ON THE PATH TOWARDS THE CRYSTAL STRUCTURE OF CHOLESTEROL TRANSFER PROTEIN (CETP)

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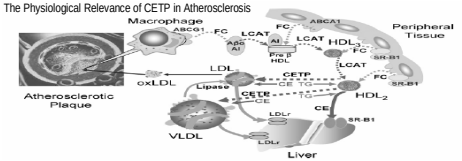
ABSTRACT

Cholesteryl ester transfer protein (CETP) shuttles various lipids between lipoproteins, resulting in the net transfer of cholesteryl esters from atheroprotective, high-density lipoproteins (HDL) to atherogenic, lower-density species. Inhibition of CETP raises HDL cholesterol and may potentially be used to treat cardiovascular disease.
Plasma CETP has 476 amino acids and a 74 kDa molecular mass, of which 28 % by mass is attributed to glycosylation at N88, N240, N341 and N396. The high content of glycosylation, hydrophobic residues (44%) and free cysteines (5) presented significant challenges for structural studies. To improve crystallizability, parallel approaches were utilized involving combinations of mutations to control glycosylation and free cysteines and complex formation with antibodies.
The first structure of CETP was solved at 3.5 Å using crystals of a CETP triple mutant in complex with an antibody Fab fragment. Another fully active triple mutant later yielded superior crystals without Fab. The higher resolution structure was solved by molecular replacement to 2.2 Å. The path towards obtaining these high resolution crystals involved a multi-construct strategy to obtain crystallizable protein. Techniques such as Light Scattering, Biacore, and Analytical Ultracentrifugation, were used extensively to characterize and compare the properties of constructs and as QC checks for multiple batches of the same construct, and, where co-complex crystallization was attempted, whether a complex was obtained in solution prior to crystallization. The techniques were used in conjunction with extensive crystal screening experiments to obtain consistently reproducible crystals of CETP.

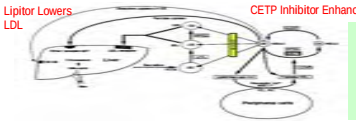
INTRODUCTION

The Physiological Relevance of CETP in Atherosclerosis



Model for CETP in reverse cholesterol transport. Free cholesterol (FC) in peripheral tissues or atherosclerotic plaque is removed via ABC transporters through their interaction with apo-AI. The esterification of cholesterol with fatty acids by the LCAT reaction drives the growth of HDL particles first via discoidal preβ-HDL and then to mature spherical HDL particles via smaller HDL3 and larger HDL2. Spherical HDL2 and HDL3 particles and mature HDL do not interact well with ABCA1 in peripheral tissue but can access additional FC through apo-AI-mediated interaction with related specific macrophage ABCG1 transporters or the SR-B1 scavenger receptors found in peripheral cells. CE's can be delivered directly to the liver by the ineteraction of HDL particles with the hepatic SR-B1 receptor or indirectly following the CETP process that transfers CE from HDL to LDL and VLDL particles in exchange for TG. The CE in VLDL and LDL particles can then be taken up in the liver by the hepatic LDL receptor (LDLr).

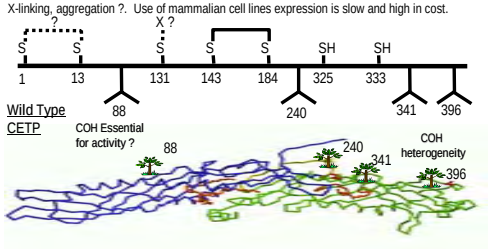
Effect of Inhibition of CETP



A combination therapy provides a comprehensive approach for the treatment of Atherosclerosis

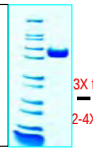
CETP: A MOLECULE WITH COMPLEX ISSUES!

A highly hydrophobic molecule: possibility of aggregation, solubility issues, low yield. Heavily N-glycosylated at 4 sites (25% of protein). Contains 7 Cysteines: possibility of issues with X-linking, aggregation ?. Use of mammalian cell lines expression is slow and high in cost.



IMPROVING PURIFICATION

Conventional (20-40% yield)
Hydrophobic Chrom.
(elute in H_2O)
Anion Exchange Chrom.
Buffer Exchange

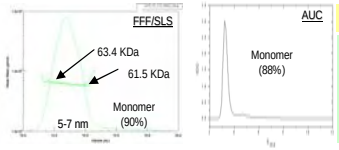


3X faster
2-4X more

Affinity (85% yield)
CNBR-X-linked 2E7
(anti-CETP mAb)
Elute: Citrate gradient pH 6-4
Buffer Exchange

EVALUATING SOLUTION PROPERTIES

FFF/Static Light Scattering and Sedimentation Velocity evaluation of native WT-CETP

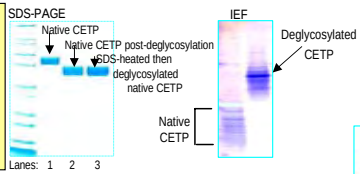


MW by MALDI MS: 62.3/60.5 KDa

- Solution properties of native CETP do not significantly change with conc. or with time.
- AUC and DLS data are consistent with an extended structure, similar to BPI molecule.

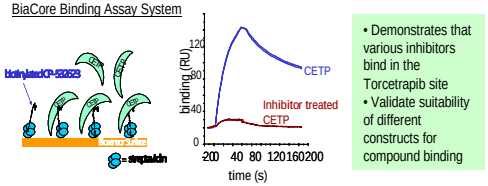
IMPROVING CARBOHYDRATE HOMOGENEITY

Deglycosylation Protocol
Purified Protein
4hrs w/PNGase-F (37ºC)
HIC
Buffer Exchange
Concentration



DEVELOPING A BIOPHYSICAL ASSAY FOR INHIBITOR BINDING

BiaCore Binding Assay System



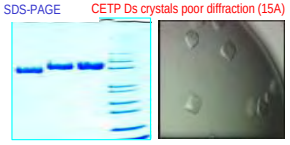
- Demonstrates that various inhibitors bind in the Torcetrapib site
- Validate suitability of different constructs for compound binding

LESSONS LEARNED

Enzymatic Deglycosylation
- Is not complete
- Protein can be De-sialydated
- Deglycosylated protein is pure (>95%)
- Homogeneous (85-90% monomer)
- Moderately Active (75-80%)
- Stable ($Tm_{1/2}$ >70C)

Important Learning:
- Sugar @ 240 & 396 removable
- Sugar @ 88 & 341 not removable

SDS-PAGE



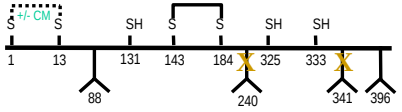
CETP Ds crystals poor diffraction (15A)

DsDg Ds WT Cys 1-13 cross-linking ?? (1-131)

IMPROVE SOLUTION PROPERTIES WHILE MAINTAINING ACTIVITY

Strategy: Mutate non-removable glycosylation, and Cys (requires additional protein constructs)

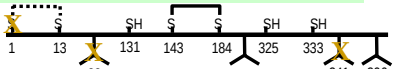
Double Mutant



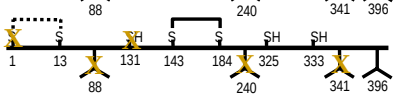
Key points in the design of new constructs using Double Mutant as the model
- N88 thought to be essential for activity and secretion
- Extensive deglycosylation of DM did not abolish activity (70%)
- Cysteine cross-linking found. Alkylation increases activity

Two, of several new constructs cloned, expressed and purified, are shown below.

CETP 434

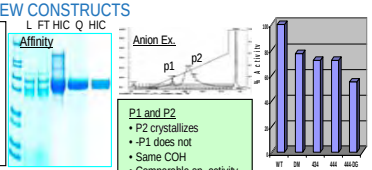


CETP 444



PURIFICATION OF NEW CONSTRUCTS

Purification Protocol
- Concentrate Medium 50X
- mAb Affinity
- HIC
- Anion Exchange
- HIC
- Concentration

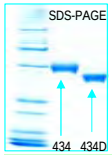


Using this mwethod protein is > 99% pure, very stable, and 70% active

P1 and P2
- P2 crystallizes
- -P1 does not
- Same COH
- Comparable sp. activity
- Charged Lipid ?
- Conformation ?

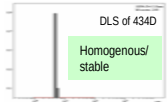
N88 is not essential for activity

DEGLYCOSYLATION; STABILITY; BINDING

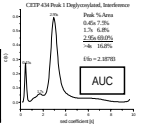


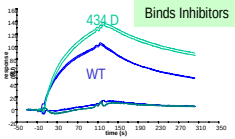
Fully deglycosylated

Sequence mass	LCMS mass
53046 Da	53048 Da

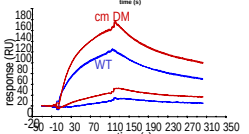


(1 week post-deglycosylation)





Binds Inhibitors



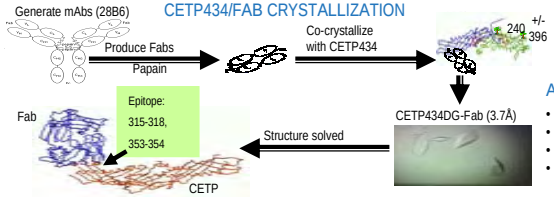
LESSONS LEARNED

- Improvements in homogeneity
- All constructs bind inhibitors
- Bound phospholipid is reduced (1:1).
- Cholesterol is non-detectable
- WT, DM, 434 crystallized with weak diffraction (10-15Å)
- Full deglycosylation of CETP434 did not improve crystal quality

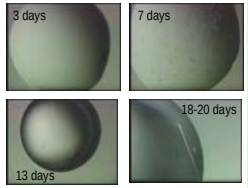
STRATEGY TO IMPROVE CRYSTAL QUALITY

- Generate monoclonal Fabs
- Evaluate compound binding to CETP-Fab complex
- Co-crystallize in the presence of Fabs (28B6)
  - With alkylated DM
  - With glycosylated and deglycosylated CETP434
- Advance CETP444 into crystallization trials
- Requires generation of mAbs and production of Fabs

CETP434/FAB CRYSTALLIZATION



CETP-444 NON-DEGLYCOSYLATED PROTEIN CRYSTALLIZATION



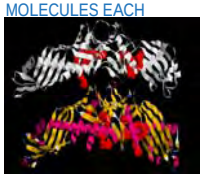
- Crystals appear in 3 days,
- Grow over another 15 days to 20-30 x 20-30 x 200um
- Diffraction = 3.2-3.4A (in house)
- Diffraction = 2.1-2.3A (IMCA APS)

2.2A Crystal Structure of Non-deglycosylated Apo-CETP-444

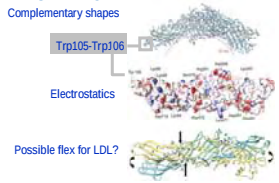
Key learnings:
- 4C is essential
- [CETP-444] > 5mg/ml (10mg/ml stock)
- Crystals are reproducible in 48-well and 24-well plates
- Seeding: Micro and Streak used for improving crystal size

No crystals if:
- Temp = 22C
- 444 is deglycosylated
- With 434 or DM
- Co-crystallized with inhibitors……so far!

BPI (WHITE) AND CETP BIND TWO PHOSPHOLIPID MOLECULES EACH



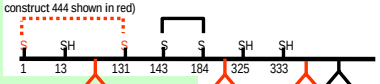
CONCAVE SURFACE AND CONFORMITY WITH SHAPE OF HDL



LESSONS LEARNED

- Multiple cell systems
- Multiple constructs
- Tech. developments
- Stability-yield issues
- Heterogeneity issues
- Doability issues

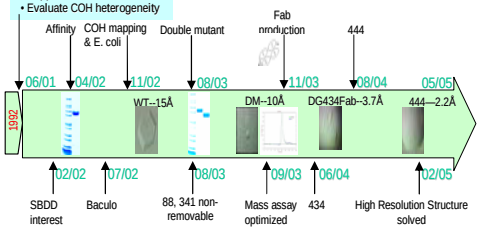
Native CETP (changes and modifications to produce crystallizable CETP, construct 444 shown in red)



- Cysteine Xlinking is 1-131
- Fully deglycosylated CETP does not produce Hi-Res crystals
- Lipid removal could improve crystal soaking

IN CONCLUSION: CETP TIMELINE

- Support CETP-WT
- Evaluate COH heterogeneity



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