Use of mass spectrometry in the study of enzymes

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MCLM 452

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Overview of class

- Modification of the enzyme to regulate its activity
- Examining the chemistry of enzyme:substrate intermediates
  - Locating the site of inactivation of suicide inhibitors
- Reaction mechanism
  - Measuring all substrates and products
  - Enzyme kinetics
  - Structural studies
  - Stopped flow
- BAT, my kinda ‘zyme - Erin Shonsey

A good review of this topic

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

Mass spectrometry and the study of enzymes

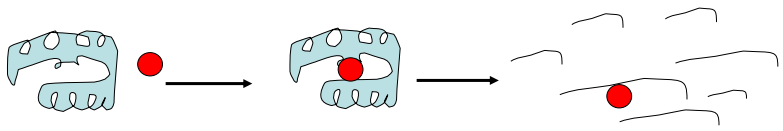
- **Enzymes often undergo posttranslational modifications in order to be active under the conditions in a cell**
  - **for example, many enzymes in the signal transduction pathways are activated by phosphorylation on serine, threonine and tyrosine residues**
    - **EGF receptor (tyrosine kinase), TGF beta type I receptor (serine kinase)**
  - **sites of phosphorylation can be determined by mass spectrometry because of the increase in mass of *m/z* 80 of peptides containing each phosphate group**

Enzymes and Mass Spec

Enzymes may undergo changes in structure once activated (see above) or during the reaction they catalyze

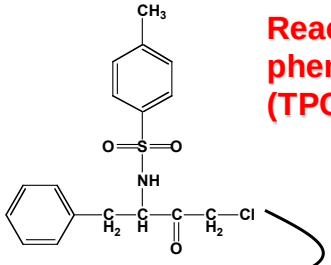
- **this could be probed by H-D exchange experiments (this is coming up in Peter Prevelige's lectures)**
- **simulation of phosphorylation may be necessary by mutating serine and threonine groups to aspartate and glutamate, respectively**

MS of enzymes



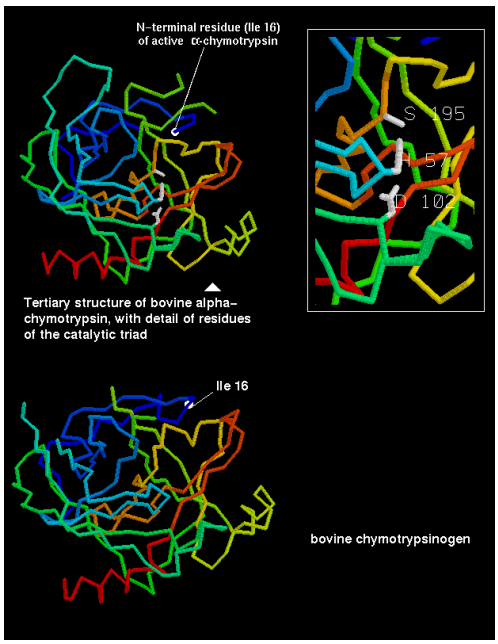
Enzymes can be inactivated by suicide substrates - these come into the active site and undergo a covalent reaction, thereby blocking the approach of other substrate molecules

- **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

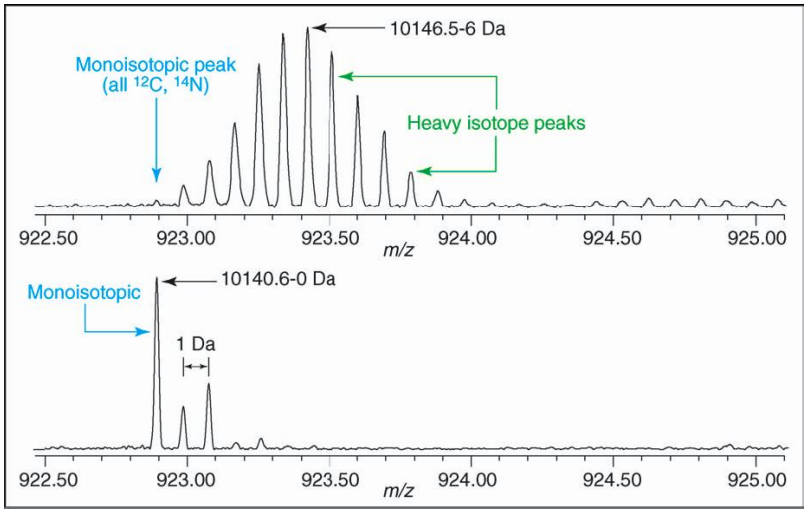
Glu-C

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

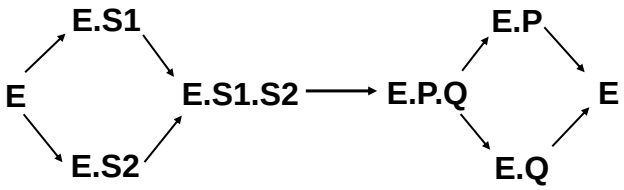
Mass spectrometry and enzyme-catalyzed reactions

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.

$$E + S \longrightarrow ES \longrightarrow EP \longrightarrow E + P$$

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

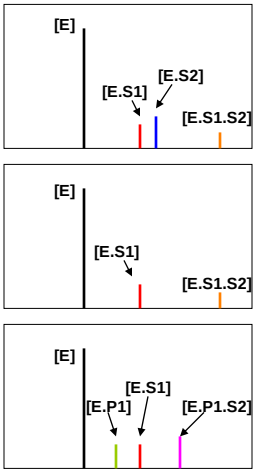
Mass spectrometry and enzyme-catalyzed reactions

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

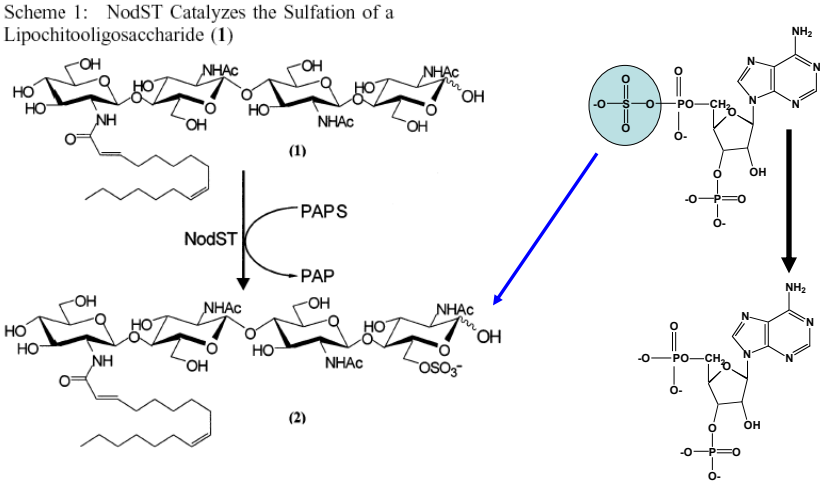


Mass spectrometry and substrates and products of enzyme reactions

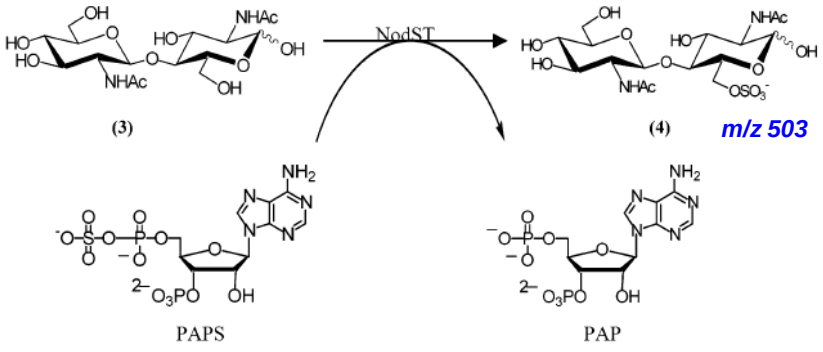
- Most enzyme reactions are studied by measuring the appearance of a product or (more rarely) the disappearance of a substrate
- If the substrate or product has a unique absorbance or fluorescence, the reaction can be followed in real time
- Some substrates have no usable absorbance or fluorescence -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

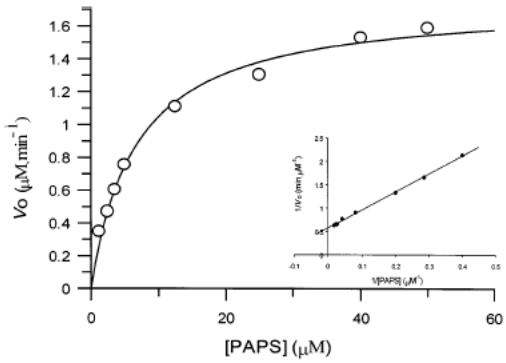


FIGURE 3: Saturation plot of V_0 vs [PAPS]. The inset is a double-reciprocal plot of $1/V_0$ vs 1/[PAPS] ([PAPS] = 1.25, 2.5, 3.5, 5, 12.5, 25, 40, and 50 μM, [chistobiose] = 1 mM, [NodST] = 90 nM, and pH 8.0).

Inhibition of ST by PAP using ESI-MS

Pi et al., Biochemistry 41:13283

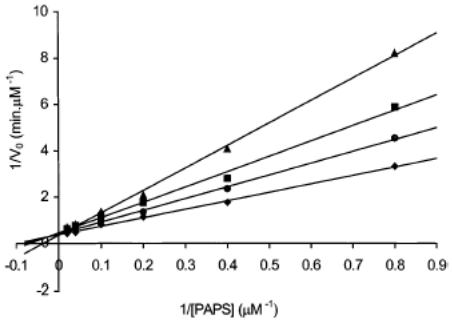
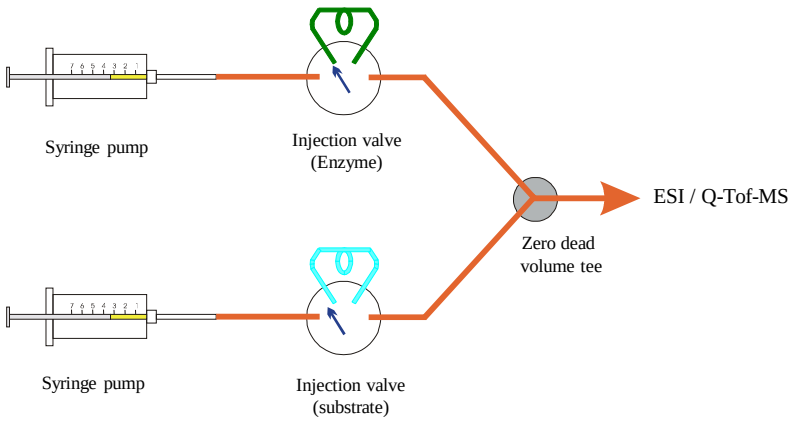


FIGURE 5: Double-reciprocal plot of $1/V_0$ vs 1/[PAPS] at different PAP concentrations: 0 μM PAP (▲), 0.75 μM PAP (■), 1.5 μM PAP (●), and 3.0 μM PAP (◆) ([PAPS] = 1.25, 2.5, 5, 10, 25, and 50 μM, [chitobiose] = 1 mM, [NodST] = 90 nM, and pH 8.0).

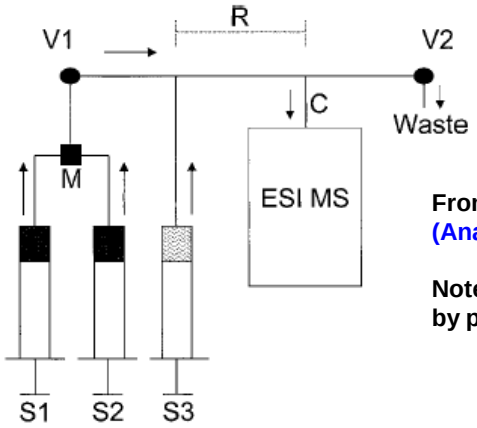
Non-covalent enzyme:substrate complexes

- **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



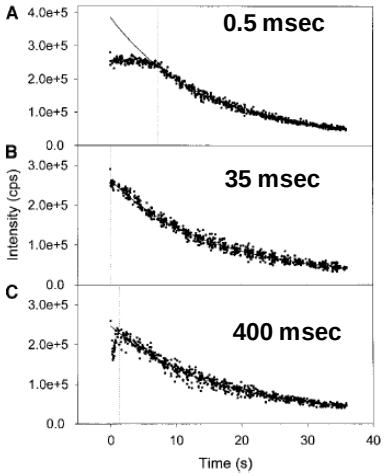
Stopped flow set up



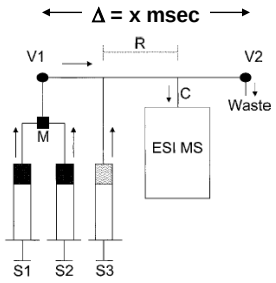
From Kolakowski and Konermann (Anal Biochem 292:107)

Note the additional flow introduced by pump S3

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

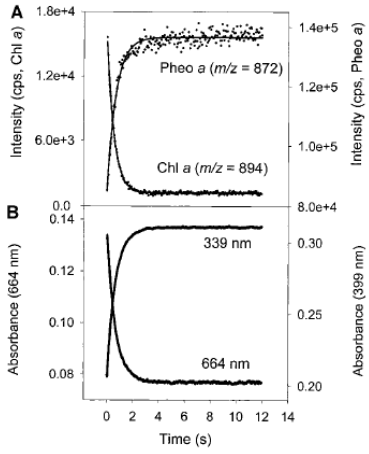


In this reaction, hydrolysis of acetylcholine in an alkaline buffer is monitored by the ion at *m/z* 146



Kolakowski and Konermann (Anal Biochem 292:107)

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

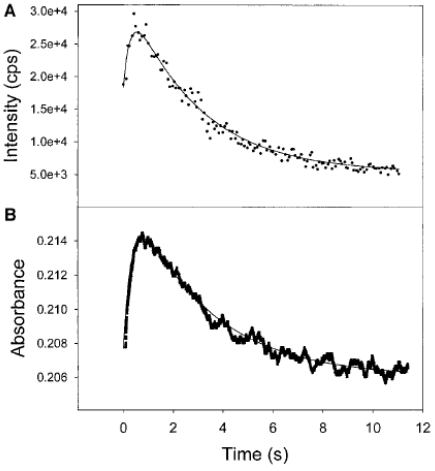


These data are from the conversion of chlorophyll A to pheophytin A (loss of Mg and gain of two protons).

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**

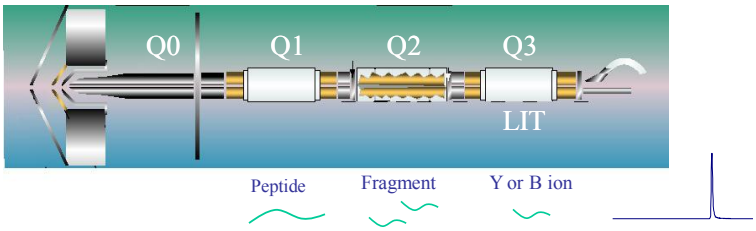
Studying multiple enzymes simultaneously

- 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)

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

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

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

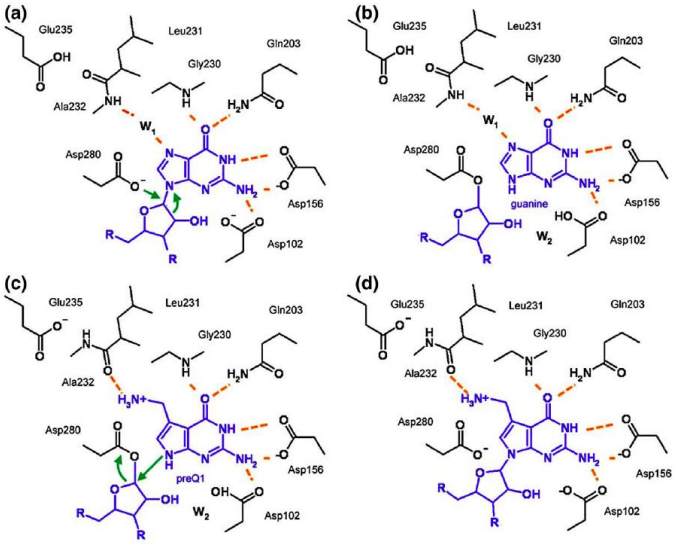
Lahoz et al., 2008

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)

Tgt enzyme reaction



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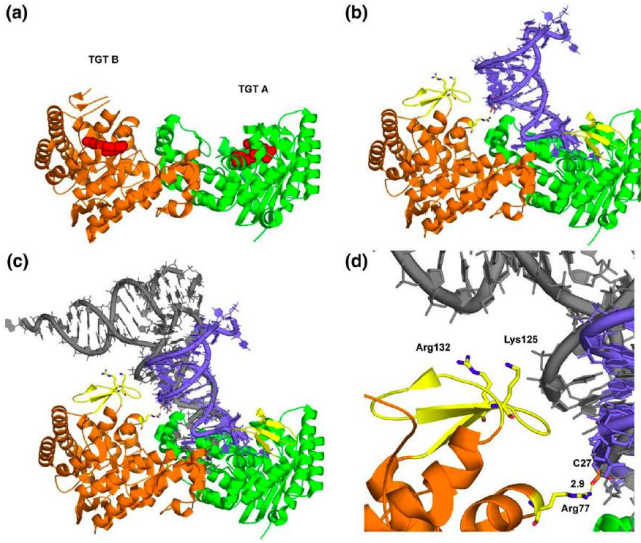
Structures of Tgt complex



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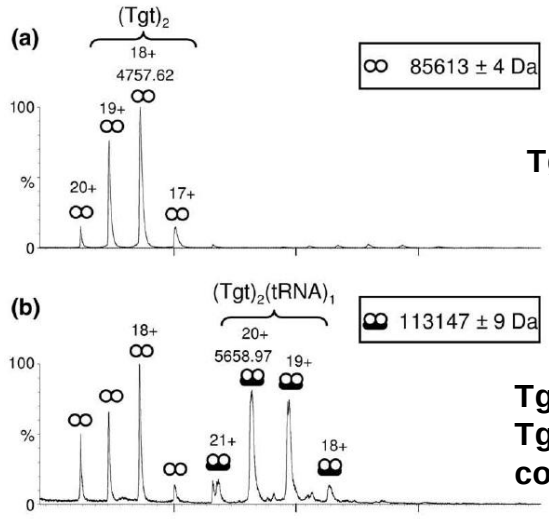
Non-covalent ESI-MS of Tgt



Tgt dimer

Tgt dimer and Tgt-tRNA complex

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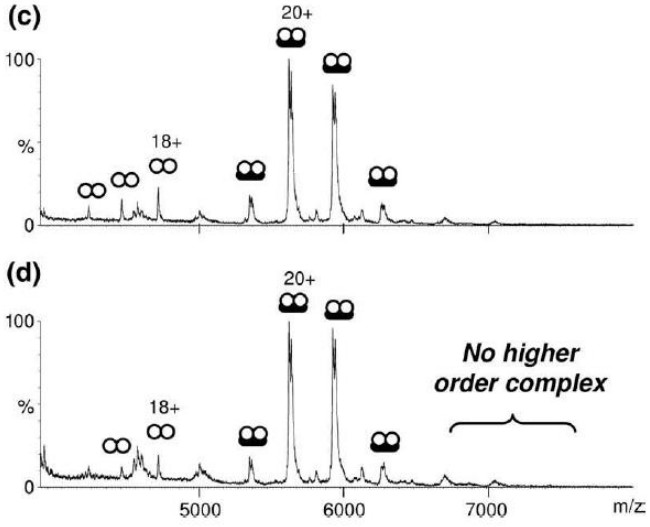
Adding more tRNA does not lead to higher order complexes



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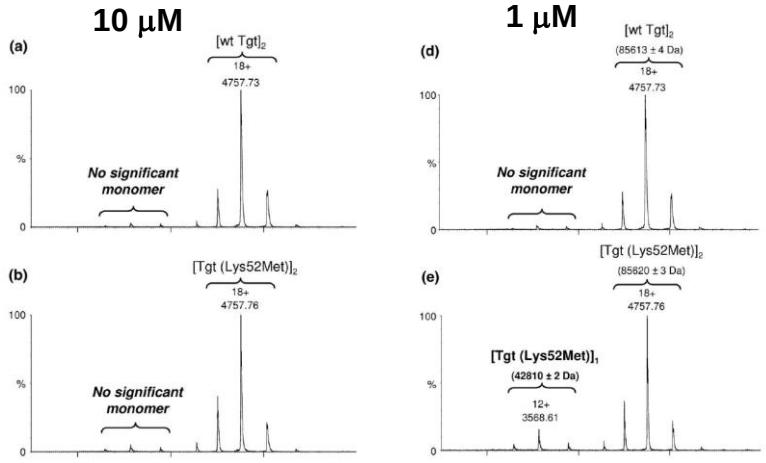
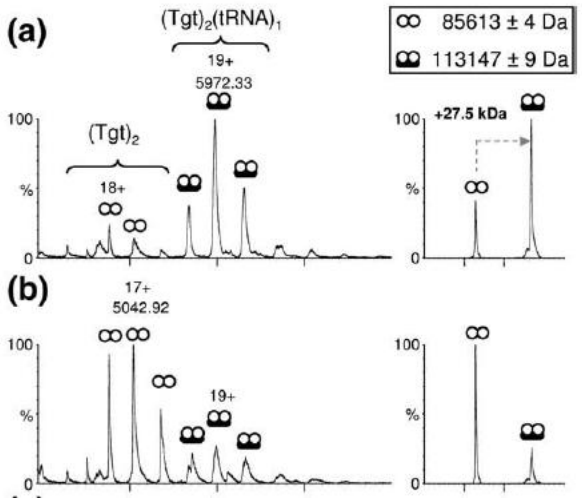
Evidence for Tgt dimer dissociation in Lys52Met mutant



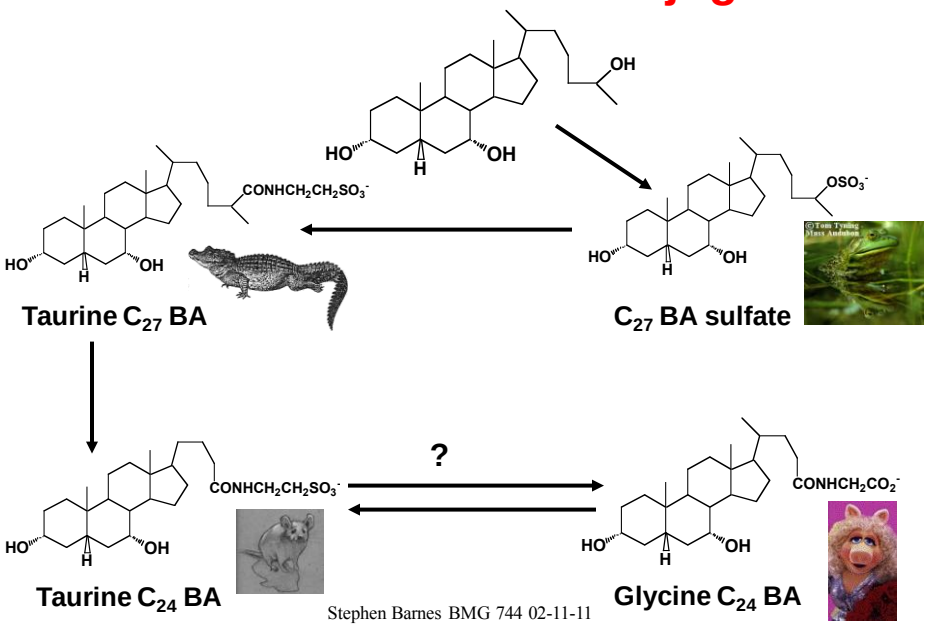
Effect of an inhibitor on formation of Tgt dimer-tRNA complex



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



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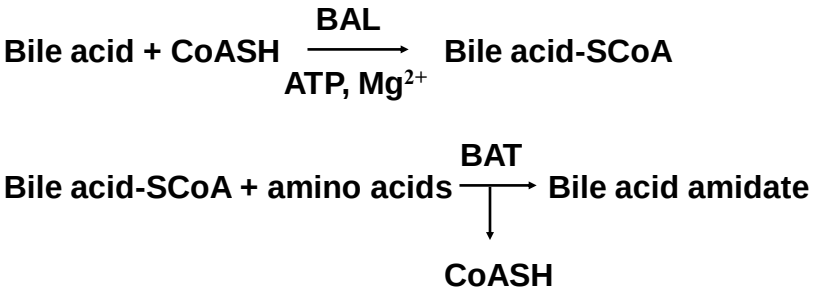
Bile acid N-acylamidate formation (in hepatocytes)



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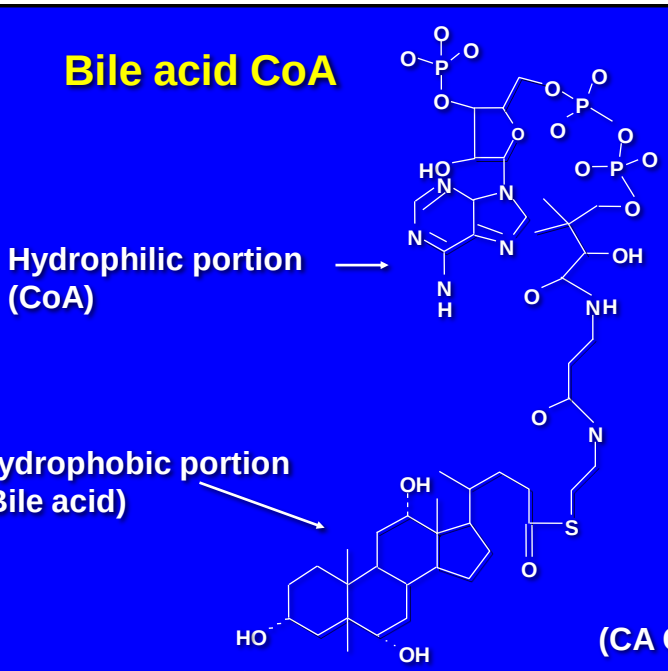
Bile acid CoA



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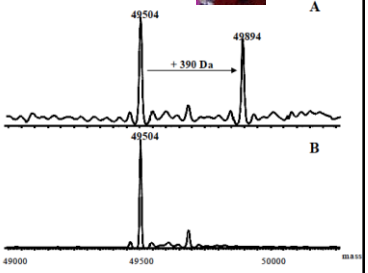
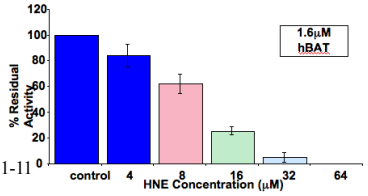
Background

- 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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Homology between BATs

```
hBAT    MIQLTATPVSALVDEPVHIRATGLIPFQMVSFQASLEDENGDMFYSQAHYRANEFGEVDL  60
Kan-1   MAKLTAVPLSALVDEPVHIRVTGLTPFQVVCLQASLKDDKGNLFNSQAFYRASEVGEVDL  60
mBAT    MAKLTAVPLSALVDEPVHIQVTGLAPFQVVCLQASLKDER-KPVSSQAFYRASEVGEVDL  59

hBAT    NHASSLGGDYMGVHPMGLFWSLKPEKLLTRLLKRDVMNRPFQVQVKLYDLELIVNNKVAS  120
Kan-1   ERDSSLGGDYMGVHPMGLFWSMKPEKLLTRLVKRDVMNRPHKVHIKLCHPYFPVEGKVIS  120
mBAT    EHDPSLGGDYMGVHPMGLFWSLKPEKLLGRLIKRDVINSPYQIHIKACHPYFPLQDLVVS  119

hBAT    APKASLTLERWYVAPGVTRIKVREGRLRGALFLPPGEGLFPGVIDLFGGLGGLLEFRASL  180
Kan-1   SSLDSLILERWYMAPGVTRIHVKEGRIRGALFLPPGEGPFPGVIDLFGGAGGLFEFRASL  180
mBAT    PPLDSLTLERWYVAPGVKRIQVKESRIRGALFLPPGEGPFPGVIDLFGGAGGLMEFRASL  179

hBAT    LASRGFASLALAYHNYEDLPRKPEVTDLEYFEEAANFLLRHPKVFGSGVGVVSVCQGVQI  240
Kan-1   LASHGFATLALAYWGYDDLPSRLEKVDLEYFEEGVEFLLRHPKVLGPGVGILSVCIGAEI  240
mBAT    LASRGFATLALAYWNYDDLPSRLEKVDLEYFEEGVEFLLRHPKVLGPGVGILSVCIGAEI  239

hBAT    GLSMAIYLKQVTATVLINGTNFPFGIPQVYHGQIHQPLPHSAQLISTNALGLLELYRTFE  300
Kan-1   GLSMAINLKQITATVLINGPNFVSSNPHVYRGKVFQPTPCSEEFVTTNALGLVEFYRTFE  300
mBAT    GLSMAINLKQIRATVLINGPNFVSQSPHVYHGQVYPPVPSNEEFVVTNALGLVEFYRTFQ  299

hBAT    TTQVGASQYLFPIEEAQGQFLFIVGEGDKTINSKAHAEQAIGQLKRHGKNNWTLLSYPGA  360
Kan-1   ETADKDSKYCFPIEKAHGHFLFVVGEDDKNLNSKVHAKQAIAQLMKSGKKNWTLLSYPGA  360
mBAT    ETADKDSKYCFPIEKAHGHFLFVVGEDDKNLNSKVHANQAIAQLMKNGKKNWTLLSYPGA  359

hBAT    GHLIEPPYSPLCCASTTHDLR--LHWGGEVIPH-AAAQEHAWKEIQRFLRKHLIPDVTSQL  418
Kan-1   GHLIEPPYSPLCSASRMPFVIPSINWGGEVIPH-AAAQEHSWKEIQKFLKQHLNPGFNSQL  420
mBAT    GHLIEPPYTPLCQASRMPILIPSLSWGGEVIPHSQAAQEHSWKEIQKFLKQHLLPDLSSQL  420
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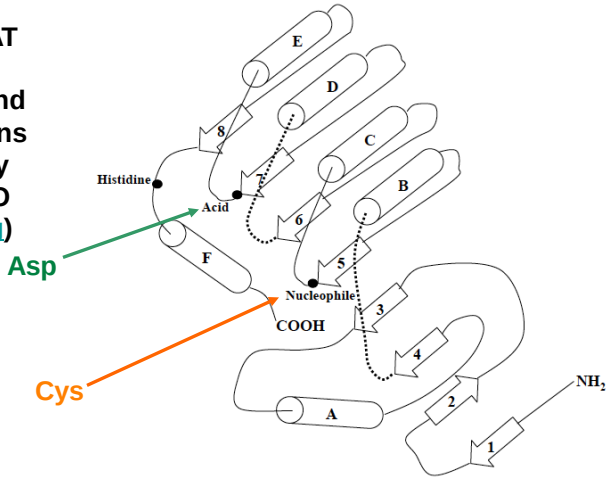
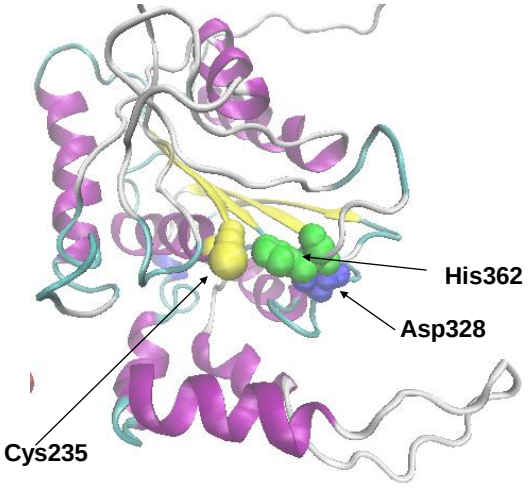
hBAT is a member of a class of proteins with an α/β hydrolase fold

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)



Modeled structure of hBAT

In the absence of hBAT crystals, structural homology experiments using FUGUE were performed. These identified two previously crystallized proteins, *dienelactone hydrolase* and *acylamino acid releasing enzyme* as structural homologs of hBAT. These two structures were used to create a threaded structure for hBAT which was refined using Modeller 9v2

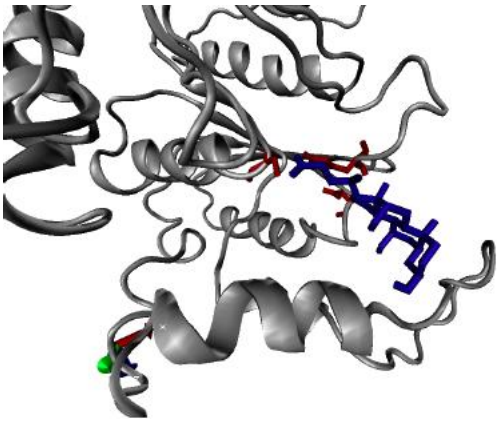


http://tardis.nibio.go.jp/fugue/prfsearch.html

Modeling the cholate-hBAT adduct

Docking of cholic acid (in blue) with hBAT was carried out with (1) an open method (GRAMM) allowing interaction at any site, and (2) docking directed at the Cys235 residue.

The latter produced low and high energy configurations. The former of these is shown here (the catalytic triad residues are in red).

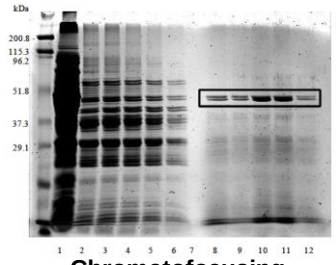


Hypothesis

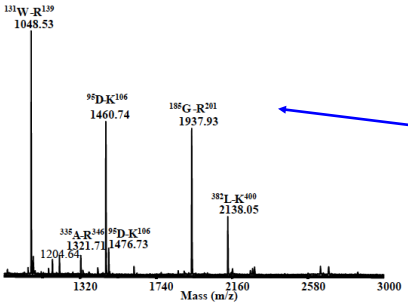
- **The ability of 4HNE to react with Cys, His and Lys residues is dependent on their accessibility to solvent containing 4HNE.**
- **Therefore, in the presence of cholyl CoA, and hence covalently linked cholate to Cys235, the accessibility of 4HNE to the active site will be blocked, thereby revealing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

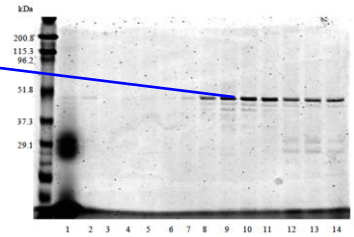
Purification of wt-hBAT

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



Chromatofocusing

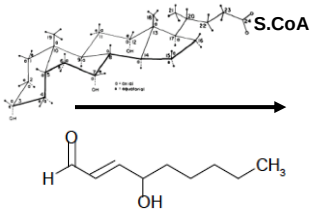




Stephen Barnes BMG 744 02-11-11 **DEAE-Sephacryl**

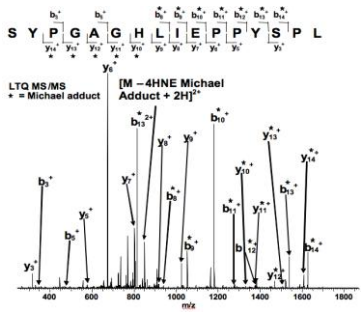
Experimental plan

hBAT -NH_2
-SH
-imidazole



hBAT adducts

Digestion with trypsin and chymotrypsin



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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

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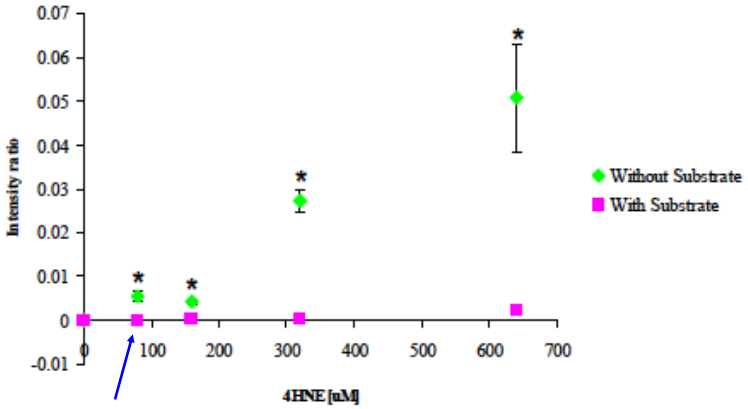
Shonsey et al., 2007

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



Concentration used for comparative analyses

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Cholyl CoA blocks 4HNE adduct formation on hBAT

- P-values for the effect of cholyl CoA on blocking 4HNE modifications (equimolar)
  - His62 0.0341
  - His194 0.0240
  - His336 0.2113
  - His362/Cys372 0.0384
  - His383 0.0063
  - His407 0.0010

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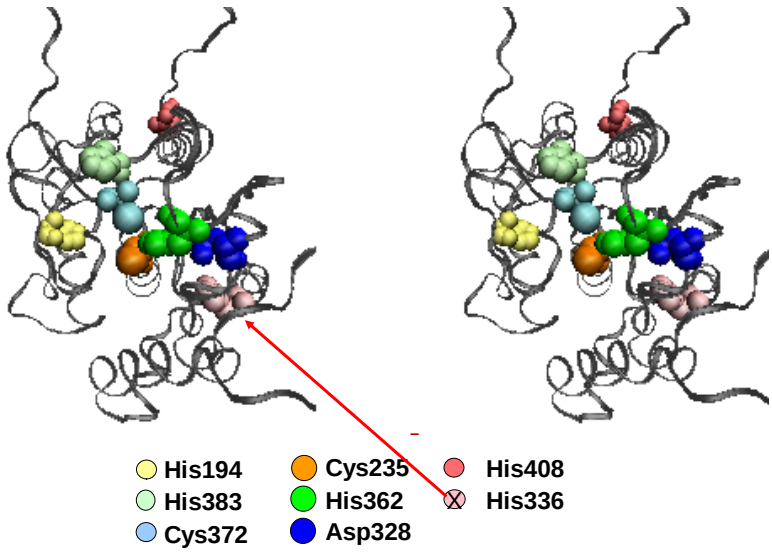
Sites of 4HNE modification of hBAT blocked by cholyl CoA



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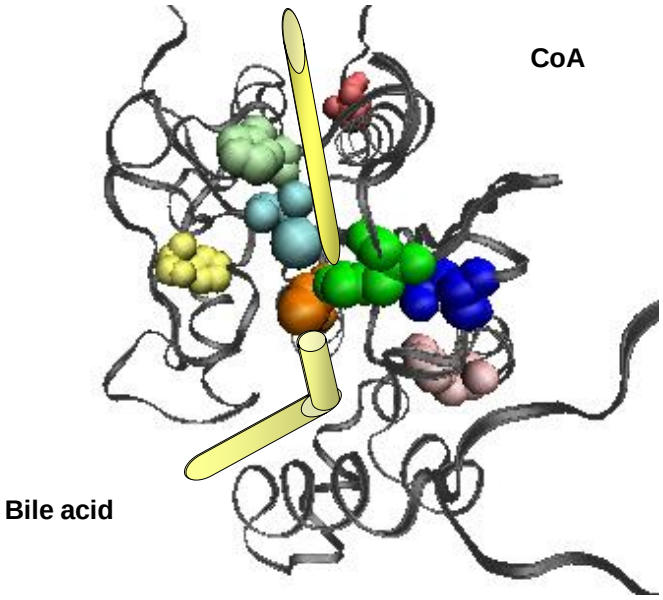
Channels in hBAT to accommodate cholyl CoA



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