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Binding site analysis of an Acyl CoA Binding Protein from *Plasmodium falciparum*

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Chapter 1
Introduction

1.1 *Plasmodium falciparum*

Plasmodium falciparum is an intracellular parasite that causes the highly infectious disease malaria. It is transmitted by the female *Anopheles* mosquito and is very prevalent in developing countries like ours. Clinical symptoms of the parasite appear during the asexual stages of its life cycle. Malaria caused by this species of *Plasmodium* is the most dangerous form of malaria with highest mortality rate [1].

The life cycle of *Plasmodium falciparum* is slightly complex. The parasite is present as Sporozoites, a spore like form of the parasite, within the salivary glands of an infected female *Anopheles* mosquito. They enter the human bloodstream upon a mosquito bite. These sporozoites then invade liver hepatocytes. The sporozoites remaining in the bloodstream are cleared within 30 minutes. Once these sporozoites enter the hepatocytes, they undergo asexual division and differentiation to form merozoites. This process takes place typically over a period of 10-14 days and results in tens of thousands of merozoites. These merozoites burst open from the hepatocytes and enter the bloodstream, where individual merozoites invade red blood cells (RBCs) and again undergo cell division via the process of schizogony. In this, first the nucleus undergoes multiple rounds of division to form smaller nuclei and then the rest of the cell divides accordingly. A total of 12-16 cells form from each mother merozoite. Some merozoites undergo division to form sexual form of the parasite, female and male gametocytes. All these cells rupture the RBCs and enter the bloodstream, this is when the clinical symptoms appear. The gametocytes enter into a female *Anopheles* mosquito when it bites a diseased person. The male and female gametocytes enter the mosquito gut where fertilization occurs, and finally lead to formation of hundreds of sporozoites which ultimately invade the mosquito's salivary glands. The whole cycle repeats when this mosquito bites a healthy person.

1.2 Acyl CoA binding proteins

Acyl CoA binding proteins are small cytosolic proteins and are approximately 10-55 kDa in size. The larger proteins have acyl CoA binding as one of the function among others due to presence of an acyl CoA binding domain. The main function of acyl CoA binding proteins is to bind to long, medium and short chain fatty acyl CoA molecules in the cell. The structure and sequence of acyl CoA binding proteins is highly conserved across various species from different kingdoms. It has a characteristic structure made by specific

arrangement of 4 alpha helices [2]. Acyl CoA binding proteins are very important for cell's proper functioning. By binding to fatty acyl CoA, these proteins regulate the pool size of free fatty acyl CoA molecules within the cell. Fatty acyl CoA is involved in regulation of various metabolic pathways. It is involved in synthesis of various types of lipids i.e. sphingolipid, glycerolipid etc [3].

It has been reported that long chain fatty acyl CoA molecules inhibit the function of acetyl-CoA carboxylases involved in the initial steps of fatty acid biosynthesis [4]. Fatty acyl CoA molecules are involved in regulation of membrane trafficking as well. It has been previously demonstrated that Palmitoyl-CoA is involved in membrane trafficking by using Triascin C, an acyl CoA synthase inhibitor, which severely hindered membrane transport [5]. The mechanism however, is still unclear.

It was found that acyl-CoA esters decreased the free Ca^{2+}concentration in steady-state in clonal permeabilized pancreatic β-cells (HIT-cells) in a chain-length and concentration-dependent manner [6]. It was demonstrated that the level of long-chain acyl-CoAs increased 2-fold when clonal β-cells were incubated with Palmitate for 18 h. It was also observed that long-chain acyl-CoA esters induced reversible, concentration dependent opening of ATP-sensitive potassium channels [7].

Palmitoyl CoA and Oleoyl CoA, and not their fatty acid counterparts have been involved in regulation of activity of cellular Protein Kinase C (PKC), an important enzyme involved in activation of various intermediary proteins of different signalling cascades. Both Palmitoyl and Oleoyl CoA increased cytosolic PKC activity by 60-70% in presence of Ca^{2+}, diacylglycerol and phosphatidylserine [8]. Long-chain acyl-CoA esters also enhance the activity of PKC from rat brain cells. This was studied both with and without added diacylglycerol but in the presence of phosphatidylserine and Ca^{2+}, in both cases [9]. The effect was less pronounced in the absence of diacylglycerol.

Hence fatty acyl CoA molecules, being involved in so many different regulatory roles are very important for proper cellular functioning. Acyl CoA binding proteins, being important regulators of concentration of free fatty acyl CoA are very tightly controlled in terms of expression and degradation and their level of expression can reveal several features of cellular physiology.

1.3 Acyl CoA binding proteins in *Plasmodium falciparum*

Acyl CoA binding proteins in *P. falciparum* are not much studied. According to PlasmoDB database, a total of six acyl CoA binding proteins have been identified in *P. falciparum* namely PF3D7_1477800 (ACBP749), PF3D7_1001100.1 (ACBP14), PF3D7_1001100.2 (ACBP15), PF3D7_0810000 (ACBP99), PF3D7_1001200 (ACBP16) and PF3D7_1119000 (ACBP197). Expression studies for all 6 have been done but only of a genomic scale via microarray analysis of the parasite at different life cycle stages.

Structural studies have been done only for one of the paralogs of pfACBPs, ACBP749. The crystal structure shows the protein bound to its ligand. Recently, it was reported that several differences exist between the binding pocket of bovine ACBP and pfACBP. This finding could be exploited for identifying drug targets against *P. falciparum*. Discovery of specific compounds that bind to pfACBP and not human ACBP can be used to hinder the parasite's metabolic processes in malaria infected patients without affecting the host's metabolism. Hence it is very important to study the characteristics of the binding pocket of pfACBPs in greater detail and compare the findings with that of human ACBPs to effectively narrow down on possible drug candidates.

1.4 Objectives of this study

The objective of my study is to first identify the binding site of ACBP16 using bioinformatics tools and then to study the importance of individual residues of the binding pocket which are interacting with the ligand Myristoyl-CoA. The importance of each residue would be studied by mutating the residue into an Alanine and then measuring the quenching constant of the following reaction:

$$P + L \rightleftharpoons P\text{-}L$$

P-L is protein-ligand complex, P is protein and L is ligand. If the mutation causes loss of binding, the quenching constant, decreases. On the other hand, if the mutation causes the binding to increase, the quenching constant value would increase. The measurement of quenching constant would be done by studying the fluorescence quenching of Trp residues of ACBP upon ligand binding.

Chapter 2
Materials and Methods

2.1 Materials

2.1.1 Bacterial strains

Strain	**Genotype**
E. coli DH5α	F– Φ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F) U169 *rec*A1 *end*A1 *hsd*R17 (rK,mK+) *pho*A *sup*E44 λ– *thi*-1 *gyr*A96 *rel*A1
E. coli BL21 (DE3)	fhuA2 [lon] ompT gal (λ DE3) [dcm] ΔhsdS λ DE3 = λ sBamHIoΔEcoRI-B int::(lacI::PlacUV5::T7 gene1) i21 Δnin5

Table 1. Bacterial strains used and their genotypes

2.1.2 Plasmids

Plasmid	**Description**	**Reference**
pET 21b	Expression vector with ampicillin resistance	Novagen
pET21b_ac16	Overexpression of ACBP16 protein	This study
pET21b_ac16_Y30A	Overexpression of ACBP mutant Y30A	This study
pET21b_ac16_Y33A	Overexpression of ACBP mutant Y33A	This study
pET21b_ac16_K34A	Overexpression of ACBP mutant K34A	This study
pET21b_ac16_K56A	Overexpression of ACBP mutant K56A	This study

Table 2. Plasmids used in this study

2.1.3 Primers

Oligonucleotide ID	Sequence 5'----3'	Purpose
AR_ACBP16_FP	CATATGATGGAAGATTTATTTCAAGC	Gene specific forward primer for amplification of ACBP16
AR_ACBP16_RP	CTCGAGTTATTCTTTATTTTGCCAAT	Gene specific reverse primer for amplification of ACBP16
AR_ACBP16_Y30A_FP	GCTAGATTTAGCTAAATATTATAAGC	Mutagenesis forward primer for creating Y30A mutant of ACBP16
AR_ACBP16_Y30A_RP	GCTTATAATATTTAGCTAAATCTAGC	Mutagenesis reverse primer for creating Y30A mutant of ACBP16
AR_ACBP16_Y33A_FP	GATTTATATAAATATGCTAAGCAAAG	Mutagenesis forward primer for creating Y33A mutant of ACBP16
AR_ACBP16_Y33A_RP	CTTTGCTTAGCATATTTATATAAATC	Mutagenesis reverse primer for creating Y33A mutant of ACBP16
AR_ACBP16_K34A_FP	GATTTATATAAATATTATGCGCAAAGTAC	Mutagenesis forward primer

		for creating K34A mutant of ACBP16
AR_ACBP16_K34A_RP	GTACTTTGCGCATAATATTTATATAAATC	Mutagenesis reverse primer for creating K34A mutant of ACBP16
AR_ACBP16_K56A_FP	GATAAAAAAGCATATGAGGC	Mutagenesis forward primer for creating K56A mutant of ACBP16
AR_ACBP16_K56A_RP	GCCTCATATGCTTTTTTATC	Mutagenesis reverse primer for creating K56A mutant of ACBP16

Table 3. Oligonucleotides used in this study

2.1.4 Enzymes

One Taq® Master Mix w/ GC buffer by New England BioLabs.

NdeI by New England Biolabs

XhoI by New England Biolabs

T4 DNA Ligase by New England Biolabs

2.1.5 Antibiotics

Ampicillin

Stock conc.: 100mg/ml

Working conc.: 100µg/ml

Nalidixic Acid

Stock conc.: 100mg/ml

Working conc.: 100µg/ml

2.1.6 Media

LB medium

Tryptone 10gm

Yeast Extract 5gm

NaCl 10gm

Volume made upto 1000ml with water and pH adjusted to 7.0-7.2 with 1N NaOH

LB agar media

LB medium 1000ml

Bacto-agar 15gm

Ladders

TrackIt 50bp DNA ladder by Life Technologies

Generuler 1kb DNA ladder by Life Technologies

Benchmark unstained protein ladder by Life Technologies

2.1.7 Buffers and chemicals

Tris-Acetate-EDTA (TAE) buffer

Tris-acetate 40mM

EDTA(pH8.0) 2mM

SDS running buffer

Tris	25mM
Glycine	92mM
SDS	0.1% (w/v)

Lysis buffer

Na_2HPO_4	50mM
NaCl	300mM
Imidazole	10mM

Wash buffer

Na_2HPO_4	25mM
NaCl	250mM
Imidazole	50mM

Elution buffer

Na_2HPO_4	25mM
NaCl	250mM
Imidazole	300mM

Fluorimetry buffer

Na_2HPO_4	20mM
NaCl	50mM

CD buffer

Na_2HPO_4	20mM
NaF	50mM

IPTG, Guanidine Hydrochloride, Ethidium Bromide

2.2 Methods

2.2.1 *Insilico* analysis

2.2.1.1 Homology modelling of ACBP16

Crystal structure of ACBP16 was required to find out the important interacting residues within the binding pocket. However, the crystal structure for ACBP16 has not been solved till now; hence an alternate approach for modelling of ACBP16 was taken. A model for ACBP16 was made based on its sequence similarity with existing crystal structures via homology modelling software modeller9.15. First, an NCBI BLAST search was run for ACBP16 protein against database of proteins with known crystal structures. The top four results with the highest sequence similarity were taken, their pdb file and FASTA file were downloaded and renamed “tseq1.pdb”, “tseq2.pdb”, “tseq3.pdb”, “tseq4.pdb” and “tseq1.fasta”, “tseq2.fasta”, “tseq3.fasta”, “tseq4.fasta” respectively. “tseq1” corresponded to the result with highest sequence similarity. Script shown in Fig.1 was written to compare models of the four results based on RMSD and to do a multiple sequence alignment of the four results with our ACBP16 protein and output it as a dendogram

```
from modeller import *
env = environ()
aln = alignment(env)
for (pdb, chain) in (('tseq1', 'A'), ('tseq2', 'A'), ('tseq3', 'A'), ('tseq4', 'A')):
m = model(env, file=pdb, model_segment=('FIRST:'+chain, 'LAST:'+chain))
aln.append_model(m, atom_files=pdb, align_codes=pdb+chain)
aln.malign()
aln.malign3d()
aln.compare_structures()
aln.id_table(matrix_file='family.mat')
env.dendrogram(matrix_file='family.mat', cluster_cut=-1.0)
```

Fig. 1. Modeller script1.

Then, modeller script 2 (shown in Fig. 2) was used to align ACBP16 with the best result of the dendogram.

```
from modeller import *
env = environ()
aln = alignment(env)
mdl = model(env, file='tseq1', model_segment=('FIRST:A','LAST:A'))
aln.append_model(mdl, align_codes='tseq1', atom_files='tseq1.pdb')
aln.append(file='qseq1.ali', align_codes='qseq1')
aln.align2d()
aln.write(file='qseq1-tseq1.ali', alignment_format='PIR')
aln.write(file='qseq1-tseq1.pap', alignment_format='PAP'
```

Fig. 2. Modeller script 2

Then, modeller script 3 was used to make a model of ACBP16 based on its sequence alignment with the best result of the dendogram. Fig. 3 shows modeller script 3.

```
from modeller import *
frommodeller.automodel import *
#from modeller import soap_protein_od
env = environ()
a = automodel(env, alnfile='qseq1-tseq1.ali', knowns='tseq1', sequence='qseq1',
assess_methods=(assess.DOPE, #soap_protein_od.Scorer(), assess.GA341))
a.starting_model = 1
a.ending_model = 5
a.make()
```

Fig. 3. Modeller script 3

A total of five models were generated and were assessed based on calculated Discrete Optimized Protein Energy (DOPE) score. The model with the best (lowest) DOPE score was selected for further analysis. The model was then validated by checking its Ramachandran plot using online server and was compared with ACBP749 using visualization program, Visual Molecular Dynamics.

2.2.1.2 Binding site analysis of ACBP749

The binding site of ACBP749 was analysed from its crystal structure which depicted the protein in its ligand bound form. Interacted residues within the binding pocket were identified and were later mutagenized via site directed mutagenesis.

2.2.1.3 Sequence alignment of ACBP749 with ACBP16

The protein sequence of ACBP749 was aligned with that of ACBP16 and the position of residues of binding site of ACBP749 were marked in both.

2.2.2 Molecular biology techniques

2.2.2.1 Generation of mutant acbp16 genes

Mutant ACBPs were generated using overlap extension PCR. Complementary mutagenic primers were used to incorporate mutations into the gene. At first, mutagenic and gene specific primers were taken (according to Table 3) and used to generate 2 smaller fragments from acbp16 gene. These fragments were then combined in another PCR reaction, in which the complementary ends, incorporated due to the complementary mutagenic primers were annealed allowing the 3' overlap of each strand to act as a primer for the 3' extension of the complementary strand. The resulting fusion is further amplified using gene specific primers giving full length mutant genes as PCR products.

Mutants were made such that each mutant protein would be an alanine mutant one of the ligand interacting residues. The final amplicons were PCR purified, quantified in NanoDrop™ and aliquoted to be subjected to Agarose gel electrophoresis to check size and purity.

2.2.2.2 Purification of amplified DNA fragments by PCR purification

Removal of residual enzyme and buffers from previous experiments is necessary for downstream processing of DNA. To do the same, commercially available kits were used. Binding buffer B2 was added to the PCR mix in 4:1 ratio and mixed well. The mixture was put in a quick spin column and centrifuged at 13000 RPM for 1 min. The flow through was discarded. After this, 500µl wash buffer W1 was added into the column, followed by centrifugation at 13000 RPM for 1 min. An additional centrifugation at maximum speed was given for 2-3 mins to remove residual ethanol. The column was transferred to an elution tube. 40µl of nuclease free water was added and the tube was incubated for 1 min at room temperature. Then, the tube containing the column was centrifuged at 13000 RPM for 1 min. The flow through collected in the elution tube was the purified DNA.

2.2.2.3 Agarose gel electrophoresis

Agarose gel electrophoresis was used to check the purity and/or size of a DNA sample wherever required. In brief, DNA samples were mixed with appropriate volume of 6x loading dye (0.25% bromophenol blue, 0.25% xylene cyanol and 30% glycerol in water) and subjected to electrophoresis through 1% Agarose gel. Electrophoresis was done in 1x TAE buffer and ethidium bromide was added to the gel during preparation to a final concentration of 0.5µg/ml for visualization of DNA bands under UV light.

2.2.2.4 Restriction digestion of acbp16Y30A, acbp16Y33A, acbp16K34A, acbp16K56A and pET 21b

acbp16Y30A, acbp16Y33A, acbp16K34A, acbp16K56A and pET 21b vector were digested with restriction enzymes *NdeI* and *XhoI* in Fast Digest buffer and incubated for 3-4 h. The digested products, were subjected to Agarose gel electrophoresis and the corresponding bands were cut from the gel, purified via gel elution protocol and quantified using NanoDrop™.

2.2.2.5 DNA purification from gel via gel elution protocol

Whenever DNA of high purity was to be obtained, DNA was run on gel and purified from the gel slice using gel elution protocol. To do the same, commercially available kits were used. Solubilization buffer L3 was added to the gel slice in 3:1 ratio and incubated at 50^{o}C for 10 mins with frequent mixing. After that, it was mixed well with 1 volume of isopropanol. The mixture was put in a quick spin column and centrifuged at 13000 RPM for 1 min. The flow through was discarded. After this, 500µl wash buffer W1 was added into the column, followed by centrifugation at 13000 RPM for 1 min. An additional centrifugation at maximum speed was given for 2-3 mins to remove residual ethanol. The column was transferred to an elution tube. 40µl of nuclease free water was added and the tube was incubated for 5 mins at room temperature. Then, the tube containing the column was centrifuged at 13000 RPM for 1 min. The flow through collected in the elution tube is the purified DNA.

2.2.2.6 Ligation of acbp16Y30A, acbp16Y33A, acbp16K34A, acbp16K56A and pET 21b

A total of four ligation reactions were set up each with pET 21b and one of the PCR amplicons to give pET21b_Y30A, pET21b_Y33A, pET21b_K34A and pET21b_K56A respectively. 70ng of vector was taken and the molar ratio of vector:insert was kept as 1:3 for the ligation reaction. T4 DNA ligase was used and the mixture was incubated at 16^{o} C overnight after which *E. coli* DH5α cells were transformed with the ligation mixture.

2.2.2.7 Transformation of *E. coli* DH5α with the ligation mixture

E. coli DH5α ultra competent cells were used for transformation. 250µl of -80^{o}C stored DH5α ultra competent cells were taken and thawed on ice. The ultra competent cells were aliquoted equally into five tubes, each containing 50µl of cell suspension. Four of the tubes were used for transformation of the four ligation mixes of acbp16Y30A, acbp16Y33A, acbp16K34A and acbp16K56A respectively and one was used as a negative control (without ligation mixture). Half (10µl) of each ligation mix was added to its respective tube of ultra competent cells and incubated in ice for 30 mins followed by heat shock at 42^{o}C for 90 s. The vials were immediately kept on ice for 1 min and incubated at 37^{o}C, 200 RPM for 1 h after addition of 1ml LB media. Cells were pelleted by centrifugation at 5000 RPM for 5 mins, resuspended in 100µl LB media and plated on an LB agar plate containing ampicillin as a selection marker at the final concentration of 100µg/ml of LB agar. The plates were incubated at 37^{o}C overnight and the colonies obtained were patched on an LB agar plate containing ampicillin and Nalidixic acid both at final concentration of 100µg/ml. Ampicillin is a selection marker for the pET 21b vector while Nalidixic acid is that for DH5α strain of *E. coli*. Afterwards, the patched colonies were screened for the presence of the insert by colony PCR.

2.2.2.8 Colony PCR of patched colonies

Patched colonies of each clone were screened by colony PCR. For each clone, a small amount of cells from a single patched colony were taken and suspended into a tube containing PCR master mix and gene specific primers. The PCR was performed and run

on an Agarose gel to check for amplification of the gene. A total of 5 patched colonies were screened for each clone. Following this, plasmid was isolated from the positive clones for each of pET21b_Y30A, pET21b_Y33A, pET21b_K34A, and pET21b_K56A and sent for DNA sequencing with T7 forward and reverse primers to confirm the presence of respective mutations.

2.2.2.9 Transformation of acbp16Y30A, acbp16Y33A, acbp16K34A and acbp16K56A mutants and wild type ACBP in *E. coli* BL21 (DE3)

E. coli BL21 (DE3) ultra competent cells were transformed with recombinant plasmids pET21b_Y30A, pET21b_Y33A, pET21b_K34A and pET21b_K56A according to the protocol mentioned above.

2.2.2.10 Overexpression of wild type and mutant ACBP proteins in *E. coli* BL21 (DE3)

Four test tubes containing 10ml LB media and ampicillin to a final concentration of 100µg/ml each were inoculated with respective BL21 transformants of the wild type and mutant ACBP16s and grown overnight at 37^{o}C at 200RPM. 10ml of each of these cultures were used to inoculate secondary cultures in 1ltr LB media containing ampicillin to a final concentration of 100µg/ml. The cultures were grown at 37^{o}C and 200 RPM till A_{600} reached 1.0 and then induced with 100mM IPTG for 4 h, after that the cells were harvested by centrifuging at 5000 RPM for 5 mins. Pelleted cells were resuspended in 120ml of lysis buffer, along with lysozyme and protease inhibitor PMSF and incubated on ice for 30 mins. The cells were further lysed by sonication for 10 mins at 30 amplitude, $PULSE_{ON}$ time 30 s and $PULSE_{OFF}$ time 40 s and the cell debris was pelleted down by centrifuging at 14000 RPM at 4^{o}C for 1 h. The supernatant containing the soluble protein was incubated with Ni-NTA beads for 1 h at 4^{o}C with gentle shaking. 2ml Ni-NTA beads were used for every 1ltr secondary culture. The supernatant was transferred to the column and allowed to settle. The flow through was collected. Each column was washed with 300ml wash buffer and the elutant was collected. All proteins were eluted with 10ml

of elution buffer. Total 10 elutions of 1ml each were taken for every protein. A small aliquot for each was taken and run on SDS PAGE for checking purity. Every protein was quantified by NanoDrop™ and stored at 4^{o}C.

2.2.2.11 Sodium Dodecyl Sulphate Poly Acrylamide Gel Electrophoresis (SDS PAGE)

SDS PAGE gel consists of stacking and resolving gels. 15% resolving gel was made using the following chemicals:

- H2O 2.3ml
- 30% Acrylamide mix 5.0ml
- 1.5M Tris (pH8.8) 2.5ml
- 10% SDS 0.1ml
- 10% ammonium persulphate 0.1ml
- TEMED 0.004ml

5% stacking gel was made using the following chemicals:

- H2O 2.3ml
- 30% Acrylamide mix 5.0ml
- 1.5M Tris (pH8.8) 2.5ml
- 10% SDS 0.1ml
- 10% ammonium persulphate 0.1ml
- TEMED 0.004ml

2.2.2.12 Dialysis of mutant and wild type proteins

Elution buffer, in which the proteins were eluted in, was exchanged for fluorimetry buffer for doing fluorimetry studies. In brief, 2ml of the highly concentrated elutions of each protein ACBP16Y30A, ACBP16Y33A, ACBP16K34A, ACBP16K56A mutants and wild type ACBP16 were taken in separate dilution bags and put in 500ml fluorimetry buffer at 4^{o}C with constant stirring. The buffer was replaced after an interval of 4 h and then 10 h. 10 h after the second buffer change, the proteins were transferred to separate microcentrifuge tubes and centrifuged at 14000 RPM at 4^{o}C for 30 mins to pellet down

any precipitated protein. The samples were then quantified by Bradford assay, aliquoted and run on SDS PAGE to check purity and stored at 4^{o}C.

2.2.2.13 Bradford assay for quantification of mutant and wild type ACBPs

Bradford assay was done to quantify protein. To do this, Bovine Serum Albumin (BSA) standards were made in the respective protein buffer of varying concentrations. Small aliquots of proteins to be measured i.e. wild type ACBP16, ACBP16Y30A, ACBP16Y33A, ACBP16K34A and ACBP16K56A mutants were taken and 3 dilutions (20x, 10x, 5x) were made. A 200µl mixture (100µl of protein + 100µl of Bradford reagent) was loaded into each well of 96 well plate and analysed in an ELISA reader.

2.2.2.14 Acyl CoA removal from purified proteins

It has been previously reported that recombinant ACBPs also come bound with fatty acyl CoA. Since for the binding studies the protein needs to be in its free form, this bound ligand was removed using guanidine hydrochloride denaturation. 500µl of 1mg/ml protein was taken and incubated with 1.5ml of 5M guanidine hydrochloride at room temperature for 30 mins. The mixture was loaded onto a PD-10 column equilibrated with the fluorimetry buffer and eluted with 6ml of the same buffer into 6 tubes, each containing 1ml of the elutant. Protein content is quantified in each using Bradford assay and protein containing samples are concentrated.

2.2.2.15 Circular Dichroism (CD) spectroscopy of mutant and wild type ACBPs

CD spectroscopy analysis was done for the mutant and wild type proteins to measure change in secondary structure if any. For CD experiments, the proteins were in CD buffer at concentration of 23µM. CD measurements were performed in JASCO-810 spectrophotometer with 0.1cm quartz cuvette. Secondary structure was analysed in far UV range (190-260 nm) with data pitch 1nm. Results obtained for mutant proteins were compared with those of wild type protein.

2.2.2.16 Fluorimetry for binding kinetics analysis of wild type and mutant ACBPs

All fluorimetry experiments were performed in fluorimetry buffer in a Hitachi F7000 fluorescence spectrophotometer, under CAT mode, with a PMT voltage of 700V and scan speed of 240nm. The binding affinity of protein for the ligand was obtained by calculating the quenching constant, K_{SV} using the equation: $(F_0/F)_{330} = 1 + K_{SV}[Q]$, where F_0 is the initial fluorescence intensity and Q is the concentration of ligand at fluorescence intensity F [10].

Chapter 3
Results and Discussions

3.1 Homology modelling of ACBP16

After performing BLAST for amino acid sequence ACBP16 protein against the PDB database the best hit obtained was ACBP749 of *P. falciparum*. Hence, ACBP16 was modelled using ACBP749 as a template (Fig. 4). The structure hence obtained was highly similar to that of ACBP749. Ramachandran plot was calculated for the modelled ACBP16 to validate the predicted structure and is shown in Fig. 5.

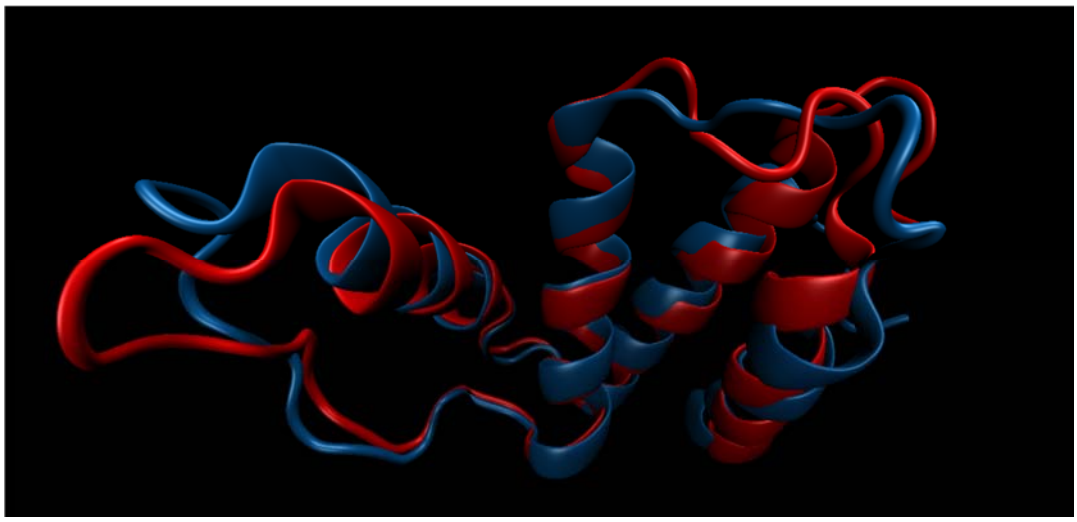
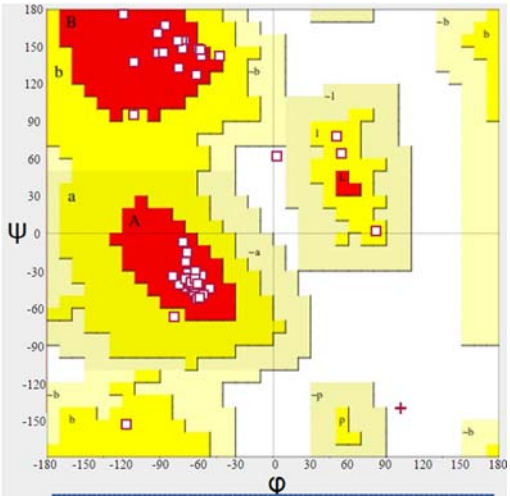


Fig. 4. Comparison of 3 dimensional structure of modelled ACBP16 (red) with the crystal structure of ACBP749 (blue).



Most favorable region:	82 residues (91.1%)
Additionally allowed region:	7 residues (7.7%)
Generously allowed region:	0 residue (0%)
Disallowed region:	1 residue (1.1%)

Fig. 5. Ramachandran plot of modelled ACBP16 and the distribution of residues among the 4 regions

3.2 Binding site analysis of ACBP749 and its sequence alignment with ACBP16

Binding site of ACBP749 was analysed in greater detail in visualization software, Visual molecular Dynamics to identify the residues involved in direct interaction with the ligand. Fig. 6 shows the binding site of ACBP749. The residues interacting with the ligand are highlighted in blue.

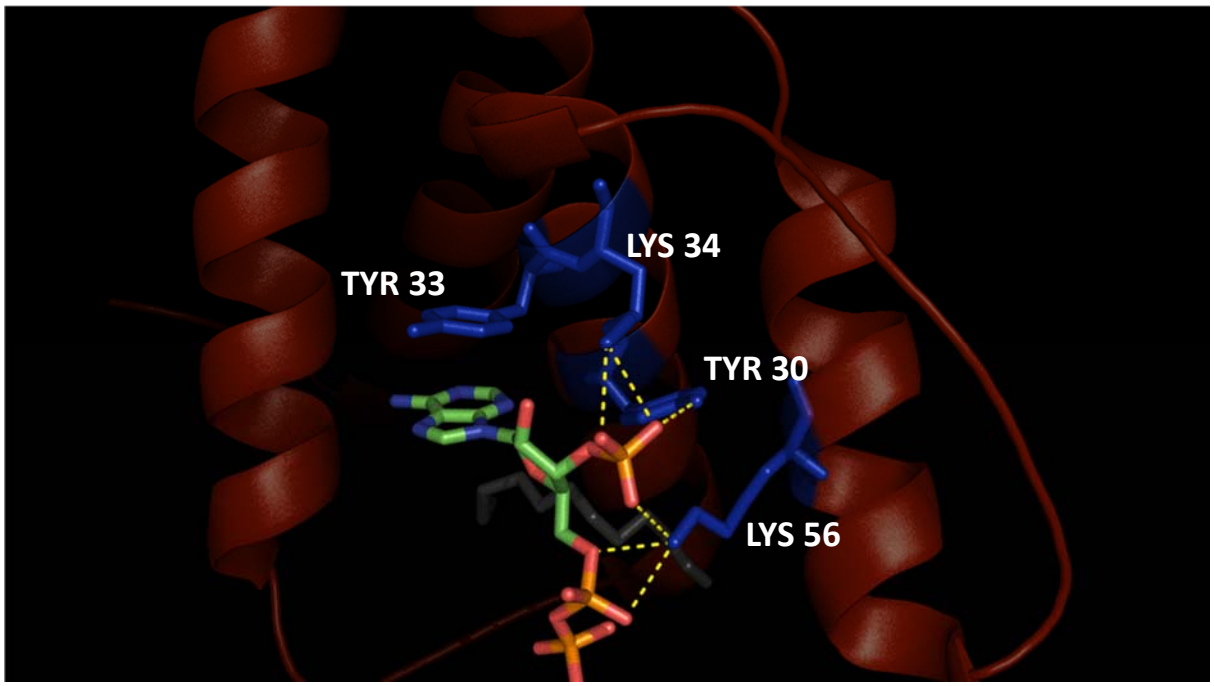


Fig. 6. Binding site of ACBP749. Tyr 33 is involved in base stacking with CoA while Lys 34, Tyr 30 and Lys 56 are involved in hydrogen bonding.

After this, sequence alignment of ACBP749 and ACBP16 was done. The residues of ACBP749 interacting with the ligand are identical in ACBP16. Hence, it was safe to assume that the residues of binding site of ACBP749 had an important role to play in ACBP16's binding with its ligand.

```
ACBP 749          1 MAQVFEECVSFINGLPRTINLPNELKLDLYKYYKQSTIGNCNIKEPSAHK     50
                    |..:|:..|:::|.||....|..|.||||||||||:|||||||||..:
ACBP 16           1 MEDLFQASVNYVNSLPNNKPLSVETKLDLYKYYKQSTVGNCNIKEPSYFQ     50

ACBP 749         51 YIDRKKYEAWKSVENLNREDAQKRYVDIVSEIFPYWQDGE     90
                    |.|:|||||||:||||:|||:|||::||:|.||.||:.|
ACBP 16          51 YADKKKYEAWKSIENLNKEDAKKRYIEIVTENFPNWQNKE     90
```

Fig. 7. Sequence alignment of ACBP749 with ACBP16. The ligand interacting residues are marked in grey.

3.3 Generation of ACBP16 mutants

Site directed mutagenesis via overlap extension PCR was used to create the four mutants, acbp16Y30A, acbp16Y33A, acbp16K34A and acbp16K56A. Mutations were incorporated in PCR using mutagenic primers according to Table 3, which generated two smaller fragments for each gene, with part complementarity. Following this, a final overlap extension PCR was carried out to generate the complete mutant gene. The results of the PCRs are shown in Fig. 8.

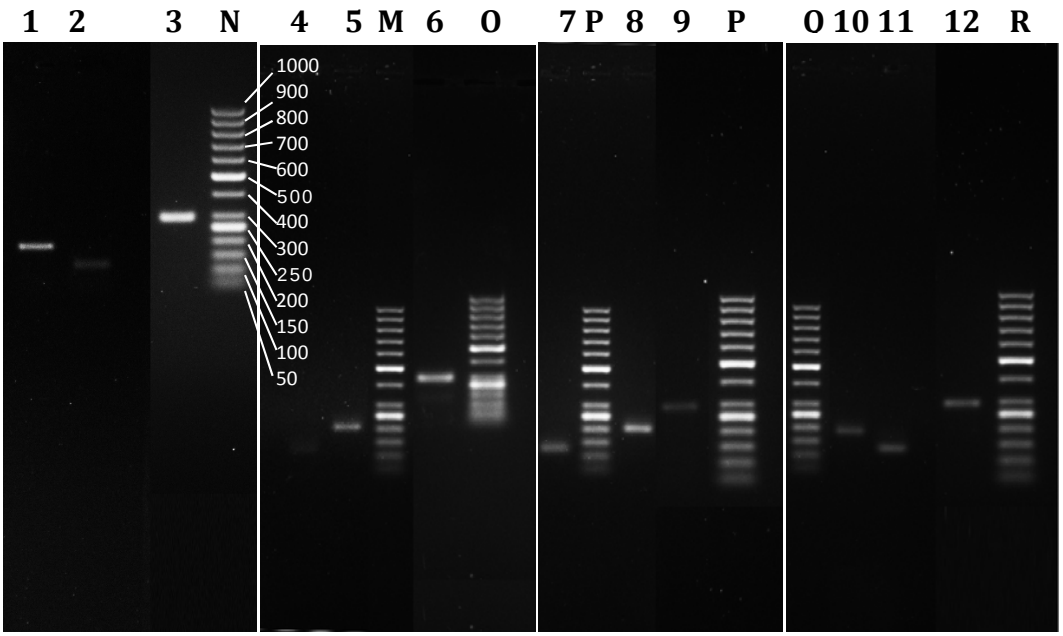


Fig. 8. Agarose gels of PCRs to carry out site directed mutagenesis. Lanes 1,2; 4,5; 7,8; 10,11 are the 2 smaller fragments of acbp16Y30A, acbp16Y33A, acbp16K34A and acbp16K56A respectively. Lanes 3, 6, 9 and 12 are the products of the overlapping PCR of acbp16Y30A, acbp16Y33A, acbp16K34A and acbp16K56A respectively. Lanes N, M, O, P, Q and R are 50bp ladders.

Clones obtained were checked for the presence of the insert via colony PCR. The result is shown in Fig. 9. The positive clones were later confirmed via DNA sequencing. All the mutants except acbp16K56A had the correct insert with the desired mutation in place. The mutant acbp16K56A had multiple insertions and deletions.

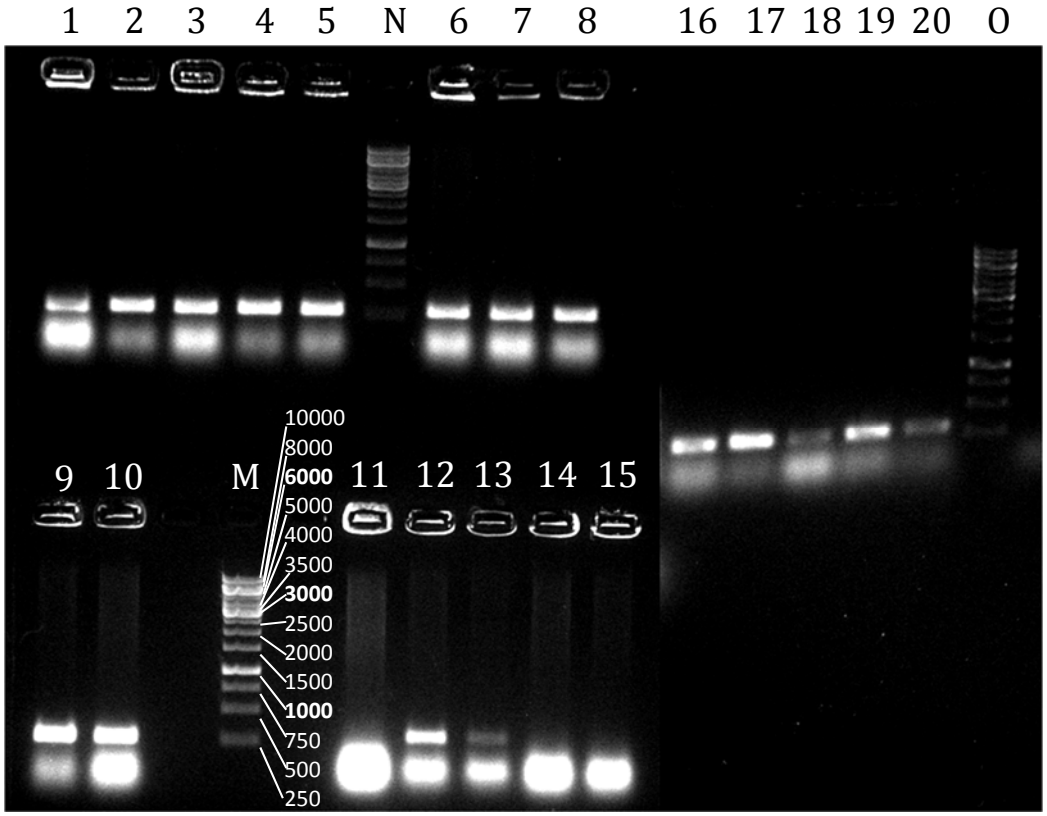


Fig. 9. Agarose gel electrophoresis of amplified product of colony PCR of the transformants of each mutant. Lanes 1-5 are of acbp16Y33A mutant. Lanes 6-10 are for acbp16K34A mutant. Lanes 11-15 are for acbp16K56A mutant and lanes 16-20 are for acbp16Y30A mutant. Lanes N,M,O are 1kb ladders.

3.4 Overexpression and purification of mutant and wild type ACBPs

Mutant and wild type ACBPs were overexpressed in *E. coli* BL21 (DE3) and checked on an SDS PAGE gel. Elutant collected after passing cell lysate (flow through) and after washing (after wash) are also run on the SDS PAGE gel. Fig. 10 shows the SDS PAGE gels for the wild type and the mutant proteins.

All proteins except Y30A mutant have relatively good purity.

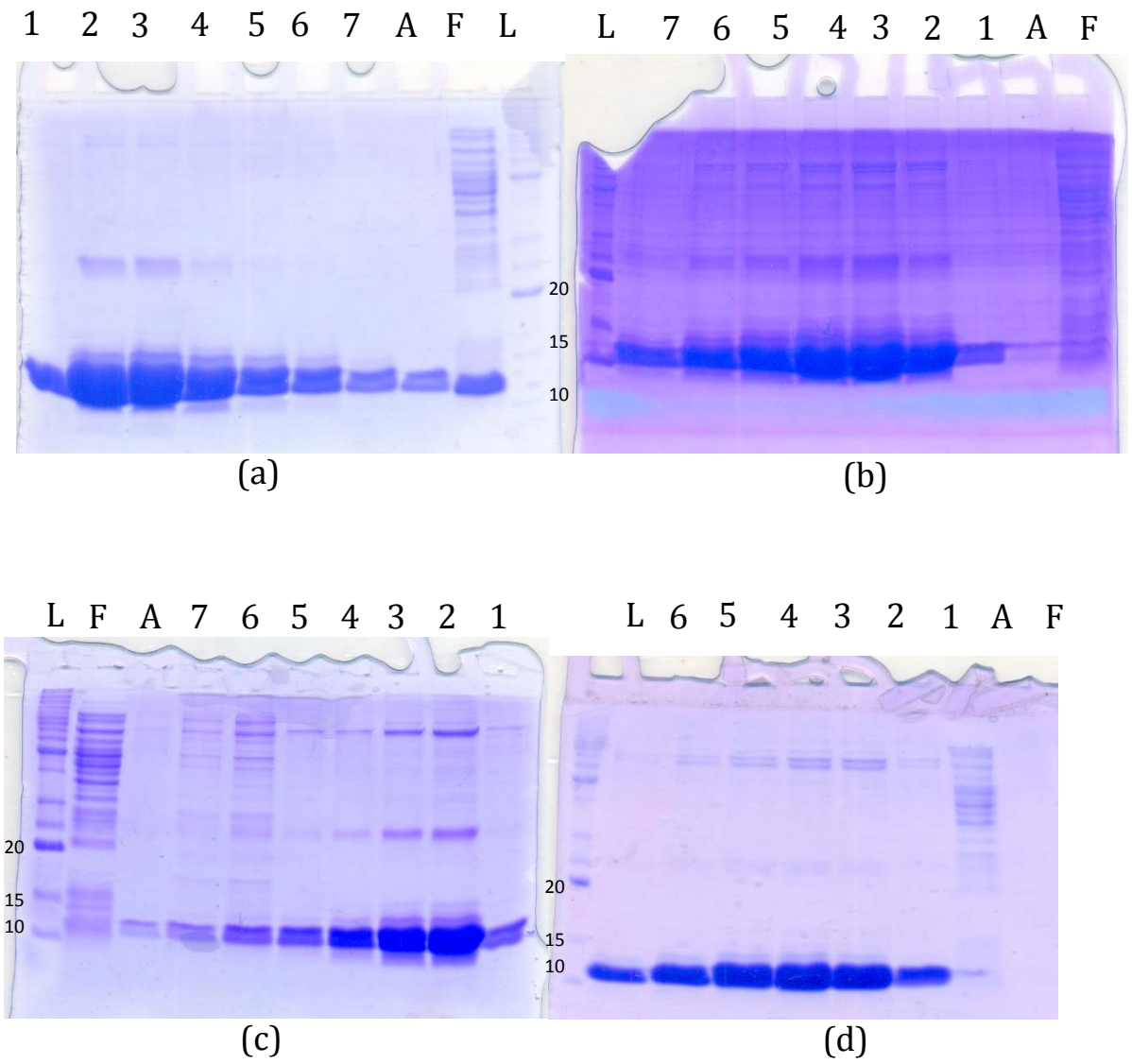


Fig. 10. SDS PAGE gels of protein purification. In each gel, lanes 1-7 are eluted fractions of the protein. Lane F,A and L represent flow through, after wash and ladder respectively. (a)Wild type ACBP (b) ACBP16 Y30A (c) ACBP16Y33A and (d) ACBP16 K34A.

3.5 Dialysis of mutant and wild type ACBPs

The purified proteins were dialysed against fluorimetry buffer and SDS PAGE was run to check the purity. Fig. 11 shows the SDS PAGE gels of the proteins after dialysis.

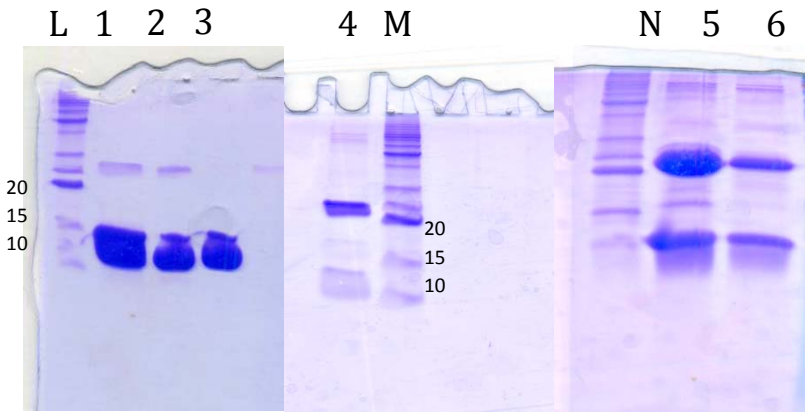
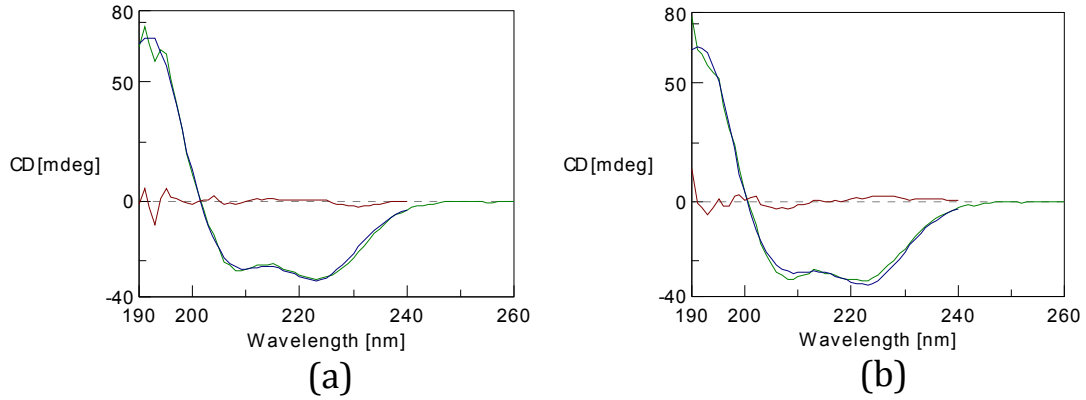


Fig. 11. SDS PAGE gels of purified proteins after dialysis. Lanes 1-3 are ACBP16Y33A mutant after dialysis. Lanes 4,5 and 6 are ACBP16K34A mutant, ACBP16Y30A mutant and ACBP16Y33A mutant after dialysis respectively. Lanes L,M and N are protein ladders.

3.6 CD spectroscopy of mutant and wild type ACBPs

CD spectroscopy results showed that ACBP16Y30A and ACBP16K34A mutants showed negligible perturbations in secondary structure even after induction of mutation. However, the mutant ACBP16Y33A did show considerable departure from wild type CD spectra. Fig. 12 shows the CD spectra of wild type and mutant ACBP16 proteins.



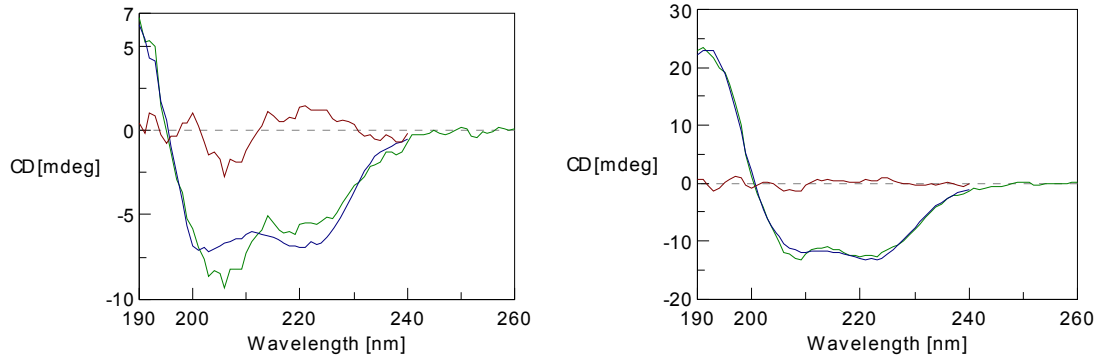


Fig. 12. Far UV CD spectra for the proteins. (a) Wild type (b) ACBP16Y30A mutant (c) ACBP16Y33A mutant (d) ACBP16K34A mutant.

3.7 Fluorimetry of mutant and wild type ACBPs

Quenching constants for the wild type and mutant ACBP proteins is given in Table 4.It is observed that the quenching constants for the mutants are less than that of wild type ACBP16. The mutant ACBP16Y33A has the lowest quenching constant which was expected as it was involved in strong base stacking with CoA. The others were involved in hydrogen bonding, a weaker interaction as compared to base stacking; hence their mutants do retain some binding capabilities. ACBP16K34A is the least affected mutant.

Hence the Tyrosine 33 is the most important residue for ACBP16 in binding to Myristoyl-CoA followed by Tyrosine 30 and Lysine 34.

Protein name	**Quenching constant (K_{sv}) (M^{-1})**
Wild type ACBP	4164.3
Y30A mutant ACBP	2448.9
Y33A mutant ACBP	1727.58
K34A mutant ACBP	4016.9

Table 4. Calculated quenching constants for the four proteins.

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