGGEV	IPHA-AAQE	398	mouse
319	FLFIVGEGDDKNSKAHAEQATEOLKRHGKNNWTL	LSYPGAGHLEIPEPYSPLCCASMTTHDLR--LHWGGEV	IPHA-AAQE	398	mouse
320	FLFIVGEGDDKNSKAHAEQATEOLKRHGKNNWTL	LSYPGAGHLEIPEPYSPLCCASMTTHDLR--LHWGGEV	IPHA-AAQE	398	rat
319	FLFIVGEGDDKNSKAHAEQATEOLKRHGKNNWTL	LSYPGAGHLEIPEPYSPLCCASMTTHDLR--LHWGGEV	IPHA-AAQE	397	hamster
397	HSWKEIQKFLRKHLIPDVTSQI			418	human
397	HSWKEIQKFLRKHLIPDVTSQI			418	orangutan
396	HSWKEIQKFLRKHLIPDVTSQI			417	gibbon
397	HSWKEIQKFLRKHLIPDMTSPL			418	rhesus
397	HSWKEIQKFLRKHLIPDVTSQI			418	marmoset
400	HSWKEIQKFLRKHLIPVVTSQL			421	horse
399	HSWKEIQKFLRKHLIPVIPSQI			420	rabbit
399	HSWKEIQKFLRKHLIPVIPSQI			420	rabbit
397	HSWKEIQKFLRKHLIPVLTSQL			418	elephant
399	HSWKEIQKFLKQHLIPDLSQL			420	mouse
399	HSWKEIQKFLKQHLIPDLSQL			420	mouse
399	HSWKEIQKFLKQHLIPDLSQL			420	rat
398	HSWKEIQKFLKQHLIPDLSQL			419	hamster

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

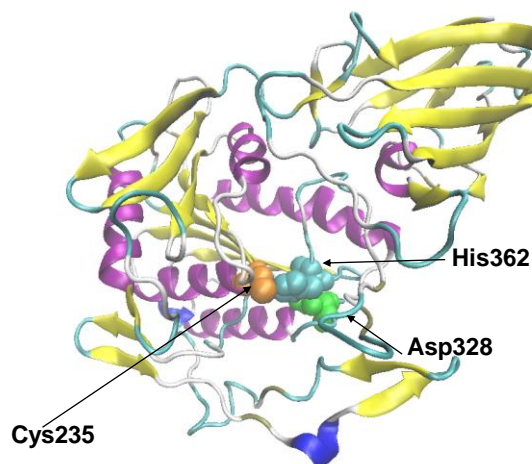
The regions of hBAT that were part of α -helices, β -sheets and unstructured regions were determined by the use of the JUFO (www.Meilerlab.org)



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Modeled structure of hBAT

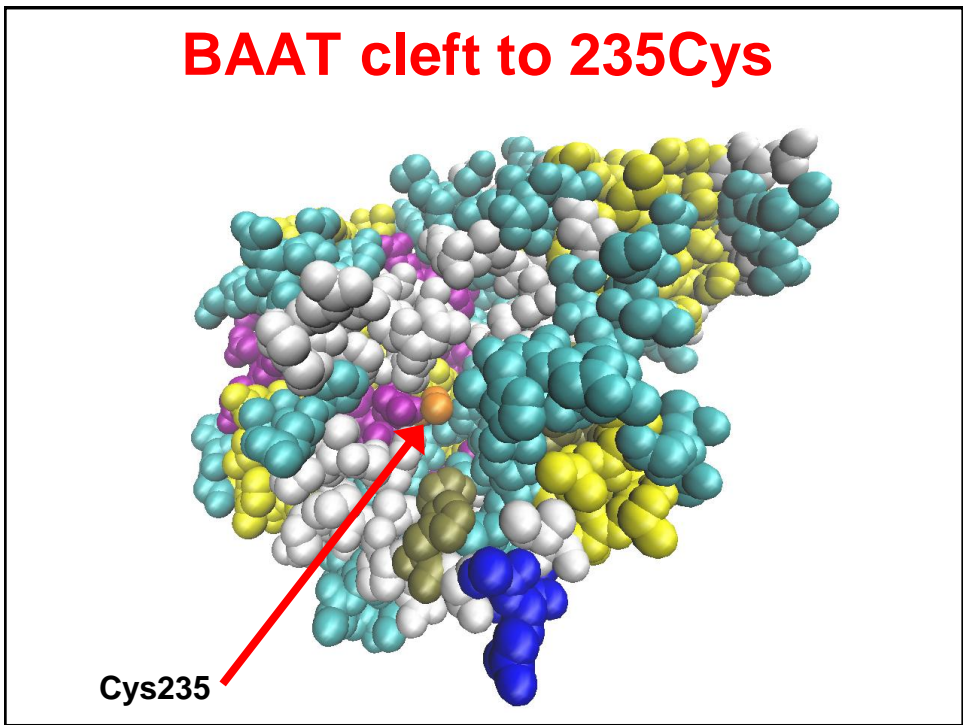
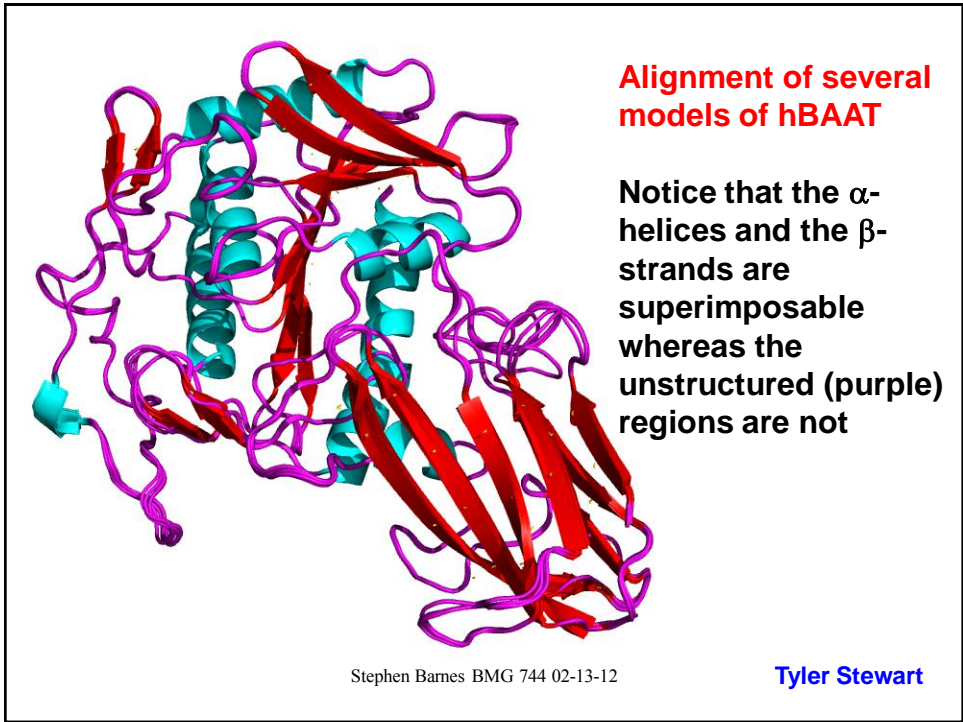
In the absence of hBAT crystals, structural homology experiments using FUGUE were performed. These identified two previously crystallized proteins, *ACOT2* and *ACOT4* as structural homologs of hBAT. These two structures were used to create a threaded structure for hBAT which was refined using Modeller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

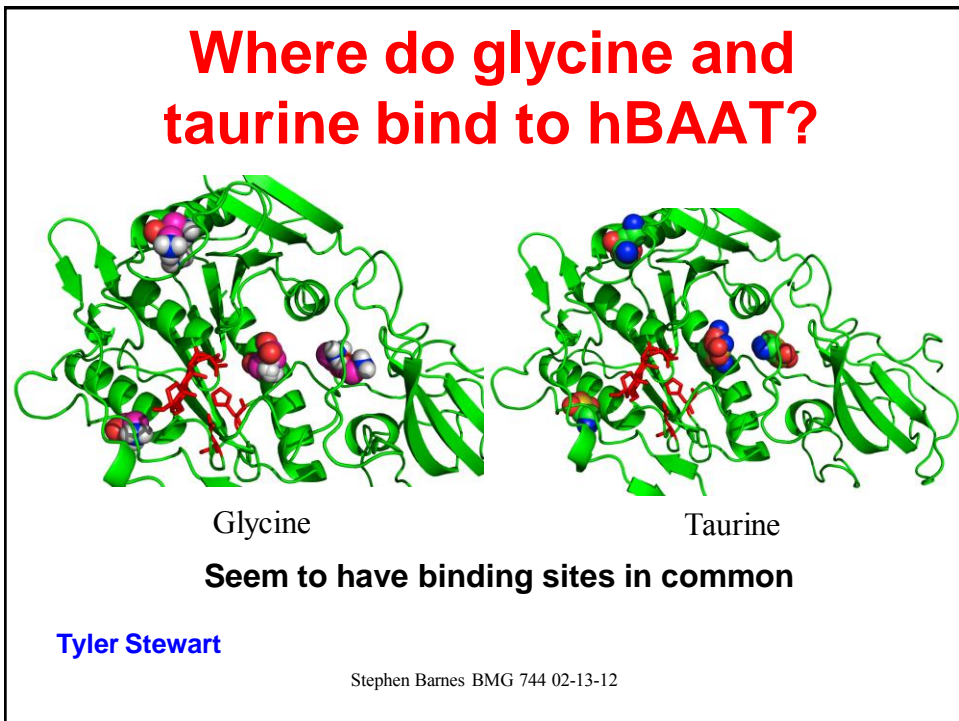
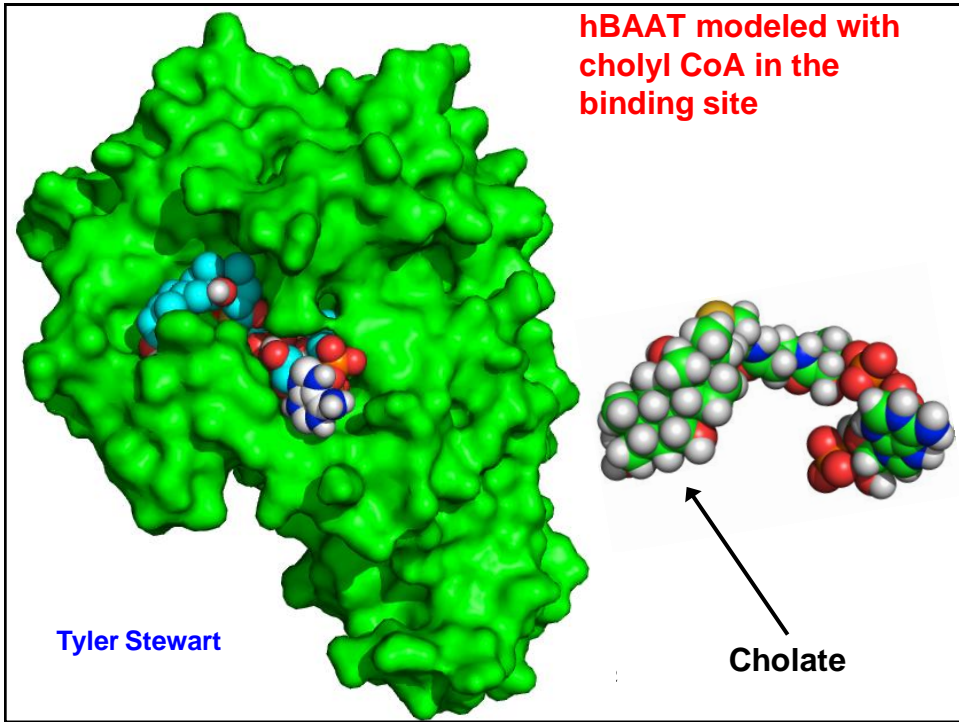


Tyler Stewart

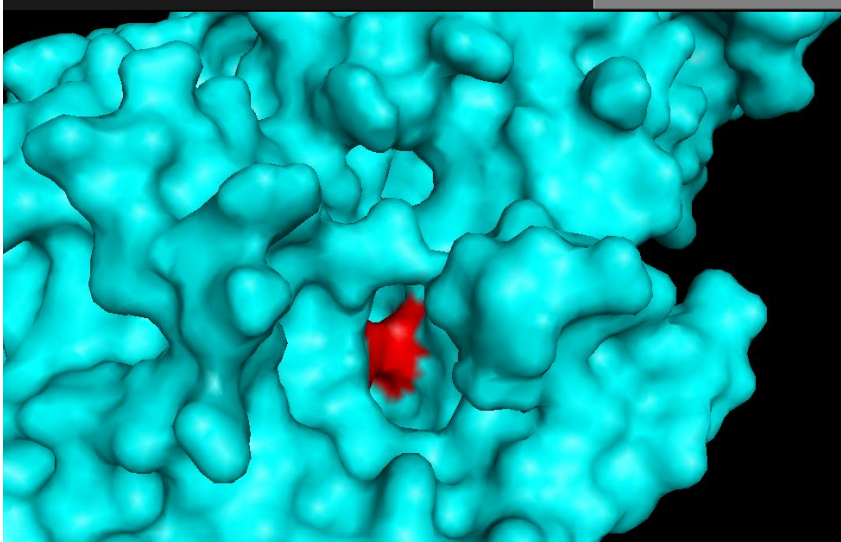
<http://tardis.nibio.go.jp/fugue/prfsearch.html>

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Visualization of the taurine “tunnel” on hBAAT



Tyler Stewart

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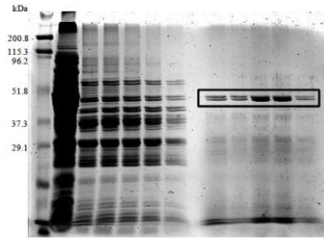
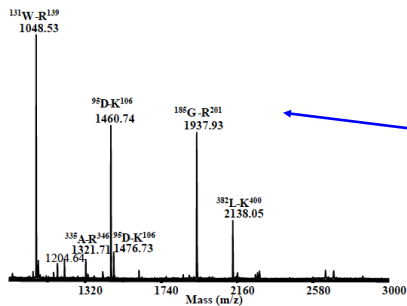
Hypothesis

- 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 choyl 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

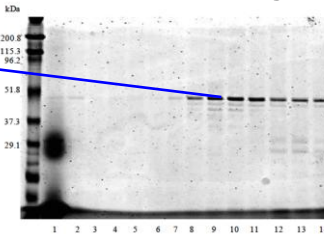
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Purification of wt-hBAT

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



Chromatofocusing

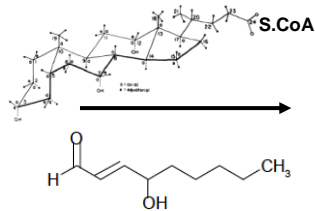


Erin Shonsey

Stephen Barnes BMG 744 02-13-12 DEAE-Sepacryl

Experimental plan

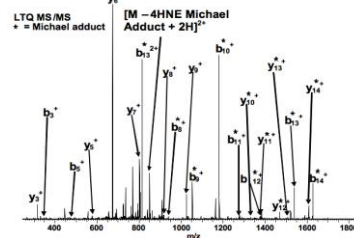
hBAT -NH₂
-SH
-imidazole



hBAT adducts

Digestion with trypsin and chymotrypsin

S Y P G A G H L I E P P Y S P L



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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

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
 (+156) (+156) (+156) (+156) (+156) (+156) (+156) (+156) (+156) (+156) (+156) (+156) (+156) (+156) (+156) (+156) (+156) (+156) (+156)

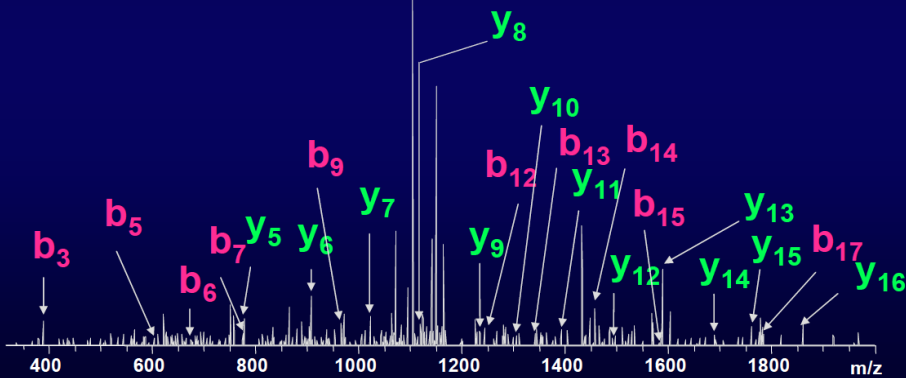
Peptide mass: 2346.3061

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
 \llcorner \llcorner \llcorner \llcorner \llcorner \llcorner \llcorner \llcorner \llcorner \llcorner \llcorner \llcorner \llcorner \llcorner \llcorner \llcorner \llcorner \llcorner \llcorner

$[M+2H]^{2+}$ - HNE
 Michael adduct



Erin Shonsey

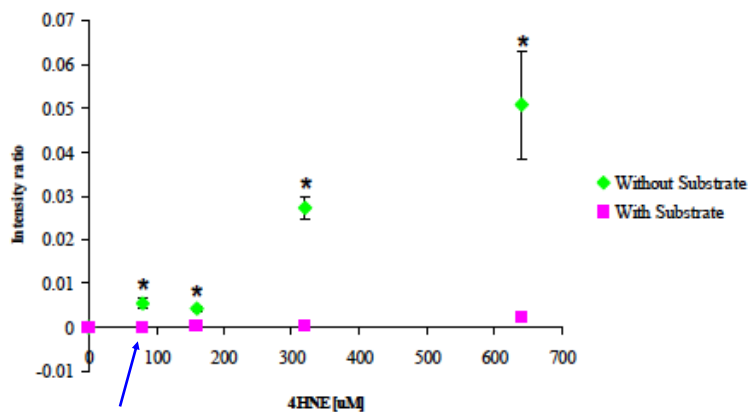
Concentration-dependent sites of 4HNE modification on hBAT

Peptide	Modified Amino Acid				
	128 μ M HNE	64 μ M HNE	32 μ M HNE	16 μ M HNE	8 μ M HNE
AHAEQAIGQLKR	H336	H336	H336	H336	H336
RLHWGGEVIPHAAAQEHAWK	H397	H397	H397	H397	
AQQQLFVIVGEGDKTINSK	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	H362, C372, C373, H378	H362	H362	H362	H362
STTHDLR					

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Shonsey et al., 2007

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



Concentration used for comparative analyses

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