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**Structural insight into the interaction of human
carbonic anhydrase II and human anion
exchanger 1 c- terminal region**

Diplomarbeit

Vorgelegt am Institut für Biomolekulare Strukturchemie
an der Universität Wien

von

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Zusammenfassung:

Das 29 kDa Metalloprotein humane Carbonische Anhydrase (HCAII) katalysiert die Reaktion $\text{H}_2\text{O} + \text{CO}_2 \leftrightarrow \text{HCO}_3^- + \text{H}^+$. In menschlichen Erythrozyten bindet sie an den humane Anionen Transporter 1 (HAE1), der HCO_3^- gegen Cl^- Ionen durch die Zellmembrane der roten Blutkörperchen schleußt, und bildet so ein Metabolon. Ein Metabolon ist ein Komplex aus schwach interagierenden Bindungspartnern. Dadurch können Metabolite ohne Verlust von einer „aktive site“ zur nächsten geleitet werden, wodurch der Prozeß an Effizienz gewinnt. Das HCAII – HAE1 Metabolon wurde in verschiedenen Geweben nachgewiesen, wobei es auch außerhalb respiratorischer Prozesse Schlüsselfunktionen in der Sekretion von Chlorid oder Hydrogencarbonat Ionen einnimmt.

HCAII interagiert auch mit anderen Mitgliedern der drei HCO_3^- - Transporter Familien. Diese Tatsache unterstreicht die Wichtigkeit dieser HCO_3^- - Transport Metabolons in verschiedenen Geweben.

Vorhergehende Studien haben bewiesen, dass HCAII durch seine N- terminale helix mit der C- terminalen cytoplasmischen region von HAE1 interagiert. Die C- terminale Sequence L886DADD des HAE1 spielt dabei eine wichtige Rolle. Ob andere Motive für die Komplexbildung wichtig sind, war unklar. Außerdem konnte die Bindungsstelle auf HCAII noch nicht bestimmt werden.

Durch NMR- Messung konnte nachgewiesen werden, dass die Aminosäuren E882 und E899 zusätzlich zur Sequence L886DADD auf dem C- Terminus von HAE1 für die Komplexbildung mit HCAII wichtig sind. Außerdem kann aufgrund von NMR Titrations- Experimenten angenommen werden, dass die Bindungsstelle des C- terminus von HAE1 nahe dem Zugang zur „aktive site“ von HCAII liegt.

Abstract:

The 29 kDa metalloprotein human carbonic anhydrase II (HCAII) catalyzes the reaction $\text{H}_2\text{O} + \text{CO}_2 \leftrightarrow \text{HCO}_3^- + \text{H}^+$. In erythrocytes it binds to the HCO_3^- transporter human anion exchanger 1 (HAE1) forming a metabolon. A metabolon is a complex of weakly interacting binding partners passing on very efficiently substrates from one active site to another and thereby facilitating the process dramatically. This metabolon formed by HCAII and HAE1 was detected in a variety of tissues involved in Cl^- and /or HCO_3^- secretion and respiratory processes. Moreover HCAII was reported to interact with members of all HCO_3^- transporter families. Therefore the interaction site of HCAII and HAE1 is in the focus of studies concerning HCO_3^- transporter metabolons.

Based on previous studies the C-terminal cytoplasmic region of HAE1 (HAE1ct) interacts with the basic N- terminal helix of HCAII by the hydrophobic - acidic motif L886DADD. If other motifs on HAE1 ct are also involved was unclear. Moreover the binding site of HAE1ct on HCAII was not known. However it was discovered that the truncation 17 amino acids at the N- terminus is connected with disruption of the complex.

Based on NMR titration studies we discovered that E882 (upstream the acidic motif) and E899 (downstream the acidic motif) on HAE1 C-terminal region are also involved in complex formation with HCAII and even more essential. Additionally the binding site of HAE1 C-terminal region to HCAII was mapped close the entry to its active site.

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List of abbreviations

aa	amino acid
AE	Anion exchanger
Amp	ampicillin
APS	ammonium persulfate
BCIP	5-bromo-4-chloro-3-indoyl phosphate p-toluidine salt
bp	base pair
Cam	chloramphenicol
CA	Carbonic anhydrase
cDNA	complementary DNA
CRP	cysteine-rich protein
CV	colume volume
Da	dalton (1/12 of the mass of a C12 atom)
DMSO	dimethylsulfoxid
DNA	desoxy-ribonucleic acid
dNTP	desoxy-nucleotide triphosphate
DTT	dichloro-diphenyl-trichloroethane
EDTA	ethylenediaminetetraacetic acid
fw	5'-priming oligo-nukleotide
GST	gluthatione-S-transferase
HAE 1	Human anion exchanger1
HAE 1 ct	Human anion exchanger 1 C- terminal region
HCAII	Human carbonic anhydrase
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
His ₆ tag	six consecutive histidine residues
Ig	immunglobuline
IPTG	isopropyl-β-D-thiogalactopyranosid
ITC	isothermal titration calorimetry
Kan	kanamycin
kb	kilo base

LB media	Luria-Bertani media
MES	2-(N-morpholino)ethanesulfonic acid
NBT	p-nitro blue tetrazoliumvchloride
NBT	p-nitro blue tetrazoliumvchloride
Ni	nickel
OD ₆₀₀	absorbance at 600 nm
pBpa	p-bezoyl-L-phenylalanine
PCR	polymerase chain reaction
PEG	poly ethylene glycol
PIP ₂	phosphatidylinositol-4,5-biphosphate
ppm	part per million
rpm	rounds per minute
rv	3'-priming oligo-nukleotide
TEMED	N,N,N',N'-tetramethylethylenediamine
TEV	<i>Tobacco Etch Virus</i>
Tm	melting temperature
Tris	trishydroxymethylaminomethane
Trx	thioredoxin
U	unit

1. Introduction

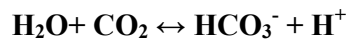
1.1 The capno metabolon; an example for the HCO_3^- transport metabolon

1.1.1 Human carbonic anhydrase I and II (HCAI and HCAII) in red blood cells

16 different isoforms (cytosolic and membrane-bound) of carbonic anhydrase were found in mammalian tissue. We focus on the isoforms I and II which are predominantly expressed in erythrocytes (Sly and Hu 1995).

Patients lacking isoform I do not have any serious symptoms (Kendall and Tashian 1977) while the lack of HCAII is not lethal but causes autosomal recessive osteopetrosis with renal tubular acidosis and cerebral calcification (Sly, Whyte et al. 1985).

This is surprising because HCAI is 6 times more abundant in erythrocytes (130 μM) than CAII (20 μM) and both catalyze the same reaction (Vince and Reithmeier 2000):



They also share 60% sequence identity and have a similar 3D structure (Liljas, Kannan et al. 1972; Kannan, Notstrand et al. 1975).

However the turnover rate of isoform II is 7 times higher than that of isoform I (Reithmeier 2001). Moreover HCAI is highly inhibited by the concentration of Cl^- in red blood cells (Reithmeier 2001).

Why is HCAII therefore so important in red blood cells at difference to HCAI ? It is part of a metabolon.

HCAII can interact with HCO_3^- while HCAI is unable to do so.

1.1.2 The Capno Metabolon

It was proved that HCAII can interact with human anion exchanger 1 (HAE1; Band 3). This anion antiporter transports HCO_3^- against Cl^- ions. The interaction between HCAII and this transporter allows the transport of substrate HCO_3^- from and to HCAII without any loss, which is dramatically enhancing the efficiency of the

process. Human carbonic anhydrase II is located close to the membrane by this interaction,

The interaction of HCAII with HAEI localizes HCAII close to the membrane where high pools of CO_2 are found. The diffusion of CO_2 through the unstirred water on the cell membrane would be rather difficult (Reithmeier 2001).

Counter current studies also suggested that HCAII can also bind to hemoglobin (Backman 1981). This means that the free H^+ produced in the interconversion of H_2O and CO_2 could be passed directly to hemoglobin and thereby decrease its affinity for O_2 . Hemoglobin seems to have a role in enhancing the catalytic activity of HCAII by exchange of the H_2O produced in the interconversion of HCO_3^- (Silverman, Backman et al. 1979).

Aquaporin 1 (AQP1) could be involved in this complex and therefore directly deliver H_2O to HCAII (Reithmeier 2001).

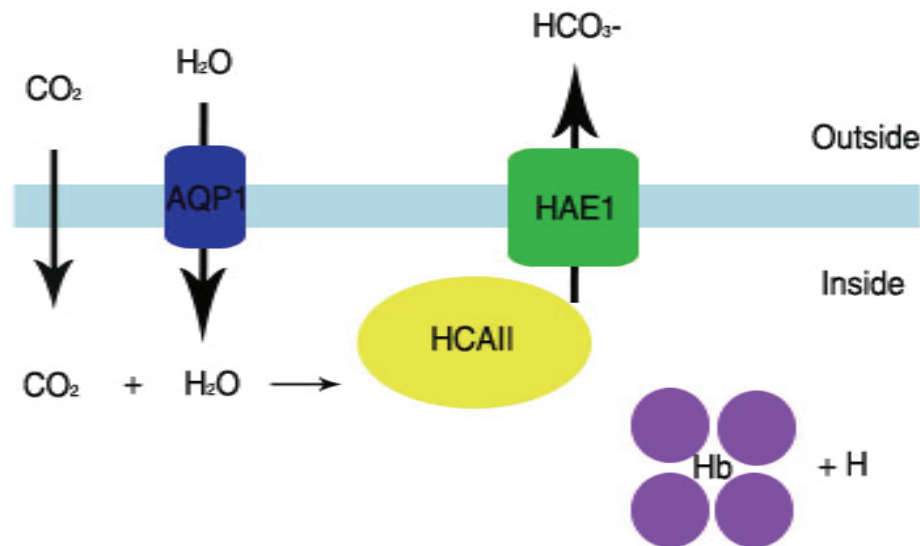


Figure 1: The Capno Metabolon (Reithmeier 2001)

In tissue, CO_2 diffuses through the lipid bilayer while H_2O is passed through AQP1 to the inside of the cell. These substrates are converted by HCAII to HCO_3^- which is transported outside the cell by HAE1 and H^+ which binds to hemoglobin. This binding induces a conformational change in hemoglobin leading to a decrease of affinity for O_2 . In the lungs HCO_3^- is reconverted to H_2O and CO_2 which diffuses out

of the red blood cells and is expired inducing a conformation change in hemoglobin increasing its affinity for O₂ (Reithmeier 2001) (Figure 1).

This series of interactions that channel substrates and products of carbonic anhydrase defines the capno metabolon. Capno is a greek word meaning smoke or CO₂. A metabolon is a complex of weakly interacting binding partners which allow substrates to be transported from one active site to the next and are therefore responsible for the fine tuning of the whole process.

In the following we shall take a closer look at the interaction partners HCAII and HAE1.

1.1.3 HCAII

It is a 29 kDa protein with overall dimensions of 55×42×41 Å (Reithmeier 2001). In similarity to HCAI it has a Zn- Ion buried deep in its active site coordinated by 3 histidine residues and one hydroxide ion. However, unlike in HCAI, the 20 N-terminal residues fold to an α - helix independently from the rest of the protein. Moreover, this region contains histidine residues which cannot be found in the sequence of HCAI (Reithmeier 2001).

N- terminally truncated versions of HCAII partly lose their catalytic activity enzyme in complex with HAE1, indicating that this region is involved in binding to HAE1, which in turn localizes HCAII close to the membrane (Sterling, Reithmeier et al. 2001).

HCAII is widely distributed in tissue. In osteocasts, kidney tubules and parietal cells it is responsible for acid secretion while in choroid plexus, pancreatic duct cells and salivary gland acinar cells it promotes HCO₃⁻ secretion (Reithmeier 2001). In red blood cells it has an important role in blood buffering and is essential for respiratory processes. This important role in a variety of processes the severe damage of its absence can be explained.

1.1.4 Human anion exchanger (HAE1)

Human anion exchanger, also termed human band 3, is a 911- amino acid glycoprotein. Its C- terminal region (55 kDa) spans the cell membrane 12 – 14 times and ends as a 33 amino acid peptide in the cytoplasm. The N- terminus forms a cytoplasmic domain (43 kDa) which binds glycolytic proteins and anchors HAE1 to the membrane cytoskeleton (Zhang, Kiyatkin et al. 2000).

The anion exchanges (AE) family comprises additional two members AE2 and AE3. AE1 is abundantly expressed in erythrocytes and a truncated version is also present in the kidney and the heart (Sterling, Alvarez et al. 2002). AE2 is almost ubiquitous while AE3 is expressed in the heart, brain and retina (Sterling, Alvarez et al. 2002). That means that AE family can be found throughout the body.

1.1.5 Interaction between HCAII and HAE1

The interaction between HCAII and HAE1 was proved by immunofluorescence, co-immunoprecipitation and micro- titer assays (Vince and Reithmeier 1998).

HAE1 C- terminal region blocked the interaction of GST- ct with an affinity of 100 μ M. The micro titer plate assays indicated a binding constante of 100 nM. However an interaction in the micromolar range seems to be more probable (Reithmeier 2001). It was also shown that inhibition of HCAII by azetolamide decreased the activity of HAE1 to approximately 70%. The loss of interaction by truncation of N-terminal region or mutations in this region had the same effects (Sterling, Reithmeier et al. 2001).

It was also found out that the 33 C- terminal amino acids of HAE1 are sufficient to interact with HCAII (Vince and Reithmeier 1998).

1.1.5.1 The acidic-basic motif of the interaction between HCAII and HAE1

The interaction is dramatically dependent on pH and ionic strength. The binding rate was rather slow ($t_{1/2}$ = 12h) at physiological pH and ionic strength (50 mM Tris pH7.5

100 mM NaCl). By lowering the pH and ionic strength, the time to reach saturation of binding was decreased (Vince and Reithmeier 1998). It is therefore rather probable that the interaction is electrostatic, based on acidic and/or basic motifs.

The N- terminal helix of HCAII contains basic amino acids and 5 histidine residues, which are not present in isoform I. Truncation of this region results in loss of binding while the enzymatic activity is still preserved (Vince, Carlsson et al. 2000). In contrast the 33 amino acid C-terminal region of HAE1 is highly acidic.

In order to map the binding site different N-terminally truncated versions of HCAII were investigated if they can still bind the 33 amino acid C-terminal region of HAE1 (Vince, Carlsson et al. 2000).

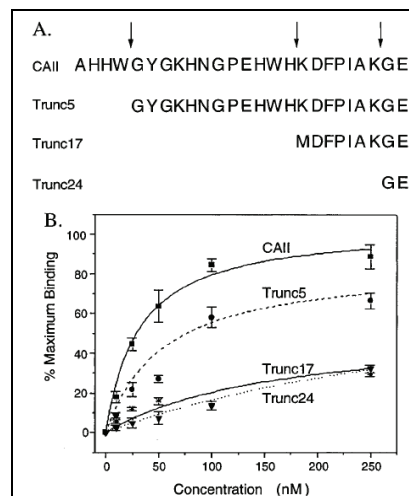


Figure 2: Effect of N- terminal truncations of HCAII on the binding with the c- terminal 33 amino acid peptide of HAE1 (Vince, Carlsson et al. 2000)

The truncation of 17 or 24 N- terminal amino acids lead to a loss of binding while the truncation of the first 5 residues resulted in 20% decrease in binding (Figure 2).

Furthermore, the role of the histidine residues in this region was tested. They are highly conserved throughout all organisms.

Combinations of mutations were done but only the mutation of all positively charged residues (5 histidine residues and lysine 9) resulted in a complete loss of binding.

That indicates that these histidine residues are important for structure or that they

form an “electrostatic platform” for the interaction between HAE1 and HCAII, facilitating therefore exchange of HCO_3^- with HAE1 (Vince, Carlsson et al. 2000).

The C-terminal region of HAE1 contains 3 patches with high content of acidic amino acid residues. Again, truncation experiments were performed, showing that the acidic patch D887ADD was essential for binding to HCAII. By mutations it was found out that the sequence of these four amino acids can be changed but it has to include at least two acidic amino acids (Vince and Reithmeier 2000).

It has additionally been shown that HAE2 can as well bind to HCAII. Here the sequence D887ANE, which again contains two acidic amino acids, seems to be important for binding (Vince and Reithmeier 2000).

L886 is conserved in HAE1 and HAE2, therefore its role was investigated by the mutation to alanine. The interaction was completely loss indicating that L886 has an important role in interaction.



Figure 3: Comparison of primary sequences of HAE1 and HAE2 (Vince and Reithmeier 2000)

Consensus sequence (orange) other acidic motifs (green)

L886DADD was proposed to be a consensus sequence for interaction of HAE1 with HCAII while a similar sequence is found in HAE2 (Figure 3). This sequence motif can also be found in the primary sequence of Aquaporin 1 (AQP1) (Vince and Reithmeier 2000), suggesting a similar binding mode.

Putative carbonic anhydrase binding sites on HAE1 therefore consist of a hydrophobic amino acid followed by four amino acid residues, of which at least two have to be acidic. This motif was also found in HAE2 (intra- and extracellular), aquaporin 1, SLC26A6 (anion transporter) and others. This proposes that the bicarbonate transport metabolon is essential in different tissues and for different purposes. Hereafter we shall take a look at proposed components in these metabolons.

1.2 Components of HCO_3^- transporter metabolons throughout the body

1.2.1 HCO_3^- exchangers

There are three groups of exchangers which transport HCO_3^- through the membrane:

1. SLC4a Cl-/ HCO_3^- exchangers (AE family)
2. SLC4a sodium coupled transporters
3. SLC26a family

The members of the SLC4A anion exchanger (AE) family and the SLC26A anion transport family mediate anion exchange at the plasma membrane of mammalian cells while the SLC26a family is involved in channeling different anionic substrates for example sulfate, formate, oxalate, nitrate, iodide etc (Esbaugh and Tufts 2006).

Within all these groups there are now examples (AE2, NHE1, kNBC1, NBC3, DRA) for interaction with carbonic anhydrases (Alvarez, Vilas et al. 2005), employing a variety of possible interaction sites apart the interaction motif of HAE1. For kNBC1 it is believed that two acidic motifs are involved in the binding (Pushkin, Abuladze et al. 2004). However, NHE1 seems to interact with HCAII *via* S796 and D797, constituting a novel binding motif (Li, Liu et al. 2006).

The metabolon of SLC26A6 with HCAII is disrupted by phosphorylation near the carbonic anhydrase binding site. This could be a universal mechanism of metabolon regulation (Alvarez, Vilas et al. 2005) as also the binding of kNBC1 to HCAII is altered by phosphorylation (Gross, Pushkin et al. 2002).

1.2.2 Carbonic anhydrases (CA)

Carbonic anhydrases are metalloenzymes carrying a Zn- ion in their active site. The dominant secondary structure motives are β -sheets (Figure 4).

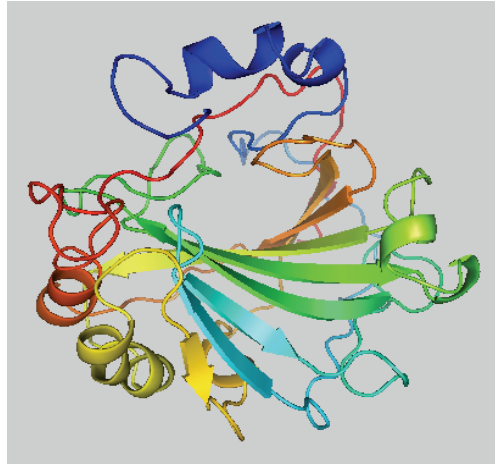


Figure 4: Structure of human carbonic anhydrase II

All of them are catalyzing the de- and rehydration of CO_2 (Figure 5).

CO_2 undergoes a nucleophilic attack by Zn- bound hydroxide. HCO_3^- is directly formed bypassing the slow reaction over carbonic acid and its dissociation. HCO_3^- on the Zn- ion is replaced by a water molecule. The Zn- binding hydroxide is regenerated by remove of a proton from the water molecule by the proton shuttling mechanism.

The catalytic function of CA is based on four main structural components.

1. Zn- binding site
2. substrate association pocket
3. threonine 199 loop
4. proton shuttling mechanism

The Zn- ion is coordinated by three histidine residues (His 94; His 96; His 119) and a hydroxide molecule. Each histidine residue has an associated ligand (Gln 92; Asn 244 and Glu 117) which stabilizes these residues by a hydrogen bond.

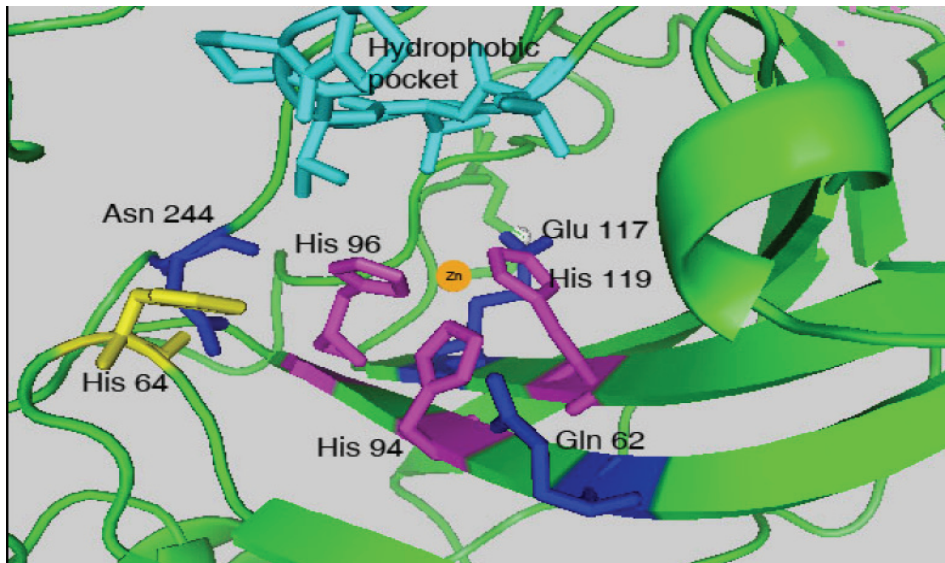


Figure 5: Active site of HCAII

The substrate binding pocket consists of six non- polar amino acid residues which channel CO_2 to the Zn- ion.

The main function of the Thr199 loop is to keep the Zn- bound hydroxyl group in the right orientation for catalysis. This is accomplished by the formation of a hydrogen bonding network.

The proton produced by the reaction is moved through a shuttle chain consisting of three H_2O molecules (Figure) before it guided by the proton shuttling mechanism. In HCAII the shuttling is highly dependent on His64, which mutation results in dramatical decrease of catalytic function.

16 isoforms of carbonic anhydrases are known. The family comprises cytoplasmic (CAI, II, II, V, VII, and XIII), membrane- bound (VI, IX, XII, XIV and XV) and CA- related proteins which have lost their catalytic function(Esbaugh and Tufts 2006).

1.2.2.1 Cytoplasmic CA

Out of six CA isoforms two are high turnover (CAII and CAVII), three are low turnover (CAI, V and XIII) and CAII has an extreme low turnover rate. This is due to structural modifications of the proton shuttling mechanism or reduction of the active site cavity (isoform I).

Phylogenetic analysis revealed that CAV and VII are the most ancestral enzymes and seem to have limited roles in vertebrates (Esbaugh and Tufts 2006).

CAV is expressed in liver and muscle mitochondria while CAII and CAI are widely distributed in all tissues (Esbaugh and Tufts 2006).

1.2.2.2 Membrane bound CA

Among the membrane bound CAs, three (IX, XII and XIV) have transmembrane domains while the two others (IV and XV) are bound to the membrane *via* glycosyl-phosphatidyl-inositol (GPI) - anchors. Nearly all of them (with exception of CAXV) have a high turnover. The reason for this is a disulfide bridge between Cys23 and Cys203 residues, which stabilizes the Thr199 loop (Esbaugh and Tufts 2006).

The central role of HCAII in interaction with HCO_3^- transporters has been clearly demonstrated additionally it has been shown that also the extracellular isoform CAIV can bind the AE1, AE2 and AE3. HAE1 together with CAIV and CAII on both sides of the membrane generate a “pull- push” mechanism for bicarbonate transport (Sterling, Alvarez et al. 2002) (Figure 6).

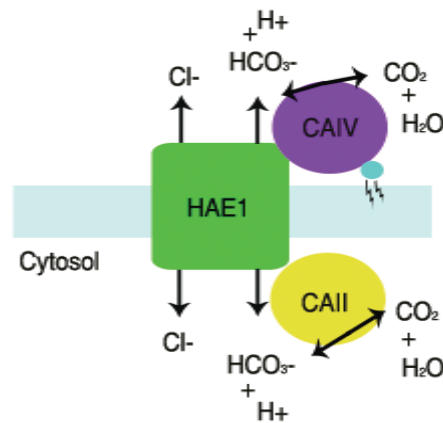


Figure 6: “Pull- Push” mechanism of CAII and CAIV (Sterling, Alvarez et al. 2002)

CA- mediated production of bicarbonate presents the “push” while the conversion of bicarbonate to CO_2 provides the “pull”- mechanism.

It is rather surprising that the lack of CAII is characterized by renal tubular acidosis and osteopetrosis while a sufficient CO_2 transport capacity is still maintained. This

could be due to the expression of CAIV in erythrocytes. Osteoclasts do not express CAIV and in kidney the presence of CAIV is not sufficient to reabsorb bicarbonate from the renal tubular lumen and prevent tubular acidosis (Sterling, Alvarez et al. 2002).

1.2.3 The Capno metabolon as a model for bicarbonate transport metabolons

CA and bicarbonate transporters have a central role in a variety of tissues. Miss-regulation of bicarbonate transporters can be the reason for serious diseases like cystic fibrosis (SLC26A6 bicarbonate Transporter).

It is rather likely that not all HCO_3^- transport metabolons are have been discovered so far. The majority of the interaction studies focused on CAII, but it is highly probable that also other carbonic anhydrases like the membrane- bound CAIV are involved in such systems.

The role of the binding site concerning the shuttling of HCO_3^- to the transporter or HCAII is still not clear. Therefore structural insight into the capno metabolon could lead to a deeper understanding of bicarbonate transporter metabolons in general.

1.3 Objectives for this study

The aim of this study is structural investigation of the binding of human carbonic anhydrase II (HCAII) to the C-terminal cytoplasmic region (33 amino acid residues) of human anion exchanger 1 (HAE1). We employed as principal structural biology techniques crystallography and NMR. Co- crystallization and crystal soaking of HCAII as well as titration studies followed by NMR will be performed. Sample preparation comprised expression and co-expression in *E. coli*, purification and complex formation.

2 Methods and Materials

2.1 Cloning

2.1.1 PCR

The primers were designed with BsaI restriction sites:

HCAIIF1 (forward)

5' 3'
AATT**GGTCT**CCCATGTCCCATCACTGGGGGTAC

HCAIIR2 (reverse)

5' 3'
GGTACCG**GAGAC**CTTATTTGAAGGAGGCTTTGATTG

The cursive letters show the BsaI restriction sites.

PCR- Mixture:

1µl template (40 ng/µl)

0.5µl Primer 1 (10 pM; Sigma)

0.5µl Primer 2 (10 pM; Sigma)

0.2µl Taq (1u/µl; Fermentas) or Pfu (1u/µl; Fermentas)

2µl 10×Polymerase Buffer (Fermentas)

1.6µl MgCl₂ (25 mM; Fermentas)

2µl dNTPs (2 mM; Fermentas)

12.2µl H₂O

20µl

PCR- Program:

Step	Temperature	Time	Cycles
Initial Denaturation	94°C	4min.	1
Denaturation	94°C	30sec.	24
Primer Annealing	55°C	30sec.	24
Elongation	72°C	1min.	24
Final Elongation	72°C	5min.	1

The most efficient temperature of Primer Annealing was defined by gradient PCR (50, 55 and 60°C).

2.1.2 DNA- Purification after PCR

The Purification of PCR- Product was done with a PCR- Purification Kit (Quiagen). DNA was eluted in 30µl H₂O.

2.1.3 Double Digest of pETM 13 with Acc65I and NcoI

5µl plasmid pETM-13 (200 ng/µl)

0.5µl NcoI (10u/µl; Fermentas)

1µl Acc65I (10u/µl; Fermentas)

4µl 10×Tango Buffer (Fermentas)

9.5µl H₂O

20µl

The double amount Acc65I was used because it has only 20-50% activity in Tango Buffer. The mixture was put 3h on 37°C. No heat inactivation is necessary because this digest is followed by a gel extraction.

2.1.4 Digest with BsaI

30µl PCR – Fragment (directly from PCR- Purification)

1µl BsaI (10u/µl; BioLabs)

4µl NE 3 Buffer (BioLabs)

5µl dH₂O

40µl

The mixture was put 3h on 50°C and was followed by 20min. on 60°C (heat inactivation).

2.1.5 Digest with EcoRV

5µl pETM- 13

0.5µl EcoRV (10u/µl; Fermentas)

1.5µl NE 3 Buffer (BioLabs)

9.5µl dH₂O

16.5µl

The mixture was put 3h on 37°C and was followed by 20min. on 85°C (heat inactivation).

2.1.6 Determination of DNA concentration

DNA- concentration was measured with a UV- spectrometer at 260nm. It gives the value in µg/ml. One absorption unit corresponds to 50 µg/ml. The absorption has to be divided with the absorption at 280nm. The ratio of pure DNA is >1.9.

2.1.7 Ligation

8µl fragment (molar ratio between vector and fragment is always 1 : 3)

1µl vector (60 ng)

1µl 10×ligase buffer (Fermentas)

0.5µl ligase (5 Weiss u/µl; Fermentas)

10.5µl

The ligase- mixture was put on 16°C over night. The ligase was inactivated by incubation for 20min. at 60°C.

2.1.8 Preparation of competent cells

1. Prepare a preculture with adequate antibiotics and let it grow over night.
2. Prepare a cell culture using LB medium and add 10 mM MgCl₂, 0.2% Glucose and adequate antibiotics.
3. Inoculate the with preculture adding 1/100 of the new culture volume.
4. Grow the cells to OD~0.4.
5. Centrifuge the cells gently and resuspend the pellet in ice cold RF1 of 1/3 of the original volume.
6. Incubate on ice for 15' up to 2h.
7. Centrifuge the cells gently and resuspend the pellet in ice cold RF2 of 1/12.5 volume of the original cell culture and store on ice for 15min.

8. Aliquote the cell culture in 100µl Eppendorf tubes and freeze then in liquid nitrogen. Store on -80°C.

2.1.9 Transformation

The whole ligation- mixture was added to 50µl of competent cells. For positive control the same was done with 1µl (~100ng/µl) of empty vector.

1. 30 min. on ice
2. 90 sec. on 42°C (heat shock)
3. 1 min. on ice
4. add 1ml of LB media and shake 1h on 37°C
5. spin 5min. at 6000rpm
6. remove 1ml and gently resuspend the pellet
7. plate the cells on Agar plates with the proper antibiotics and let grow over night on 37°C in the incubator

2.1.10 Agarose- Gel

Always 1% Agarose- Gels (Standard Agarose) were used which can efficiently separate fragments between 250bp and 12kbp.

Pouring the gels:

1. Add 0.3g Agarose to 30ml TAE – Buffer and heat it in a microwave till the agarose is completely dissolved
2. Enclose 1µl of Ethidiumbromid per ml Agarose and pour the gel

After the gels have cooled down they can be used. All gels were run in TAE- Buffer on 90V for ~ 25min. DNA- bands can be visualize by intercalated Ethidiumbromid under UV.

Sample preparation:

2µl DNA

3µl H₂O

1µl 6×Loading dye (Fermentas)

6µl

To estimate the size a 1kbp Marker (Fermentas; O'Gene Ruler 1kbp DNA Ladder ready- to use) was used.

2.1.11 Gel Extracation

A Kit (Qiagen) was used for this purpose. DNA was eluted in 30µl H₂O.

2.1.12 Preculture

Inoculate a single culture from a plate or a small clump of glycerol stock in 20ml of LB media or strike out on a plate and let it grow over night on 37°C. Use the adequate antibiotics.

2.1.13 Colony PCR

It is a method to prove if a colony has the plasmid with the right insert.

Add a colony to 50µl H₂O and put it for 5min. on 96°C. Then use this as template for PCR.

2.1.14 Mini- Prep

A Kit (Fermentas) was used.

2.1.15 Freezing cells

Glycerol was added to the cell to a final volume of 25%. The cells were stored at -80°C.

2.2 Proteinexpression and Cell lysis:

2.2.1 Expression Screen

The expression of a protein differs dependent on the used cell strain. Therefore it can be really important to choose the right bacterial strain to get a sufficient amount of protein. The expression screen makes it possible to do small scale expressions a high-throughput way (Groves and Lodge, manuscript in preparation) by usage of autoinduction- media. The bacteria are induced after they have used all the glucose and therefore have to switch to lactose.

A. Transformation:

The Transformation is performed in a 24- deep well plate. A typical set up could look like this.

	Strain 1	Strain 2	Strain 3	Strain 4	Strain 5	Strain 6
Vector 1						
Vector 2						
Vector 3						
Vector 4						

Therefore four different vectors coding for different proteins or the same proteins with different tags could be combined with six different bacterial strains.

Do the following procedure for every well of 2 plates because one is put on 20°C and one on 37°C.

1. 1µl DNA is added to 50µl of competent cells
2. Put it 30min. on ice
3. 45-60 sec. on 42°C (do this in water bath)
4. 5min. on ice
5. add 1ml of autoinduction media
6. Put 1h on 37°C
7. Add the proper antibiotics (dilution 1:1000)
8. One tray on 37°C and one on 21°C and let them grow over night

B. Harvesting and dot blot

The bacteria are harvested and by a dot blot the amount of expressed protein is estimated.

1. The bacteria are transferred to a 96- well- filter plate in duplicates (so there is enough space for two 24well plates on one 96- well format)
2. Centrifuge for 5min. at 500g in a swing- plate rotor
3. Add 25µl of lysis buffer
 - a. Bug buster (Novagen) in first of duplicates;
 - b. Denaturing buffer in second of duplicates;
4. Shake 20min. at room temperature on a gel shaker at 200rpm
5. Centrifuge at 500g for 5min. and collect the filtrate in a new plate
6. Add 2µl of the filtrate to a nitrocellulose membrane and let the membrane air dry
7. Incubate the membrane in 20ml blocking solution for 1h (1%BSA in TBST)
8. Remove blocking solution
9. Incubate the membrane with 1st antibody diluted 1:2000 in TBST for 1h
10. Remove antibody solution
11. Wash the membrane 3× 10min. in TBST
12. Incubate membrane with 2nd antibody (conjugated to alkaline phosphatase) diluted 1:3000 in TBST for 1h
13. Remove the antibody solution
14. Wash the membrane 3× 10min. in TBST
15. Develop the membrane in 10ml of colour- development buffer containing 100µl NBT and 100µl BCIP
16. Incubate and shake gently until colour develops, wash with water to stop the reaction

The first of the duplicates will show the soluble part of protein because the bug buster mix only opens the outer membranes and the cytosolic protein can leave the cell during centrifugation. The second duplicate shows the complete amount of protein because soluble as well as insoluble protein is extracted by denaturing buffer.

2.1.2 Large Scale expression and harvesting

20ml Preculture and antibiotics (dilution 1:1000) were added to 1l 1× (his-trx-HAE1) or 2× LB (HCAII). For the expression of HCAII 0.5 mM ZnSO₄ has to be added. (Zn²⁺ is in the active site of HCAII). The initial OD should be ~0.05. The bacteria were grown at 37°C till they reached an OD around 0.6. Then they were induced with 0.5 μM IPTG and were incubated 4h more on 37°C. An alternative way was to grow them over night on 30°C. Then the cells were spun for 15min. at 10 000g. Then the bacteria were resuspended in 20-40ml buffer for the following first purification step and stored on -80°C.

2.2.3 Large Scale expression and harvesting of labeled protein for NMR

The same protocol like for unlabeled proteins but expression in M9- Minimal media.

2.2.4 Cell lysis

One part of cell lysis was to freeze the cells on -80°C and thaw them again. (Freeze-thaw- cycle) This was followed by 10 min. sonication with pulses of 2 sec. with a pause of 8 sec with strength of ~60%. After this the cell-lysate was spun for 30 min. at 20 000g. The supernatant was used for purification.

2.3 Purification

All columns (accept the gravity columns) were connected to ÄKTA Purifier (Amersham Bioscience).

2.3.1 Affinity chromatography

Affinity chromatography is based on a matrix- bound molecule which can “fish out” its specific binding partner. The affinity should be between 10^{-5} and 10^{-7} M. Stronger binding makes it hard or even impossible to elute the binding- partner from the resin. This is normally done by a competitive partner or conformational changes of the binding partner due to changes in pH or ionic change.

2.3.1.1 IMAC

A special sort of affinity chromatography is the IMAC (immobilized metal chelating affinity chromatography). Here a metal chelating- group is bound to the resin. In this way different metals like Cu^{2+} , Ni^{2+} , Zn^{2+} , Co^{2+} , Fe^{2+} or Fe^{3+} can be immobilised to the matrix. The most common method of IMAC is the purification of his- tagged proteins on a nickel column. The 6 histidines are coordinated by the nickel and can bind in this way to the column. The tagged protein is eluted with imidazol which competes with the his - tag for Ni. It is advised to add some imidazol during equilibration and washing of the column to exclude unspecific interaction. Intensive used resin can be regenerated with NiSO_4 after removing the used nickel with an EDTA- containing buffer (stripping buffer).

2.3.1.1.1 Gravity flow nickel column

A gravity nickel column (Biorad) was used which was manually loaded with nickel sepharose. (2ml/1l expression) The protein was loaded 3 times and washed once with lysis buffer. 6-12 Fractions were eluted with elution buffer depending on the visible colour- change of the resin. For buffers see Buffer list.

2.3.1.1.2 Nickel column on ÄKTA

A His Trap HP 5ml (Amersham Bioscience) column was used. The column was equilibrated with Buffer A. The protein was eluted over 20 column volumes with a gradient up to 80% Buffer B. For buffer see Buffer list.

2.3.2 Ion- Exchangers

Ion- Exchangers are based on the amphoteric character of amino acids. In acidic environment lysine, arginine and histidine are positive charged whereas aspartate and glutamate are negatively charged in basic pH-environment. The isoelectric point is defined as the pH at which the proteins has no charges at all. Therefore a protein is positive charged in an acid buffer ($\text{pH} < \text{pI}$) and negative charged in a basic buffer ($\text{pH} > \text{pI}$) (Figure 7).

This is exploited by ion- Exchangers. In cation exchangers positive charged proteins bind to the negative charged resin and are eluted by an increasing gradient of cations in salt. For anion exchangers the principle is the same but here the protein is competing with anions.

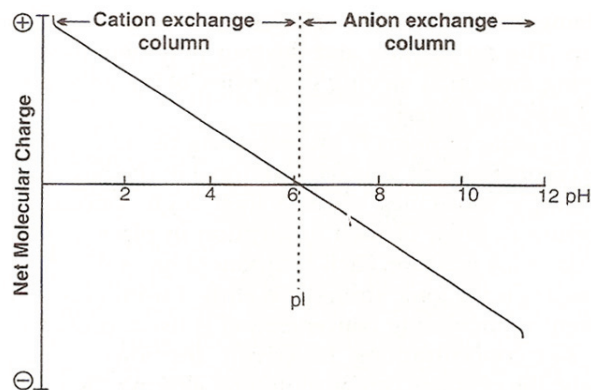


Figure 7: Use of anion- or cation exchangers dependent on the pH of the buffer (Rosenberg 2005)

The more unequal the pH of the buffer is to the pI, the stronger the protein can bind to the resin. The pH should at least be +1 or - 1 of the pI. Posttranslational modifications and conformational changes can have effect on the elution volume because they can charge distribution on the surface of the protein.

2.3.2.1 Cation- exchanger

A SP Sepharose FF (Amersham Bioscience) was used.

The column was equilibrated with Buffer A + 20 mM $(\text{NH}_4)_2\text{SO}_4$. The protein was eluted by an increasing salt gradient up to 1M over 10 column volumes.

For buffers see Buffer list.

2.3.2.2 Anionexchanger

A Hi Trap Q FF 1ml (Amersham Bioscience) column was used. The sample was always loaded two and sometimes even three times when the column was still overloaded.

The protein was eluted over 10 column volumes with a gradient starting with Buffer A and ending with 50% Buffer B.

For buffers see Buffer list

Resource Q (5ml; Amersham Bioscience) was also run. Same buffer where used like for HiTrap Q FF 1ml but without β - Mercaptoethanol. A gradient over 25 column volumes up to 100% Buffer B was used.

2.3.3 Hydrophobic Interaction Chromatography

HIC is a combination of a non- denaturing salt precipitation and a chromatographic separation-method. The surface tension is increased by chaotropic salt and therefore the hydrophobic regions of the protein are exposed to the surface. These hydrophobic areas can interact with the hydrophobic resin. Normally alkyl- or phenyl groups are used for this purpose. The proteins are eluted by a decreasing gradient of salt.

Normally $(\text{NH}_4)_2\text{SO}_4$ is used as salt- component because it has a chaotropic and “salting out” effect and is in this way supporting the hydrophobic interaction.

Protocol:

A Phenylsepharose – column (Amersham Bioscience) was used for the second step of HCAII purification

For this the concentration of $(\text{NH}_4)_2\text{SO}_4$ had to be increased to 2 M. The $(\text{NH}_4)_2\text{SO}_4$ has to be added slowly and on ice. Then the sample was centrifuged for 30min. 20 000g to remove precipitate.

$$\frac{(V_1 \times c_1) + (V_2 \times c_2)}{(V_1 + V_2)} = c_3$$

V_1Sample Volume
 c_1Concentration of $(\text{NH}_4)_2\text{SO}_4$ in the sample
 V_2Volume of $(\text{NH}_4)_2\text{SO}_4$ with concentration c_2
 c_2Concentration of $(\text{NH}_4)_2\text{SO}_4$
 c_3Final concentration of $(\text{NH}_4)_2\text{SO}_4$ in the sample

The column was equilibrated with Buffer B. The protein was eluted by a decreasing gradient of salt to 0 M over 10 column volumes. For buffers see buffer list.

2.3.4 Size exclusion chromatography (Gelfiltration)

SEC can separate proteins due to their size. The column is filled with a densely packed porous matrix. Proteins larger than these pores do not enter them and will be eluted at the dead volume. Smaller proteins penetrate the pores and will be eluted later. The smaller the protein the bigger is the maze of pores and the longer it will take them to leave the column. Therefore it is important to choose a column with the right size of pores (Table 1).

Table 1: Types of Gelfiltration columns and their separation ranges; (biotech)

Gel type	Bead size μm	Fractionation range Globular proteins	Fractionation range Dextrans
Superdex peptide	11 – 15	100 – 7 000	–
Superdex 30 prep grade	24 – 44	– 10 000	–
Superdex 75 prep grade	24 – 44	3 000 – 70 000	500 – 30 000
Superdex 75	11 – 15	3 000 – 70 000	500 – 30 000
Superdex 200 prep grade	24 – 44	10 000 – 600 000	1 000 – 100 000
Superdex 200	11 – 15	10 000 – 600 000	1 000 – 100 000

Protocol:

A Sephadex 200 26/60 column (Amersham Bioscience) was used for the final steps in purification of his- trx – HAE1 and HCAII. 5ml sample were loaded with a sample

loop on the equilibrated column and elution was done over 1.5 column volumes. For buffers see Buffer list.

2.3 Analysis

2.3.1 Determination of Protein concentration by UV- Spectrometry

Different dilutions of protein in 6 M Guanidine- hydrochlorid were prepared. Then the samples are measured with a UV- spectrometer at 280nm. 6 M Guanidine- hydrochlorid was used as blank.

Following Lambert- Beers- Law:

$$E = \epsilon \times d \times \epsilon$$

E.....Absorption
d.....Optical path- length in cm
 ϵExtinction coefficient in mg/ml or mM/ml

Measured values smaller then 0.1 or bigger then 1 were not used because out of these borders Lambert- beers- Law is not valid any more.

2.3.2 SDS- PAGE

For casting gels see Materials and Methods

The Gels were run at 190V for 30-45min. till the blue front has reached the end of the glass plate. Then they were stained with Coomassie or the more sensitive Zn- Imidiazol- Staining.

2.3.2.1 Coomassie- Staining

Coomassie binds at acidic environment to the positive charged amino acids lysine, histidine and arginine. In the rest of the gel coomassie diffuses out when treated with destaining solution. Around 100ng /band can be detected. For buffers and solution see Buffer list.

2.3.2.1 Imidazol- Zink- Staining

This reverse staining method is around 10× times more sensitive than Coomassie staining and much less time consuming than silver staining. Imidazol- Zink- staining is a very gentle method that does not require strong acid solutions, organic dyes and chemical modifiers. So the proteins can be eluted and used in biological and enzymatic assays.

The method exploits the ability of proteins to bind Zn^{2+} and of imidazol to react with unbound Zn^{2+} to produce insoluble zinc- imidazolate.

The background of the gel is therefore deep- white while the protein bands are transparent.

1. Rinse the gel for a few seconds in dH_2O
2. Equilibrate the gel in 50 to 100ml 0.2 M Imidazol- solution; time of this step depends on the percentage of the gel (10%PAGE 5min.; 12.5%PAGE 10min.; 15%PAGE 30min.)
3. Discard the imidazol- Solution
4. Stain the gel in 50- 100ml 0.3 M ZnCl_2 - Solution
5. Discard ZnCl_2 - Solution when the background becomes deep white or dark blue with transparent protein bands
6. Store the reverse- stained gels in dH_2O

2.3.3 SDS- Urea- Gels

Urea- Gels can be useful when the mobility of small proteins on SDS- gels is no longer proportional to their molecular weight.

	X% Separation Gel	X% Stacking Gel
30% Acrylamid- Mix	X/3 ml	X/7.5ml
1.5 M Tris- HCl pH=8.8; 0.4% SDS	2.5ml	0ml
0.5 M Tris-HCL pH=6.8; 0.4% SDS	0ml	1ml
Urea	4.8g	1.9g
H ₂ O	3.9 - X/3ml	1.6- X/7.5ml
10% APS	50μl	30μl
TEMED	5μl	5μl

All buffers and procedures are the same as with SDS- PAGE.

2.3.4 Western – Blot

A SDS PAGE is blotted on a nitrocellulose- membrane. With antibodies a special protein or a part of it can be detected.

1. Run one gel for Western blotting and one for Coomassie- staining
2. Equilibrate a suitable size of membrane and soak 2 sponges and 2 pieces of filterpaper in Western- Transfer- Buffer (Touch the membrane only with tweezers)
3. Now build the Transfer- “Sandwich” beginning from the anode in the following order together:
 - a. Sponge
 - b. Filter
 - c. Gel
 - d. Membrane
 - e. Filter
 - f. Sponge

The proteins travel from the cathode (black) to Anode (red).

4. Place the cassette in the holder with ice cold Transferbuffer and run the blot for 1h at 100V while stirring; disassemble the sandwich and stain the gel as a transfer control
5. Block the membrane at least for one hour in 10-20ml in Blocking solution
6. Wash 3×10min. in 20ml TBST
7. Incubate for 1h with 1st antibody (anti- his- tag antibody)
8. Wash 3×10min. in 20ml TBST
9. Incubate for 1h with 2nd antibody
10. Wash 3×10min. in 20ml TBST
11. Develop the blot in 10ml Development buffer containing 100μl NBT (stored on –20°C) and 100μl BCIP (stored on –80°C)
12. Shake gently till the colour develops and the wash with water to stop the reaction

2.3.5 Expression and Purification of TEV

1. Transformation of Vector in BL21plysS
2. Prepare 5ml overnight culture with LB containing 1% Glucose, Kanamycin and Chloramphenicol
3. Inoculate overnight culture in 1l LB
4. Grow to OD ~0.5-0.8
5. Cool down and then induce with 1ml 0.5 M IPTG
6. Harvest cells and weight pellet
7. Dissolve cells in 10ml Lysisbuffer/g wet pellet
8. Sonicate 10min. 2cycles at 40% power on ice
9. Spin down 20min. 5000g

Nickel column

1. Add 1/1000 Expression Volume of Ni Sepharose to a gravity column
2. Wash with water and equilibrate with lysis buffer
3. Decant supernatant an Ni column
4. Reload flow through twice on Ni column
5. Wash with 10 column volumes lysis buffer, wash1, wash2 and wash 3
6. Elute with 5 column volumes elution buffer

7. Measure the concentration of TEV and run a gel
8. Dilute it to 1mg/ml with glycerol
9. Freeze aliquots in liquid nitrogen and store them on -20°C

2.3.6 Cleavage with TEV- protease

The samples were cleaved with 100µl TEV (1mg/ml) over night at 4°C. Sometimes the buffer conditions were really unfavourable (pH < 8) but could not be changed. Therefore more TEV was added or the cleavage was begun with 15min. on 37°C or RT and completed on 4°C.

If the sample contained imidiazol cleavage was done during dialysis.

2.3.7 Protein- Concentration

The Concentration – Steps were done with Centriprep – Devices (Millipore). For volumes smaller than 1ml “Ultrafree- MC” (Millipore) were used.

2.3.8 Dialysis

Volumes bigger then 3ml were dialyzed with “molecular porous membrane tubings” (Spectrumlabs), while for smaller volumes the Slide-.A- Lyzer – Kit (Pierce) was used. Always a cut- off at least 3 times smaller then the protein was used. A typical Dialysis protocol was to begin the dialysis in the morning change the buffer in the evening and dialyze over night.

2.3.9 Aceton- Concentration

300µl sample were mixed with 1.5ml ice- cold acetone and put for 30min. on -20°C. Then the sample was centrifuged for 15min. on maximal speed on 4°C. The supernatant was discarded and the pellet was spun in a speed vac for 15min. to remove the rest of acetone. The pellet was dissolved in 10µl dH₂O and 10µl 2×sample buffer and loaded on a SDS PAGE.

2.3.10 Thermofluor

Thermofluor is a high through put method to determine the conditions (buffer, pH, additives...) in which a protein is most stable. Thermostable proteins have a lower propensity to aggregate, unfold or undergo proteolysis. Changes of conditions can dramatically increase or decrease thermostability. It was proved that thermostable proteins also have a higher probability to crystallize (Ericsson, Hallberg et al. 2006; Vedadi, Niesen et al. 2006).

The method is based on the properties of the hydrophobic fluorescent dye SYPRO ORANGE. It is quenches by water unless interacting with hydrophobic components like the hydrophobic core of unfolding protein (Figure 8).

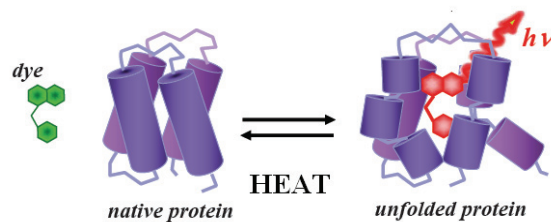


Figure 8: Dye unbound to native protein; Dye bound to heat denaturated protein

(http://lab-robotics.org/Presentations/Kranz%200509/05LRIG_JKKranz-seminar.pdf (2.07.07))

The protein is heated in descent steps and the fluorescence is measured. As long as the protein is stable, low fluorescence should be detected. Sooner or later the increase in temperature leads to heat denaturation of the protein and to sigmoid rise of fluorescence because the dye can now bind to the exposed hydrophobic regions. The inflection point of this curve is defined as the melting point (Figure 9).

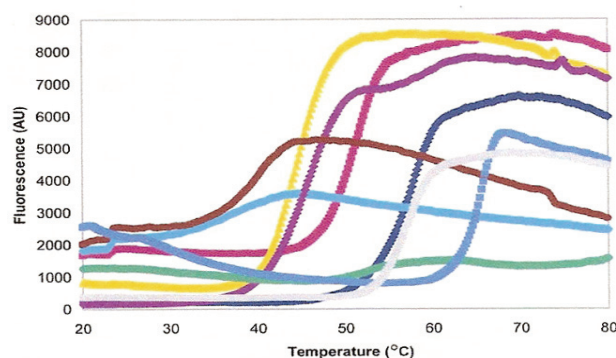


Figure 9: Melting curves measured by thermofluor (Ericsson, Hallberg et al. 2006)

The melting points (T_m) of protein in different conditions are determined and compared. The farther the curve is shifted to the right (higher temperature) the more stable the protein is in that condition. The condition with the highest T_m is most suitable to stabilize the protein.

The method requires a machine which can measure fluorescence and can heat the sample in well defined steps. The most suitable system is a RT-PCR machine.

Because there is no fluorescent dye used in RT-PCR with similar extinction and emission, therefore a combined filterset had to be used (Figure 10). The excitation filter of FAM (490nm) and the emission filter of ROX (575nm) are used.

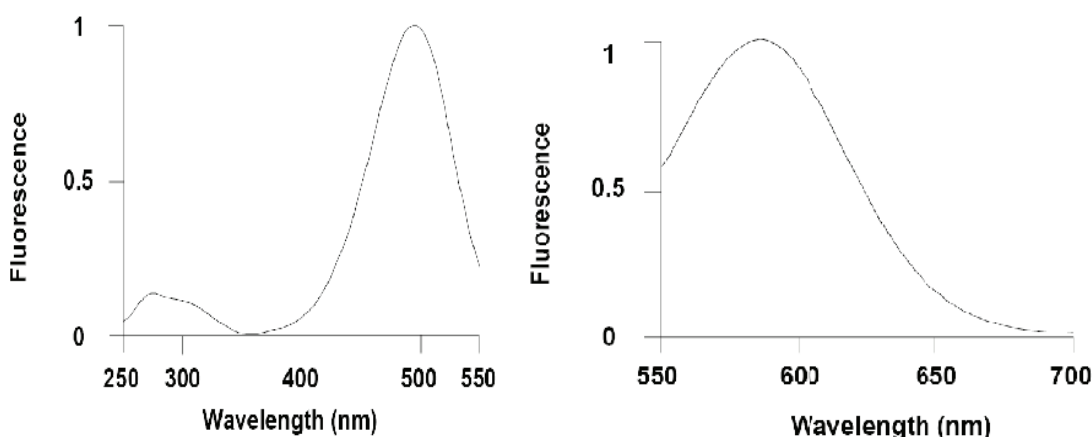


Figure 10: Excitation- (left) and Emissionspectrum (right) of SYPRO Orange

(<http://www.bio.indiana.edu/~ybelab/procedures/SyproOrange.pdf> (2.07.07))

Protocol:

The method is done with 96- well RT- PCR plates. Transparent or white ones can be used. Masterplates with different buffers, ionic strength or additives can be prepared and transferred to the 96 wells sample plate.

5 μ l protein (2.5mg/ml)
 7.5 μ l SYPRO ORANGE 5000 100 $\times$ diluted
12.5 μ l 2 $\times$ Masterplate condition
 25 μ l

The proteins were heated in steps of 1°C from 25°C to 95°C. In every step the fluorescence was measured.

2.3.11 Crosslinking

Two ways were tried.

2.3.11.1 Crosslinking with BS²G

This crosslinker can connect all different Lysines and Aminotermini of proteins and Peptides as long they come close enough. The crosslinking agent is available in the heavy and the light form that means that in the heavy form two carbons of CH₂ – groups are converted to CD₂ (Figure 11). The deuterated and the non- deuterated form are used for crosslinking in a ratio 1:1. Tryptic digested crosslinking products analyzed by mass spectrometry contain peptides showing a difference of 4 units. This difference results from the mass difference of the crosslinkers and makes it possible to identify the sites of intra- and intermolecular interactions.

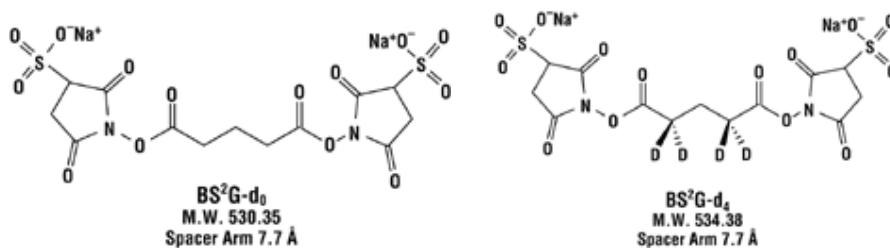


Figure 11: BS²G-d₀ and BS²G-d₄;

(<http://www.piercenet.com/products/browse.cfm?fldID=3F8F9D3C-2764-44DE-A005-CCB856AB5696> 5.11.07)

Protocol:

The reaction is pH sensible and will be impeded at a $\text{pH} < 6$. It is also important to use no buffer components with NH_2 - or SH - groups.

→200×crosslinker: 2mg heavy and 2mg light crosslinker in 40μl DMSO

For the crosslinking of the complex (HCAII and HAE1ct) 10 times higher protein concentration and crosslinker were used than for the positive control because otherwise the complex would not be visible on a coomassie- stained SDS- PAGE. The complex was formed in Gelfiltration buffer and then diluted with crosslinking buffer for the reaction.

α- actinin (103 kDa) was used as a positive control because it is known to form a homodimer and it is very likely that in this case Lysines or Amino- termini come near enough to be crosslinked.

HCAII instead of the complex was used as a negative control because it should not interact with itself.

Sample:

3μl Complex (Final conc. = 20 μM) or HCAII

3μl 200× Crosslinker

94μl 20mM HEPES pH=7.5 20mM NaCl

100μl

Positive control:

17μl α- actinin (0.6 μM)

3μl 20×crosslinker

80μl 20mM HEPES pH= 7.5 20mM NaCl

100μl

The samples were put over night on 4°C. Next day the reaction was stopped by adding 5μl of Ammonium bicarbonat and the samples were put 15min. on ice. Afterwards the crosslinking was checked on 10 and 12% SDS- PAGE.

The reaction can be optimized by changing time, concentration of crosslinker and temperature.

2.3.11.2 Crosslinking with Formaldehyd

Formaldehyd is used for unspecific crosslinking of cells and tissue slices. In lower concentration it can also be used to crosslink interacting proteins in solution (Figure 12).

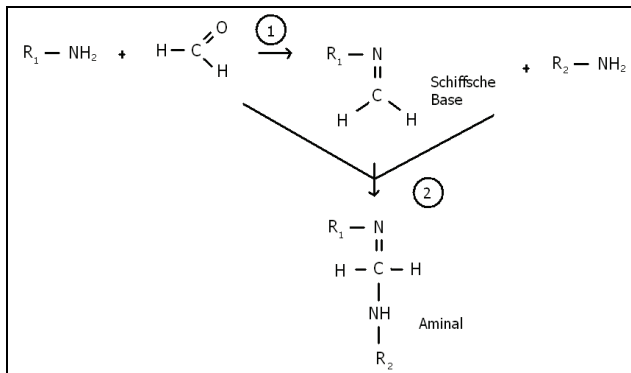


Figure 12: Mechanism of Crosslinking by Formaldehyd

Protocol:

The Products of this reaction are acid- instable and therefore a basic buffer should be choosen. To compare the results with the BS²G crosslinking a rather similar buffer (20 mM HEPES pH=8; 20 mM NaCl) was chosen. Again α - actinin (0.6 μ M) was used as a positive control.

The complex of HCAII and HAE1ct was formed in same concentration in Gelfiltration buffer and then diluted to 20 μ M with the crosslinking buffer.

Concentrations between 0.25 and 2% of Formaldehyd were added and after 2h on RT aliquots were taken and loaded on a 10 and on a 12% SDS PAGE. α - actinin was crossinked with 1% and HCAII with 2% of Formaldehyd.

2.4 Determination of Structure

2.4.1 Crystallography

Macromolecular crystallography is a method to determine the 3- dimensional structure of proteins and other macromolecules by the visualisation of the electron density. The first and maybe most challenging barrier are to get crystals. They are built of symmetrical units highly ordered in three dimensions. Every crystal can be reconstructed by its unit cell as a basic module and some clearly defined symmetrical operations. Because of their high symmetry crystals behave in the x- ray beam like an amplifier of the unit cell and make it possible to get enough information for structure determination. In difference to salt crystals protein crystals are not rigid because they contain 20-80% water and the proteins are still relative flexible.

2.4.1.1 Growing crystals

The principle of growing crystals is to decrease the solubility of the protein in the way that it forms nuclei (seeds for crystals) but does not precipitate. In the small zone between solubility and precipitation crystal seeds can form and grow. Also the right equilibrium between seeding and growing is required. An overhang of nucleation gives rise to a lot of small useless crystals (Figure 13).

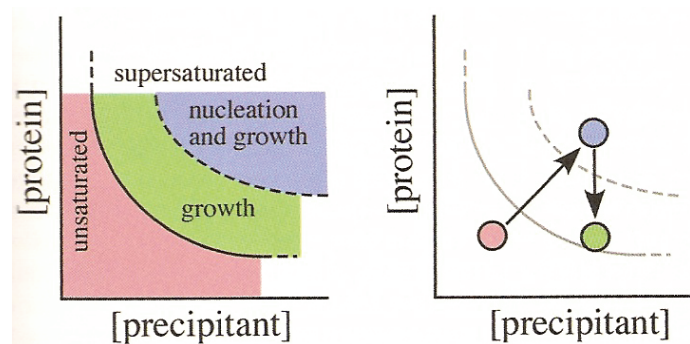


Figure 13: Phasediagram of nucleation and crystal growth (Gale 2006)

The most common used precipitants are ammonium sulphate and PEG (available in different length). Salts like ammonium sulphate precipitate proteins by competing

with them for the water shell. This is a process called salting out. Organic components like PEG lower the dielectric constant in solution and precipitate proteins in this way. Methods of setting crystallization drops are sitting drop, hanging drop, under oil batch or “dialysis”. The most popular are sitting and hanging drop (Figure 14).

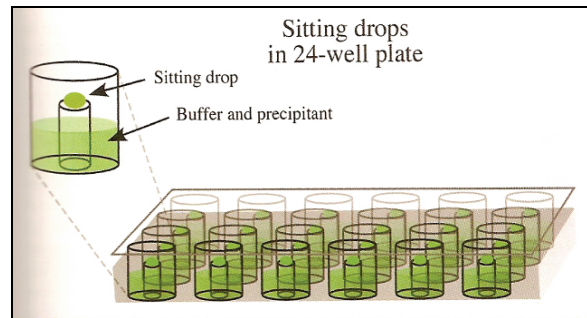


Figure 14: Sitting drop method;(Gale 2006)

2.4.1.2 Phasing

The second barrier is to determine the phases of the structure factors which are measured by the x-ray experiment. They are a necessary input for the Fourier-Transformation which transforms the reciprocal space (experimental x-ray pattern) into the real space (electron density of protein). Different methods can be used:

1. Isomorphous replacement:
Heavy atom derivatives of the crystals have to be produced and are compared to the native dataset. From their difference the phases can be extracted.
2. Anomalous scattering:
Variable wavelength synchrotron radiation is used to collect dataset at different wavelength. The different datasets are treated like native protein and derivatives. Like in isomorphous replacement this can be used to determine the phases.
3. Molecular replacement:
The structure of a homolog protein or a domain of the protein of interest has to be known. This can be used to extract the phases.

The Fourier- Transformation based on the input of structure factors and phases gives an electron density map (Figure 15). The amino acids can then be fitted in.

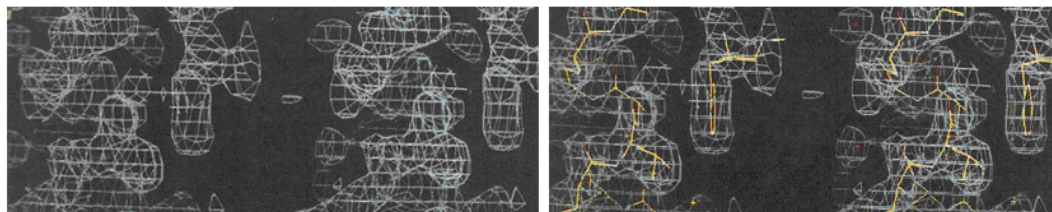


Figure 15: Electron density (left); amino acid structures fitted in electron density (right);(Gale 2006)

Protocol:

The initial screens were done with the crystallization robot. Crystalquick Plates 96 wells (Greiner) were used. It is possible to set 3 drops in these plates. A program was used which set drops of 100nl in ratio (protein: Crystal screen) 1:1 or 1:2. One place stays free. For the Refinement EasyXtal tool (NeXtal) 24 well plates were used. 1 μ l of Protein- solution was mixed with 1 μ l Crystallization Solution. The volume in the reservoir was 1ml. The data was measured with our home facility (Bruker proteum X8) or at ESRF Grenoble.

2.4.2 NMR: (Nuclear magnetic resonance)

Molecules placed into a magnetic field have a bulk magnetization along the z- axis (direction of the magnetic field). This magnetization consists of small magnetic fields due to nuclei which also have nuclear spin magnetic moment μ . When these nuclear moments experience the magnetic fields they can have high or low energy orientations depending on their angle to the magnetic field. In equilibrium the net magnetization of the chaos of “tiny nuclear magnets” is pointing into the z- direction (direction of the magnetic field) because it is energetically more favourable (Figure 16).

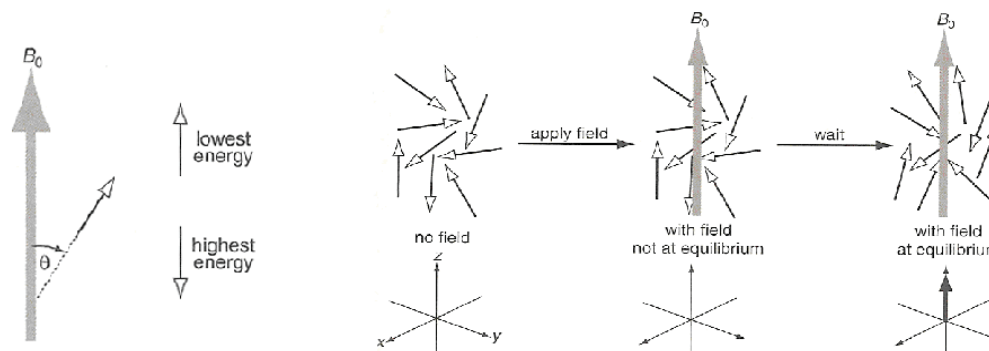


Figure 16: Dependence of energy on orientation to magnetic field (left); Assembling of net magnetization over time (right); (Keeler 2005)

This equilibrium can be disrupted by the application of a Radiofrequency- pulse. Dependent on the duration and frequency of the pulse and strength of the magnetic field, the net magnetization can be “turned” into the y- axis and a signal can be detected by coils. This signal is called FID (= Free induction decay). The FID can be processed by the Fourier- Transformation that the time domain is changed into frequency domains which can then be assigned to specific groups of the molecules (Figure 17). In this way their structure can be determined.

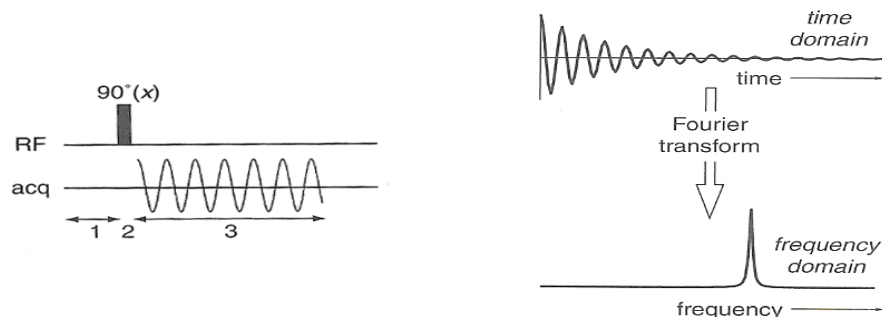


Figure 17: 1D- pulse sequence (left); Fourier- transformation of time domain (FID) to frequency domain (right); (Keeler 2005)

Not every nucleus is sufficient to contribute to the net magnetization. It is necessary that they have a nuclear spin quantum number I . This is normally the case when the nucleus has an unequal number of protons. Typical elements used for macromolecular NMR (proteins, RNA, DNA) are H^1 , N^{15} and C^{13} . Therefore for 2- or 3 dimensional NMR studies proteins are expressed in minimal medium containing the rare isotopes like N^{15} and C^{13} .

2.4.2.1 Two dimensional NMR- experiments for proteins

It is also possible to record multi- dimensional spectra. These NMR experiments have more complex pulse- sequences than 1D- experiments which include evolution times, where the signal that is recorded in t_2 is affected by what happens in t_1 .

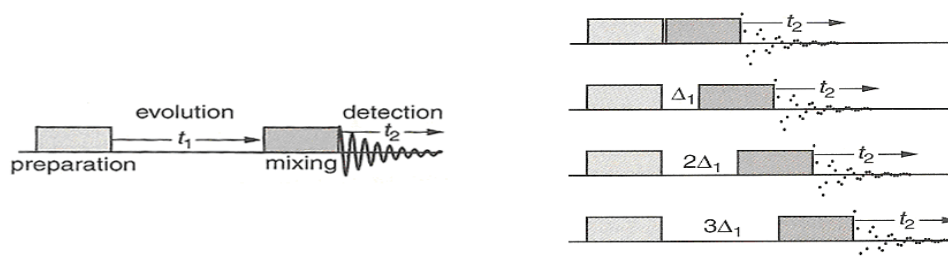


Figure 18: 2D- pulse sequence (left); Increasing t_1 in defined increments (right); (Keeler 2005)

A 2D- spectra is produced by recording an array of 1D FIDs, where i.e. t_1 is successively extended by the increment Δ_1 (Figure 18), and performing Fourier- transformation in both directions (t_1 and t_2).

A common used 2D- method is the HSQC (= Heteronuclear Single- Quantum Correlation). Here magnetisation is first transferred from H^1 to N^{15} and allowed to evolve (t_1), then magnetization is transferred back to H^1 where it is allowed to evolve while the FID is recorded (t_2). Only parts of the protein where hydrogen and nitrogen are coupling are detectable. So it is possible to see the NH- group of the backbone of every amino acid, despite of prolines, as well as amino groups of asparagines, glutamine and Lysine (Figure 18).

2.4.2.1.1 Binding Titrations

Since the chemical shift is very sensitive to differences in electronic structure, binding studies are straight forward in NMR- spectroscopy. Peaks that change their position (either upfield -to low ppm -or downfield - to high ppm) due to titration with an unlabeled ligand are therefore affected by changes in their electronic environment. Therefore, it is possible to determine the interaction site, as well as determination of binding constants

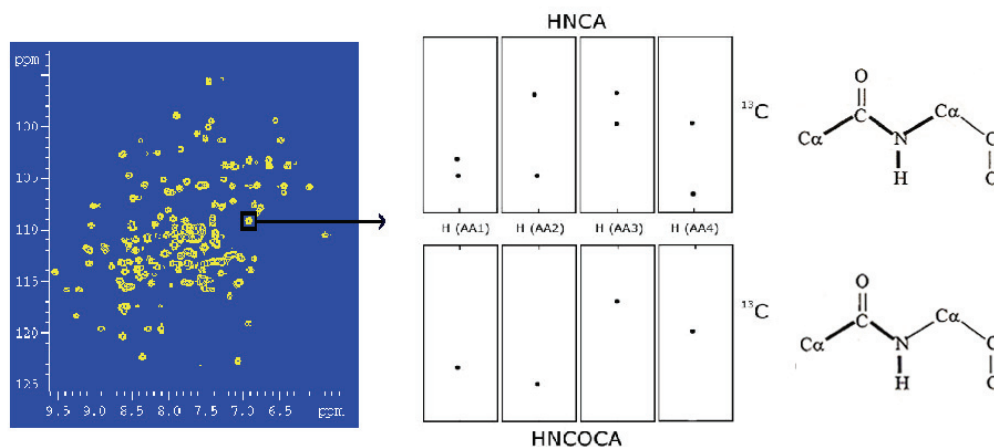


Figure 19: HSQC (left); HNCA or HNCOCA – spectra in 3rd dimension of HSQC (middle); path of magnetization transfer in HNCA and HNCOCA (right)

(http://www.public.iastate.edu/~bfulton/instructions/hsqc_3919.html (3.7.07))

(<http://en.wikipedia.org/wiki/Image:Hncanhcoca.jpg>) 3.7.07))

2.4.2.2 Three dimensional NMR- experiments

3D NMR experiments are similar working like 2D but here one evolution time more is introduced and the result is a 3- dimensional cube of information. Here the coupling

between N^{15} , C^{13} and H^1 is for example detected. Therefore the HSQC just gets a new dimension (C^{13} - shifts). These experiments differ by the “path” of magnetization transfer in the backbone which is controlled by the used coupling constants. For example in hncoca the magnetization is transferred for hydrogen to nitrogen then to the carbonyl- groups and finally to the $C\alpha$ - carbon.

By the combination of different 3D experiments (for example HNCA + HNCOCA or CBCACONH and HNCACB) and the primary sequence of the protein, every peak in the HSQC could be assigned to a position in the primary sequence of the protein (= backbone assignment). In this way it is possible to identify the amino acids which are shifted by the binding of a ligand or oligopeptide. To determine the 3D structure of the protein, additional measurements are needed: first, all resonances (also from side chains) have to be assigned and finally distance measurements (NOESY spectra) are needed.

2.4.2.2.1 NOESY

Two spins can couple either via chemical bonds or directly via space. Latter is called dipolar coupling and is the basis of the so called Nuclear Overhauser Effect (NOE). This effect is dependent on distance and decreases with its power to 6. Therefore only interactions closer than $3\text{\AA}$ can be measured. Amino acids coming close to each other not due to adjacency in primary sequence but due to adjacency in space deliver therefore an not expected NOE, which can be used to figure out the three-dimensional structure.

Protocol:

For 2D Experiments 400- 500 μ l Protein ($c = 0.3$ - 0.6mM) were used. For 3D Experiments the same volume was used but concentrations between 0.5 and 0.6mM . In all NMR experiments 20mM Na-Phosphate buffer $\text{pH} = 6$, 20 mM NaCl was used.

2.4.2.3 Pulse Sequences

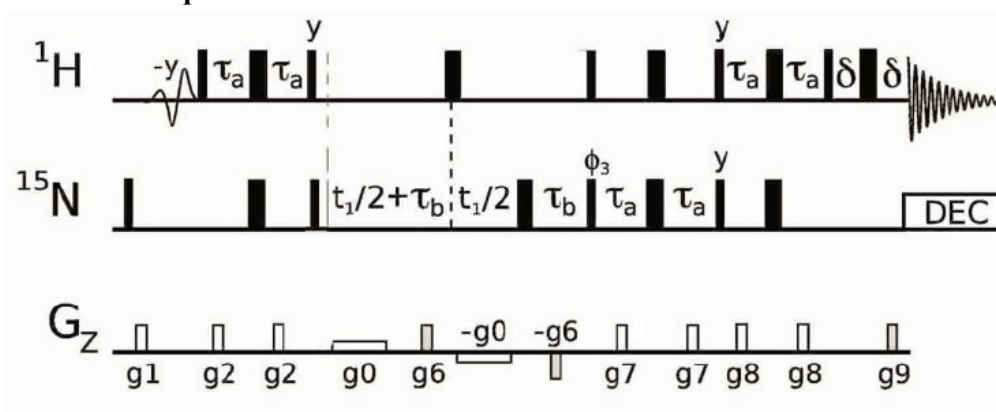


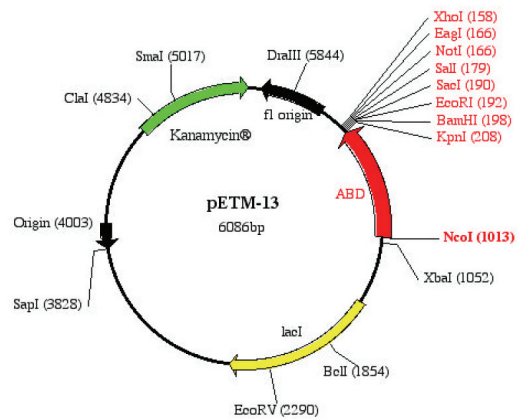
Figure 20: Sequence of PFG- PEP- HSQC (Dorigoni 2006)

The other pulse sequences were derived from the paper (Sattler Michael 1998).

2.5 Materials

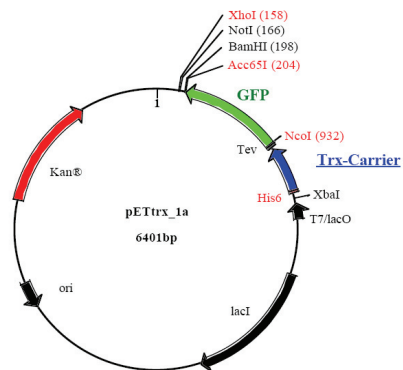
2.5.1 Used Vectors

pETM-13:



pETtrx_1a:

HAE1ct was cloned in there instead of GFP.



2.5.2 Primers

HCAIIF1 (forward)

5' AATTGGTCTCCCATGTCCCATCACTGGGGGTAC 3'

HCAIIR2 (reverse)

5' 3'

GGTACCGAGACCTTATTTGAAGGAGGCTTTGATTG

2.5.3 Cell strains

Cell Strains	Genotype	Description
DH5 α	<i>supE44 ΔU169 deoR (f80lacZAM15)hsdR17 recA1 endA1 gyrA96 thi-1 relA1</i>	High copy plasmid cell line
XL1-blue	<i>recA1, lac-, endA1, gyrA96, thi, hsdR17 (rk-, mk+), supE44, relA1, λ-, [F', proAB, lacIqZ, ΔM15, Tn10 (tetr)</i>	
BL21 (DE3)	<i>F- ompT hsdSB (rb- mB-) gal dcm (DE3)</i>	The (DE3) cells carry a chromosomal copy of the T7 RNA polymerase gene under control of the lacUV5 promoter. This strains are used for protein expression of pET vectors.
Rosetta (DE3)	<i>F- ompT hsdSB (rb- mB-) gal dcm lacY1 (DE3) pRARE6 (CmR)</i>	This strain is derived from the Tuner cell and is used to enhance the expression of eukaryotic proteins that contain rare codons. This is accomplished by supply tRNAs for AGG, AGA, AUA, CUA, CCC, GGA codons on a compatible chloramphenicol-resistant plasmid.
Rosetta (DE3) pLysS	<i>F- ompT hsdSB (rb- mB-) gal dcm lacY1 (DE3)</i>	Same as above but has an additional vector

	<i>pRARE6 pLysE (CmR)</i>	transformed that produce a larger amount of T7 lysozyme than that of pLysS. In Rosetta (DE3) pLysE the rare tRNA genes are carried on the same plasmids that carry the T7 lysozyme.
C41 (DE3)		derived from BL21(DE3) It has at least one uncharacterized mutation which prevents cell death associated with expression of many toxic proteins.
BL21* (DE3)	<i>F⁻ ompT hsdS_B (r_B⁻m_B⁻) gal dcm rne131 (DE3)</i>	The mutation in the rne gene which encodes for endonuclease RnaseE enhances mRNA stability.
BL 21 (DE3) pLysS	<i>Genotype:</i> <i>F⁻ ompT hsdSB (rb⁻ mB⁻) gal dcm (DE3) pLysE (CmR)</i>	Same as above but has an additional vector transformed that produce a small amount of T7 lysozyme that is used to suppress basal expression of T7 RNA polymerase prior to induction and thus reduces leaky expression.
C41 (DE3) pROS		C41 (DE3) transformed with pROS (Plasmid coding for t- RNAs for rare codons).
C43 (DE3)		Is derived for C41 (DE3). It can express a different set

		of toxic proteins than C41(DE3)
Tuner (DE3)	<i>F- omT hsdS_B (r_B⁻m_B⁻) gal dcm lacY1</i>	The lac permease (<i>lacY</i>) mutation allows uniformly entry and IPTG-concentration dependent low level to high level expression. Low level expression can improve the solubility and activity of difficult target proteins.

2.5.3.1 Genetic Markers

Table 2: Genetic Markers; Novagen 2004/ 2005 catalog

Genetic Markers ¹	
Marker	Description
<i>ahpC</i>	Mutation in alkyl hydroperoxide reductase conferring disulfide reductase activity.
<i>Δara-leu</i>	Unable to utilize arabinose and requires leucine for growth on minimal media.
<i>ara</i>	Unable to utilize arabinose.
<i>dcm</i>	No methylation of cytosines in the sequence CCWGG.
DE3	Contains a lambda prophage in which the gene for T7 RNA polymerase is under control of the <i>lacUV5</i> promoter.
e14	Excisable prophage element that carries the <i>mcrA</i> gene.
<i>endA</i>	Endonuclease I activity absent; thought to improve quality of plasmid minipreps.
F⁻	Strain does not contain the F episome.
F⁺	Strain contains the single copy F plasmid.
F[']	Strain contains an F plasmid which harbors some bacterial chromosomal DNA.
<i>gal</i>	Unable to utilize galactose.
<i>gor</i>	Abolishes glutathione reductase. Allows formation of disulfide bonds in the <i>E. coli</i> cytoplasm.
<i>gyr</i>	Mutation in DNA gyrase. Confers resistance to naladixic acid.
<i>hfl</i>	High frequency of lysogenization by phage λ.
<i>his</i>	Requires histidine for growth in minimal medium.
<i>hsdR</i>	Abolishes restriction but not methylation ² of certain sequences (<i>r⁻m⁻</i>).
<i>hsdS</i>	Abolishes both restriction and methylation ² of DNA at certain sites (<i>r⁻m⁻</i>).
λimm⁴³⁴	Lysogen conferring resistance to kanamycin and immunity to infection by phage 434 immunity group.
<i>lac</i>	Unable to utilize lactose.
<i>lacP⁺</i>	Produces a high level of <i>lac</i> repressor.
Δ(lac)U169	Deletion of entire <i>lac</i> operon from the chromosome.
Δ(lac)X74	Deletion of entire <i>lac</i> operon from the chromosome.
Δ(lacZ)	Deletion of β-galactosidase coding region.
Δ(lac-proAB)	Deletion of entire <i>lac</i> operon and neighboring genes for proline biosynthesis. Requires proline for growth on minimal medium.
<i>lacY</i>	Abolishes <i>lac</i> permease.
<i>lacZΔM15</i>	Lacks coding region for amino terminal portion of β-galactosidase (aa 11-41).
<i>lon</i>	Deficient for an ATP-dependent protease; thought to stabilize some foreign proteins.
<i>mcrA</i>	Does not restrict DNA modified by <i>Hpa</i> II methylase at C ⁺ CGG.
<i>mcrB</i>	Does not restrict DNA modified at <i>RmeC</i> by a variety of cytosine methylases.
Δ(mcrC-mrr)	Deletion of six genes, <i>mcrC mcrB hsdS hsdM hsdR mrr</i> , involved in methylation/restriction.
<i>met</i>	Requires methionine for growth on minimal medium.
<i>mtl</i>	Unable to utilize mannitol.
Marker	Description
<i>ompT</i>	Lacks an outer membrane protease; improves recovery of intact recombinant proteins.
<i>nupG</i>	Lacks ability to transport nucleosides.
pAR5403	Amp ^r plasmid that constitutively expresses T7 gene 10.
pAR5615	Amp ^r plasmid that expresses IPTG-inducible T7 gene 10.
P1	Lysogenic for P1 prophage; constitutively expresses <i>cre</i> recombinase.
pLacI	Cam ^r plasmid that carries the gene for <i>lac</i> repressor.
pLacIRARE	Cam ^r plasmid that carries the gene for <i>lac</i> repressor and tRNA genes for several codons rarely used in <i>E. coli</i> .
pLysE	Cam ^r plasmid that carries the gene for T7 lysozyme.
pLysS	Cam ^r plasmid that carries the gene for T7 lysozyme.
pLysSRARE	Cam ^r plasmid that carries the gene for T7 lysozyme and tRNA genes for six codons rarely used in <i>E. coli</i> .
pLysSRARE2	Cam ^r plasmid that carries the gene for T7 lysozyme and tRNA genes for seven codons rarely used in <i>E. coli</i> .
pRARE	Cam ^r plasmid that carries the tRNA genes for six codons rarely used in <i>E. coli</i> .
pRARE2	Cam ^r plasmid that carries the tRNA genes for seven codons rarely used in <i>E. coli</i> .
pMC9	Tet ^r Amp ^r plasmid (pBR322) that carries <i>lacZ</i> .
<i>proAB</i>	Requires proline for growth on minimal medium.
<i>recA</i>	Abolishes homologous recombination.
<i>recD</i>	Abolishes ExoV exonuclease activity. Useful for propagation of sequences with inverted repeats.
<i>relA</i>	"Relaxed" mutation permits RNA synthesis in the absence of protein synthesis.
<i>rpsL</i>	Carries a mutation in a ribosomal protein conferring resistance to streptomycin.
<i>srl</i>	Unable to utilize sorbitol.
<i>strA</i>	(same as <i>rpsL</i>)
<i>supE</i>	Amber suppressor strain; inserts gln suppressor tRNA for UAG codon.
<i>supF</i>	Amber suppressor strain; inserts tyr suppressor tRNA for UAG codon; required for lytic growth of <i>Sam 7</i> or <i>S100 λ</i> .
<i>thi</i>	Requires thiamine for growth in minimal medium.
Tn5	Contains the Kan ^r transposable element, Tn5.
Tn10	Contains the Tet ^r transposable element, Tn10.
Tn1000	Contains the <i>yδ</i> transposon.
<i>tonA</i>	Confers resistance to bacteriophage T1 and T5.
<i>traD</i>	Defective for ability to transfer F episome DNA.
<i>trp</i>	Requires tryptophan for growth in minimal medium.
<i>trxB</i>	Abolishes thioredoxin reductase. Allows formation of disulfide bonds in <i>E. coli</i> cytoplasm.
<i>xyl</i>	Unable to utilize xylose.

Notes

1. To denote genotype, the convention is to mention only the genes that are defective, not those that are wild type. Genes are written in lowercase italics. The symbol for a gene is usually a three-letter abbreviation that suggests the function of the gene and is followed by a capital letter if more than one gene contributes to a phenotype.

2. *EcoK* and *EcoB* refer to Type I restriction/modification systems found in *E. coli* strains K and B. The modification function methylates adenosine residues in sequences AA(N)₁GTGC for *EcoK* and TGA(N)₁TGCT for *EcoB*. The restriction activity is directed against those sequences in which the adenines are not modified.

Amp: 50 µg/ml ampicillin or carbenicillin
Cam: 34 µg/ml chloramphenicol
Kan: 15 µg/ml kanamycin
Str: 50 µg/ml streptomycin
Tet: 12.5 µg/ml tetracycline

All auxotrophs require 40 µg/ml of the appropriate amino acid for growth on minimal medium.

2.5.4 Media

1×LB	20g/l LB Broth (Sigma)
2×LB	40g/l LB Broth (Sigma)
Autoinduction Media	928ml ZY; 1ml 1000×metal mix; 50ml 20×NPS; 20ml 50×5052
1×M9 Minimal Media	6g Na ₂ HPO ₄ ×2H ₂ O+ 3g KH ₂ PO ₄ + 0.5g NaCl+1g NH ₄ Cl +1l water and then autoclaved; 10ml Trace elements; 0.3ml 1M KCl; 1ml Biotin; 1ml Thiamin; 2ml 1M MgSO ₄ ; 3-4g Glucose; For N ¹⁵ labelling 1g N ¹⁵ H ₄ Cl was used. For double labelling (N ¹⁵ and C ¹³ labelling) 1g N ¹⁵ H ₄ Cl and 3-4g C ¹³ labelled Glucose was used.

Solutions needed:

ZY (1l):

10g Tryptone; 5g Yeast Extract ad 1000 H₂O and autoclave

20×NPS (1l):

66g (NH₄)SO₄; 136g KH₂PO₄; 142g Na₂HPO₄; 900ml H₂O

Add in sequence in beaker, stir until dissolved; pH should be ~6.75; than autoclave

50×5052 (1l):

250g Glycerol; 730ml H₂O; 25g Glucose; 100g Lactose

Add in sequence in a beaker, stir until dissolved, autoclave

1000×metall mix:

50ml 0.1M FeCl₃×6 H₂O dissolved in 0.1M HCl; 2ml 1M CaCl₂; 1ml 1M MnCl₂; 1ml ZnSO₄; 1ml 0.2M CoCl₂; 2ml 0.1M CuCl₂; 1ml 0.2M NiCl₂; 2ml 0.1M Na₂MoO₄; 2ml 0.1M Na₂SeO₃; 2ml 0.1M H₃BO₃

Autoclave the Stock- solutions except FeCl₃

2.5.5 Antibiotics

Kanamycin	100mg/ml
Carbencillin	100mg/ml
Chloramphenicol	34mg/ml

The antibiotics were diluted 1:1000.

2.5.6 Agar and Pds

Agar	4,5g Agar + 300ml LB autoclaved
Kanamycin Pds	300ml Agar + 150µl Kanamycin (100mg/ml)
Kanamycin/Chloramphenicol Pds	300ml Agar + 150µl Kanamycin (100mg/ml)+ 300µl Chloramphenicol (34mg/ml)
Carbencillin Pds	300ml Agar + 300µl Carbencillin (100mg/ml)
Carbencillin/Chloramphenicol Pds	300ml Agar + 300µl Carbencillin (100mg/ml) + 300µl Chloramphenicol (34mg/ml)

Always let the agar cool down before adding the antibiotics.

2.5.7 Buffer list

2.5.7.1 Cloning

2.5.7.1.1 Agarose Gels

50×TAE	121g Tris; 28ml Acetic acid; 50ml 0.5 M EDTA pH=8; add to 500ml with dH ₂ O
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2.5.7.1.2 Preparation of Competent Cells

RF1 Buffer	12g RbCl; 9.9g MnCl ₂ ×4H ₂ O; 30ml 1M KOAc pH=7.5; 1.5g CaCl ₂ ×2H ₂ O; 150g Glycerol
RF2 Buffer	20ml 0.5M MOPS pH=6.8; 1.2g RbCl; 1.5g CaCl ₂ ×2H ₂ O; 150g Glycerol

2.5.7.2 Purification

2.5.7.2.1 Nickelcolumn

Gravitiy column

Lysis Buffer	100 mM MES pH=6; 150 mM NaCl; 10 mM Imidiazol; 2 mM Mercaptoethanol
Elution Buffer	100 mM MES pH=6; 150 mM NaCl; 400 mM Imidiazol; 2 mM Mercaptoethanol
Striping Buffer	20 mM Tris- HCl pH=7.6; 1.5 M NaCl; 50 mM EDTA

His Trap FF 5ml

Buffer A	20ml MES pH= 6; 20mM NaCl
Buffer B	Buffer A+ 500 mM Imidiazol

2.5.7.2.2 Ion exchanger

Cation exchanger

Buffer A	50 mM MES pH=6.2
Buffer B	50 mM MES pH=6.2 ; 2M (NH ₄) ₂ SO ₄

Anion exchanger

Buffer A	100 mM MES pH=6; 20mM NaCl; 2mM Mercaptoethanol
Buffer A	100 mM MES pH=6 ; 2M NaCl ; 2mM Mercaptoethanol

2.5.7.2.3 Hydophobic Interaction

Buffer A	50 mM MES pH=6.2
Buffer B	50 mM MES pH=6.2 ; 2M (NH ₄) ₂ SO ₄

2.5.7.2.4 Size Exclusion

For unlabeled Proteins (for Crystallography)

Gelfiltrationbuffer	20 mM MES pH=6; 20 mM NaCl
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For labeled Proteins (for NMR)

Gelfiltrationbuffer	20 mM Natriumphosphatbuffer pH=6; 20 mM NaCl
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2.5.7.3 Analysis

2.5.7.3.1 SDS- PAGE

2× Loading Buffer	12.5ml 1 M Tris - HCl pH=6.8; 30ml glycerol; 2g SDS; 36g Urea; 6.6ml mercaptoethanol; ~0.2% bromphenol blue
10×Running Buffer	30.25g Tris base + 144.1g glycine + 10g SDS add H ₂ O to 1l
Coomassie Staining Solution	150ml methanol; 150ml H ₂ O; 21ml Acetic acid; 9ml

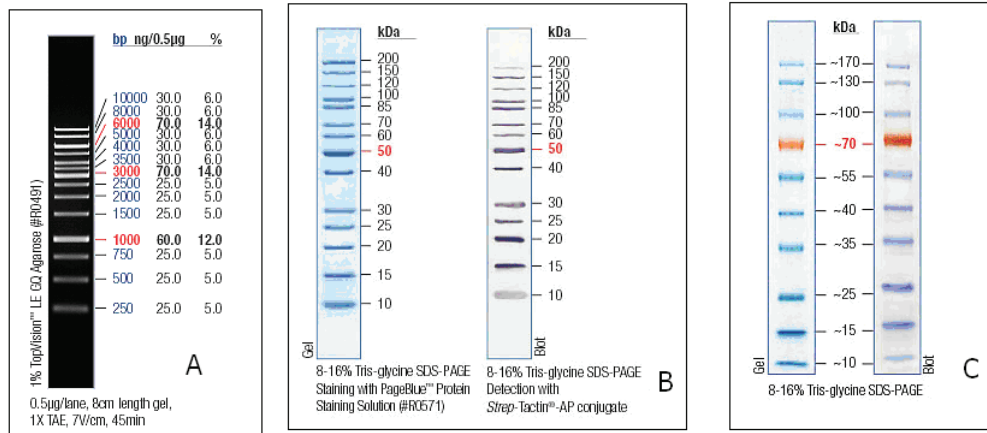
2.5.7.3.2 Westernblot and Expression Screen

10× Transfer Buffer	29g Glycin+58g Tris+ 3.7g SDS in 800ml H ₂ O and 200ml methanol
1× Transfer Buffer	80ml 10× Transfer Buffer+ 720ml H ₂ O+ 200ml methanol
TBST	20 mM Tris pH=7
1st Antibody- Solution	Rabbit anti-his antibody in TBST; dilution 1:2 000
2 nd Antibody- Solution	Mouse - Alkaline- phosphatase conjugated anti- rabbit- antibody in TBST; dilution 1: 3 000
Colour- development buffer	100 mM Tris- HCl pH= 9.5; 100 mM NaCl; 5 mM MgCl
Denaturing lysis Buffer	8M Urea; 20 mM Tris pH= 8 ; 100 mM NaCl
BugBuster Protein Extraction Reagent	Ordered from Novagen

2.5.7.3.2 Expression and Purification of TEV Protease

Lysis buffer (100ml)	20 mM Tris pH=8; 10 mM Imidiazol pH=8; 150mM NaCl; 2 mM Mercaptoethanol (always add fresh before use)
Wash 1	= Lysis buffer
Wash 2	20 mM Tris pH=8; 10 mM Imidiazol pH=8; 1M NaCl; 2 mM Mercaptoethanol (always add fresh before use)
Wash 3	20 mM Tris pH=8; 50 mM Imidiazol pH=8; 150 mM NaCl; 2 mM Mercaptoethanol (always add fresh before use)
Elution buffer	20 mM Tris pH=8; 330 mM Imidiazol pH=8; 150 mM NaCl; 2 mM Mercaptoethanol (always add fresh before use)

2.5.8 Markers



A. O'Gene Ruler 1kbp DNA Ladder ready- to use (SM 1163); Fermentas

B. PageRuler™ Unstained Protein Ladder (SM 0661); Fermentas

C. PageRuler™ Prestained Protein Ladder (SM 0671); Fermentas

3 Results

3.1 Cloning

The idea was to clone a co- expression vector for HCAII and his- trx- HAE1ct. That would have the advantage that the complex can interact from the beginning and can be purified by his tag of his- trx- HAE1.

The idea was to design forward and reverse primer which can be cut at the same time by BsaI and the result of cutting are two different overhangs. This prevents ligation into the wrong direction. The created overhangs are compatible for ligation into the NcoI and Acc65I cleaved pETM-13 vector (Figure 21 and 22).

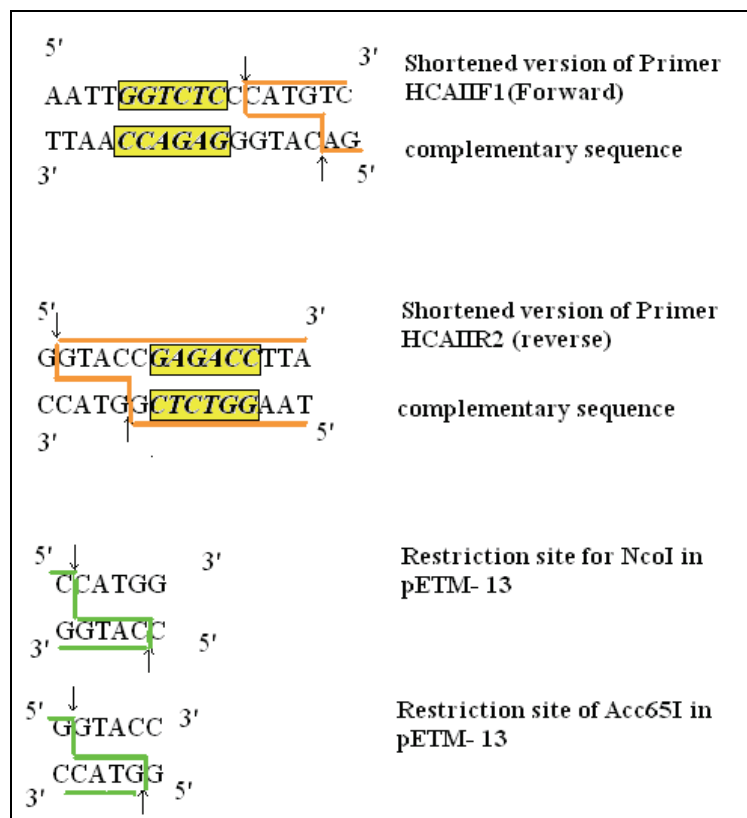


Figure 21: BsaI restriction sites

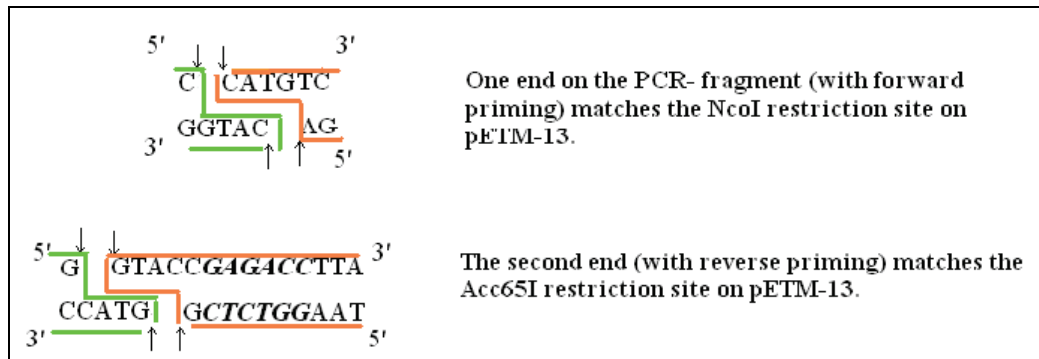


Figure 22: Ligation of BsaI restriction sites with NcoI / Acc65I cut pETM- 13

The plan was to do the following steps:

1. PCR of HCAII- template in pACA- vector with Primers containing BsaI cutting sites
2. Cut the fragment with BsaI
3. Digest of pETM- 13 with Acc65I and NcoI
4. Gelextraction bigger part of pETM-13 to remove the ABD- fragment
5. Ligation of cut fragment into cut pETM- 13
6. Transform into XL1 blue or DH5 α
7. Prepare preculture of one colony and do Miniprep
8. Cut the vector with BglII + Xho II
9. Do gelextraction for fragment (which now also carries the T7-promotor)
10. Digest pET-trx_1a HAE1ct with BamH1+ XhoI
11. Ligation the fragment into the cut vector

Steps 1-4 were performed but there were problems with ligation (Figure 21).

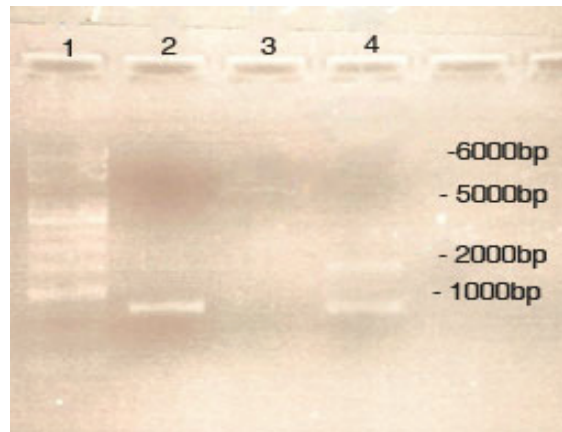


Figure 23: Ligation

1: Marker, 2: Fragment produced by PCR, 3: pETM- 13 cut with Acc65I and NcoI after gelextraction (really faint band), 4: Ligation of fragment into cut and gelextracted pETM-13

It seems that BsaI did not cut the fragment properly at one site and therefore blunt end ligation of 2 fragments has occurred. This step was tried several times but it didn't give colonies after transformation. This uncomplete cleavage can occur if there are not enough nucleotides up- and downstream of the cutting site so that the restriction enzyme cannot bind properly.

Therefore the alternative way was to ligate the fragment blunt- end into the EcoRV restriction site of pETM-13. Inserted into this vector the fragment should be cut by BsaI. PCR of the Fragment with Pfu- Polymerase (no Poly-A-Tails at 5') and digest of pETM-13 with EcoRV were performed. Then the fragment and the plasmid were ligated.

Before transformation the ligation- mixtures were cut with EcoRV because of blunt end ligation a lot of vectors would just close again. If the fragment is inserted the cutting site, it will be split by its insertion. Vectors without the fragment inserted have an intact Eco RV restriction site. Therefore they will be linearized and are excluded from transformation. The transformation gave around 20 colonies on Kanamycin plates. To check if the fragment was inserted in the plasmid, Mini Prep and PCR were done.

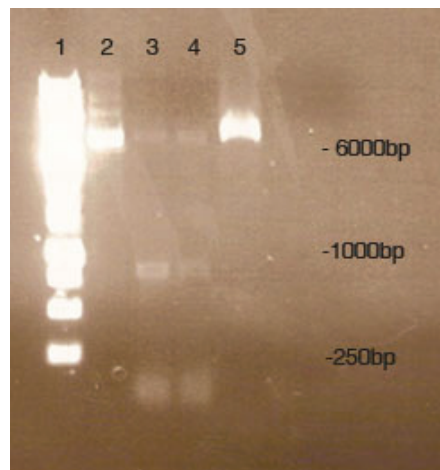


Figure 24: PCR of Mini Prep

1: Marker, 2: Miniprep, 3: PCR1 on Miniprep, 4: PCR2 on Mini Prep, 5: Mini Prep

The fragment is inserted because the PCR has produced fragments with the right length (Figure 22). The next step was to digest the plasmid by BsaI.

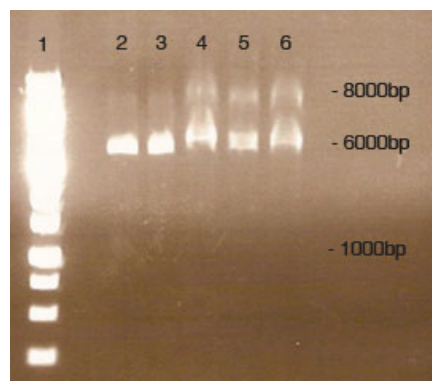


Figure 25: Plasmid of Mini Prep cut with BsaI

1: Marker, 2- 6: Plasmid with inserted Fragment cut with BsaI

By the digest of the plasmid no fragment with the right size appears (Figure 23).

There is the possibility that the PCR- Products from Miniprep have risen from unspecific interaction of the primers with the vector. It is rather improbable that this gives fragments with the right size but a negative control was done. A PCR was done on the empty pETM- 13 vector. If it gives products the primers anneal unspecifically.

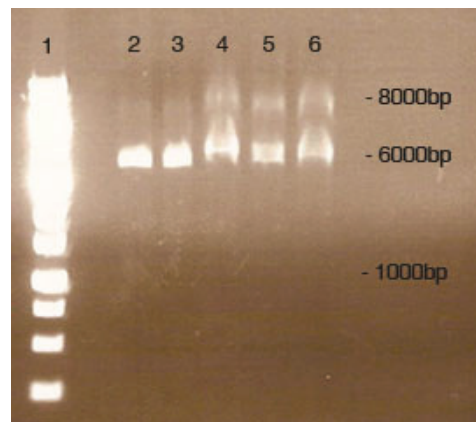


Figure 26: PCR of empty pETM- 13

1: Marker, 2: PCR of Fragment with Pfu- Polymerase, 3: same, 4: PCR of pETM-13 without insert, 5: same

The primers do not bind unspecific (Figure 24). Maybe BsaI did not work properly. In the meanwhile the proteins were expressed and purified with the already existing vectors.

3.2 Expression and Purification of unlabeled proteins

3.2.1 HCAII

3.2.1.1 Expression

HCAII was expressed with BL21. 1 l was enough to get a sufficient amount of protein. The addition of ZnSO_4 to the media is essential for the correct folding of the protein. The expression of HCAII can be increased by lowering the temperature after induction with IPTG to 30°C and following expression over night.

3.2.1.2 Purification

For the first step of purification high pI (6.5) of HCAII is exploited by a SP Sepharose FF column (Figure 27).

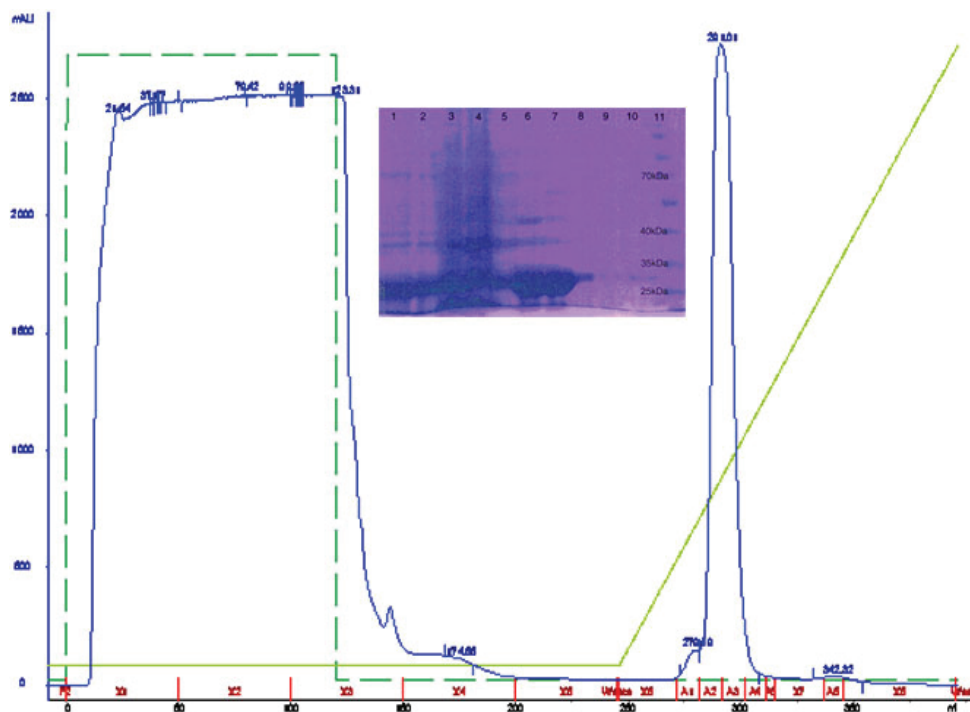


Figure 27: Chromatogram of SP Sepharose FF HCAII

Slot1: Pellet1; 2: Pellet2; 3: Supernatant1; 4: Supernatant2; 5-10: Fractions; 11: Marker

(x- axis = volume in ml; y- axis= absorbance at 280nm in mAU)

An alternative and faster way is to use a gravity Nickelcolumn (Figure 28). HCAII was not expressed with a his - tag but it was empirically proved that it binds to Nickel. This can be explained by the 5 histidines which are N- terminal and very close to each other. 2ml Nickel- resin were used per liter expression. Normally still a lot of protein was in the load fraction after the first run. For this reason the procedure was done twice. The other possibility is to increase the bed volume to 4ml but then the flow velocity is nearly decreased to zero. For every run 6- 10 fractions were eluted.

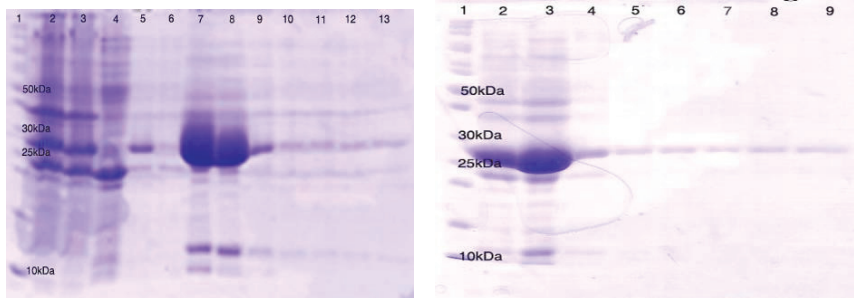


Figure 28: Fractions Nickel gravity column

Left: 1: Marker; 1: Pellet 1; 3: Pellet 2; 4: Supernatant Flow through after second run; 5: Wash first run; 6: Wash second run; 7-13: Fractions first run

Right: 1: Marker; 2-9: Fractions second run

As second step a hydrophobic interaction column (Phenylsepharose) was performed (Figure 29).

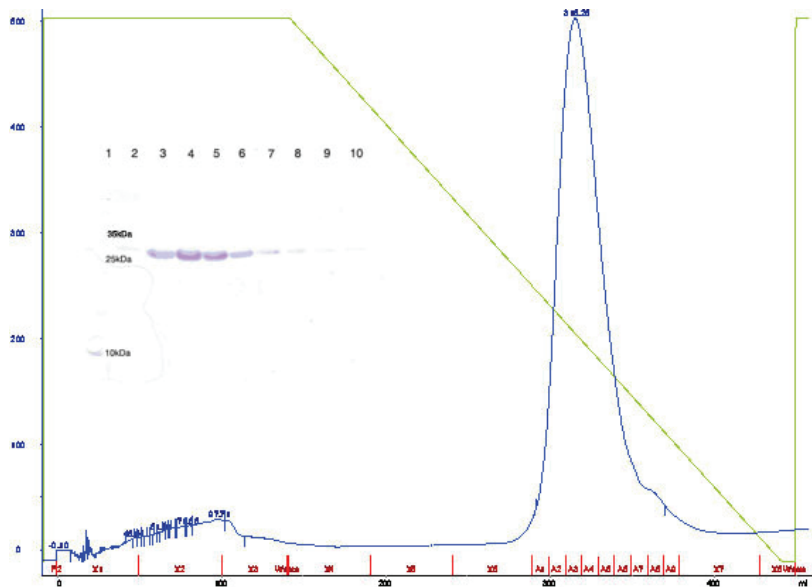


Figure 29: Chromatogram of Phenylsepharose FF – Column HCAII

Gel: 1: Marker; 2-10: Fractions of Phenylsepharose FF- column

(x- axis = volume in ml; y- axis= absorption at 280nm in mAu)

Finally Size exclusion on Superdex 200 26/60 was done to get ride of the small shoulder visible in the chromatogram (Figure 30).

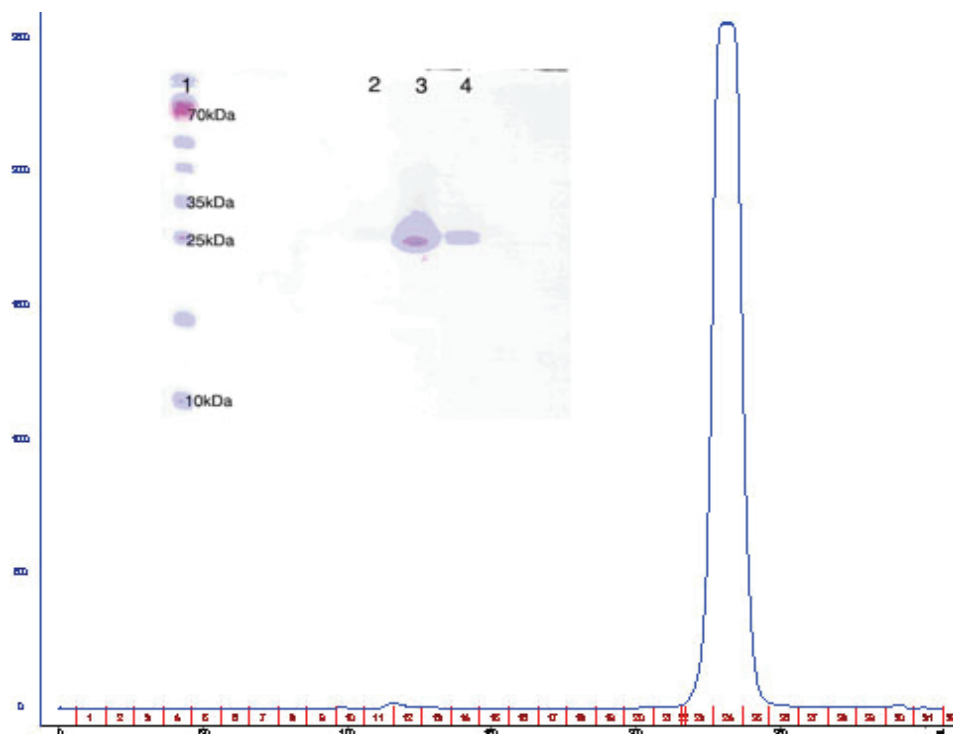


Figure 30: Chromatogram size exclusion HCAII;

Gel: 1: Marker; 2-4: Fractions Size Exclusion

(x- axis = volume in ml; y- axis= absorption at 280nm in mAu)

Per 1l of expression 25- 39mg HCAII could be purified.

3.2.2 His- trx- HAE1:

Because of its size (4kDa) the peptide HAE1 was expressed in N- terminal fusion with his-tagged thioredoxin (14kDa). The his- trx part can later be cut of by TEV (Figure 31).



Figure 31: Picture of his- trx- HAE1 with internal TEV- cleavage site.

3.2.2.1 Expression

The protein was expressed in 1 l LB. In the beginning the strain BL21 pLys was used but due to expression - studies for the labeled form (See expression of labeled proteins; his- trx- HAE1) we decided to continue with C41 pROS. This change improved the expression by around 1/3.

3.2.2.2 Purification

The first step of purification was done with a Gravity- Nickelcolumn.

Two runs were necessary because of the improved expression with C41 pROS.

6- 12 Fractions were eluted (Figure 32).

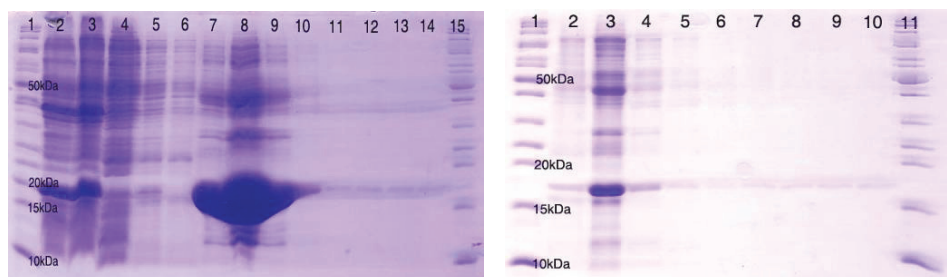


Figure 32: Fractions Nickel gravity column his- trx- HAE1ct

Left: 1: Marker; 2: Pellet1; 3: Pellet2; 4: Supernatant Flow through after second run; 5: Wash first run;

6: Wash second run; 7-14: Fractions first run; 15: Marker

Right: 1: Marker; 2-10: Fractions second run; 11: Marker

For the next step the protein was loaded on an Anionexchanger (Hi TrapQ FF 1ml).

To avoid loss by overloading the fraction X1 of the 1st run was loaded again (Figure 33 and 34).

1st. run:

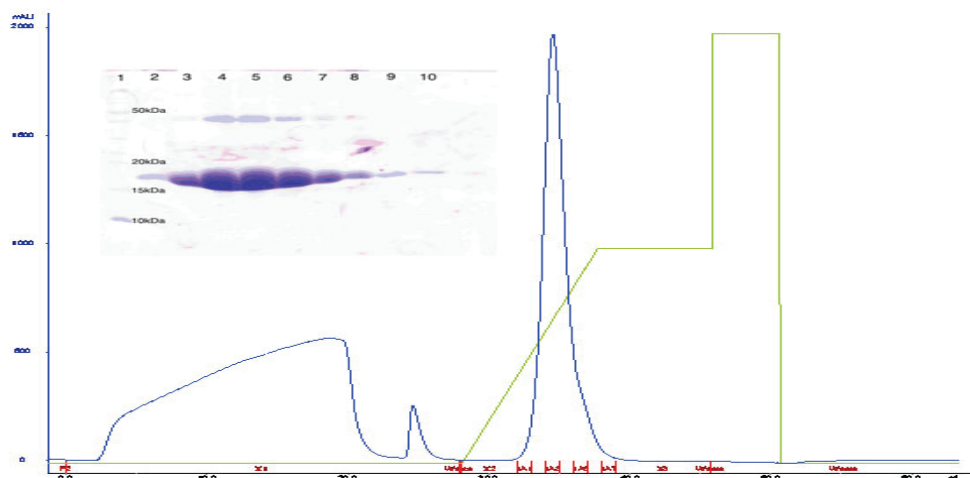


Figure 33: Chromatogram of Hi Trap Q FF 1ml 1. run his- trx- HAE1ct

Gel: 1: Marker; 2-10: Fractions first run

(x- axis = volume in ml; y- axis= absorption at 280nm in mAu)

2nd run:

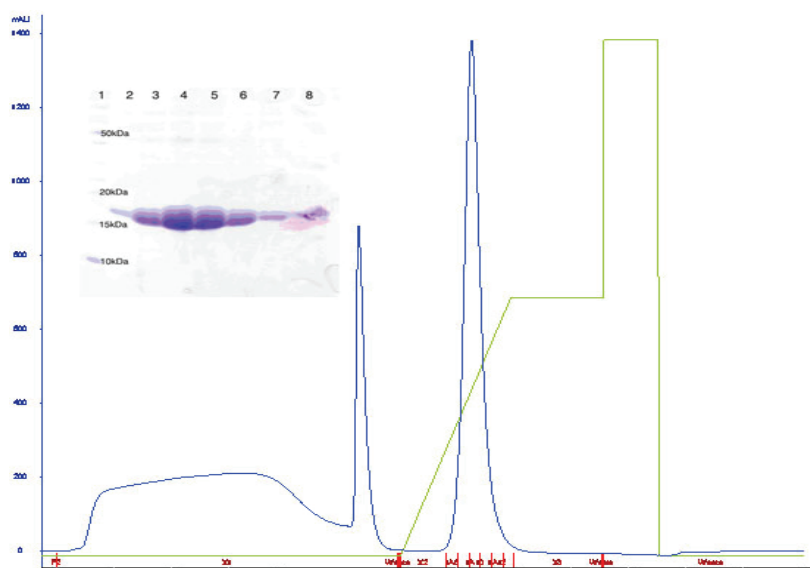


Figure 34: Chromatogram of Hi Trap Q FF 1ml his- trx- HAE1ct

Gel: 1: Marker; 2-8: Fractions

(x- axis = volume in ml; y- axis= absorption at 280nm in mAu)

To get rid of the last impurities a gel filtration on a Superdex 200 26/60 was run (Figure 35).

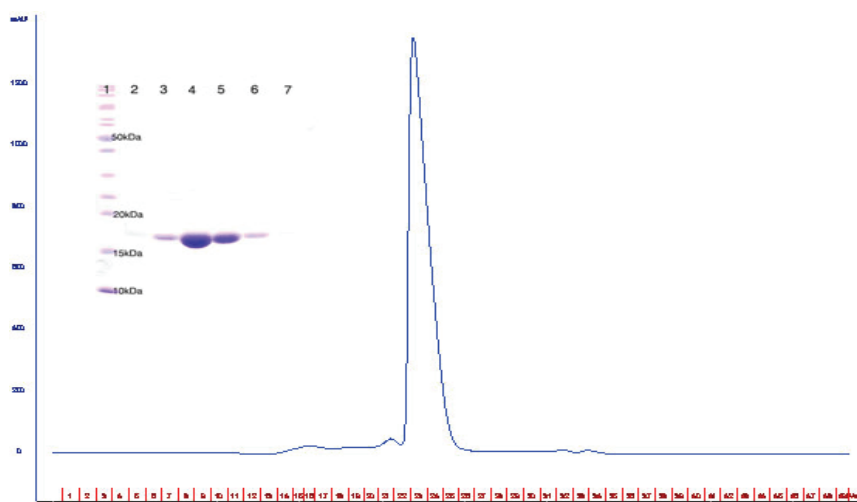


Figure 35: Chromatogram of Size Exclusion on Superdex 200 26/60

Gel: 1: Marker; 2- 7: Fractions

(x- axis = volume in ml; y- axis= absorption at 280nm in mAu)

In average 10- 20mg his-trx-HAE1 could be purified out of 1l.

3.3 Expression and Purification of labelled proteins

3.3.1 HCAII

3.3.1.1 Expression

A Pre-run in unlabeled M9- Minimal media showed that the expression is comparable to the expression in LB. Therefore no optimization was necessary.

HCAII was expressed in the N¹⁵- and the N¹⁵ C¹³ labeled form.

3.3.1.2 Purification

The purification was done the same way as with the unlabeled protein and the final amount was also 20-25 mg.

3.3.2 His- trx- HAE1

3.3.2.1 Expression

It was determined by that the expression of his- trx- HAE1 nearly goes to zero in M9- Minimalmedia. Even a screen for the optimal IPTG, Glucose- concentration or

Expression- temperature did not help. Therefore the expression in different cell lines was compared in an Expression – Screen.

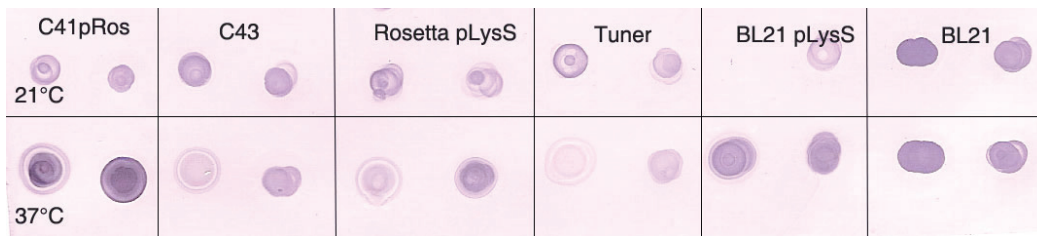


Figure 36: Dot blot of expression Screen of his- trx- HAE1 in different cell lines at 21°C and 37°C
(Left spot stands for the soluble and the right for the total amount of protein)

The dots show the soluble (left) and total (right) amount of protein expressed in the cell lines. It is clearly visible that the strain C41pRos at 37°C is expressing the highest fraction of soluble protein (Figure 36) and therefore it should be the bacterial strain of choice.

This strain is normally used for toxic proteins. It is highly probable that the peptide HAE 1 alone is unfolded and therefore it might be encountered as toxic under high pressure like in Minimal- Media.

Again a pre- run of expression in M9- Media was done and the change to the new strain led to a dramatically increase of expression (Figure 37).

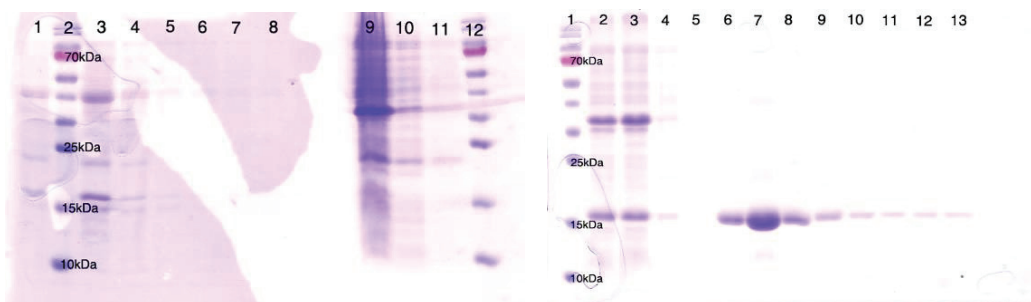


Figure 37: Expression in BL21 (left) and Expression in C41pRos (right)

Left : 1 and 3- 8: Fractions of Nickelcolumn 2: Marker; 9: Pellet; 10: Supernatant after Loading 11: Wash Fraction after loading

Right 1: Marker 2-3: Pellet 1 and 2; 4: Supernatant after Loading; 5: Wash Fraction after loading; 6- 12: Fractions of Nickelcolumn

C41pRos was used to express his- trx- HAE1 in the N¹⁵ C¹³ labeled form and the expression of unlabeled his- trx - HAE1 was also continued with this strain.

3.3.2.2 Purification

The same protocol was used as for unlabeled his- trx- HAE1ct.

For the labeled form 10-15mg of protein were purified.

3.4 TEV- cleavage and Complex Formation

The complex should be formed in a buffer with low pH and ionic strength. It was also proved that the time course of the interaction is rather slow with a $t_{1/2}$ = 12h in a buffer pH = 7.5 containing 100mM NaCl. Lower pH and ionic strength will decrease the time required for saturation (Vince and Reithmeier 1998).

To be sure that the interaction will reach saturation the complex was formed 24h on 4°C though we have chosen a buffer with lower pH and ionic strength (See buffer list Gelfiltration- Buffer). Once locked the interaction should be strong even in more unfavourable conditions.

The his- tag and the thioredoxin have to be removed from his- trx- HAE1 by TEV. This can be done before or after the formation of the complex. Both possibilities were tried.

3.4.1 Complex Formation followed by TEV- cleavage:

The complex was formed 1:1 on 4°C for 24h and then cleaved overnight by TEV on 4°C. To separate the cleaved from the uncleaved complex it was loaded on a HisTrap HP 5ml column (Figure 38).

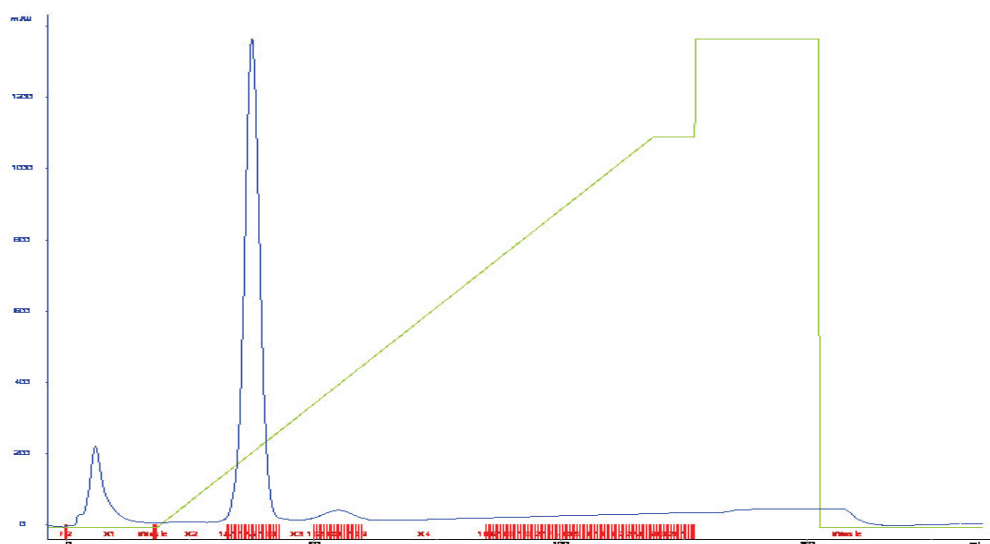


Figure 38: Chromatogram of Complex on HisTrap HP 5ml after complex formation and TEV-cleavage

(x- axis = volume in ml; y- axis= absorption at 280nm in mAu)

Two peaks are eluted with the increasing gradient of imidazole. This was expected because HCAII should also bind to Nickel (see Purification of HCAII) but in a lower extent than the his- tag of his- trx- HAE1ct. Therefore the first peak should contain the cleaved and the second the uncleaved complex.

The fractions were put on a gel. This revealed that the cleaved complex contains components of 29kDa (HCAII) and 12kDa. But HAE1ct should have a size of 4kDa. Therefore the fractions of the first eluted peak were loaded on superdex 200 26/60 to get rid of possible contaminants (Figure 39).

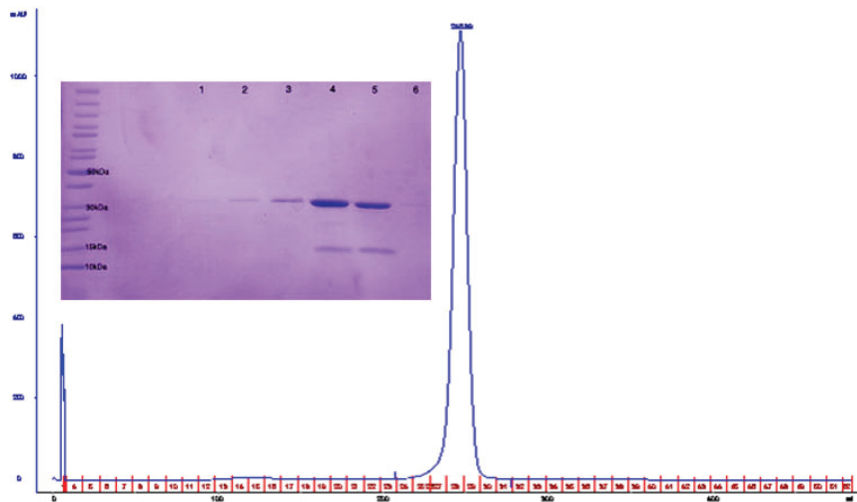


Figure 39: Chromatogram complex on Superdex 200 26/60 with Gel

1: Marker; 2-7: Fractions 1- 6

(x- axis = volume in ml; y- axis= absorption at 280nm in mAu)

It is visible on the chromatogram that there are no significant contaminations. On the SDS PAGE again a band with 29kDa (HCAII) and a band of approximately 12kDa were detected. Maybe the interaction with the Nickelcolumn was not sufficient to separate the fractions dependent on his- tag.

To verify if the 12kDa band contains a his- tag and therefore could be a cleavage product of his- trx- HAE1ct, a Western Blot with primary anti- his antibodies was done (Figure 40).

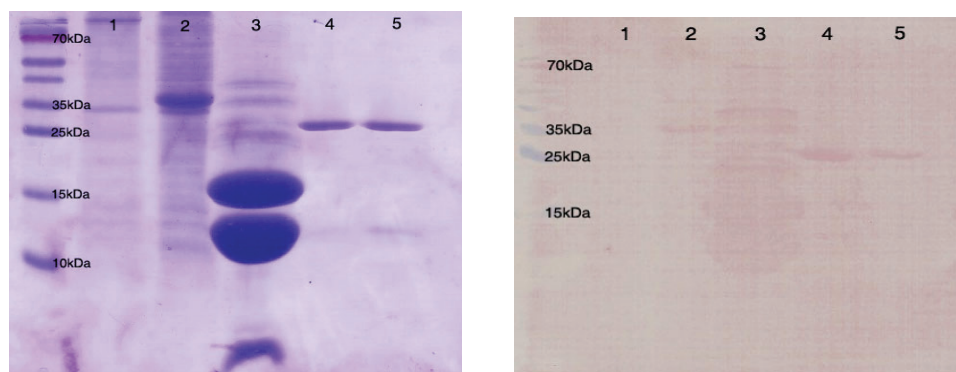


Figure 40: Parallelgel for Westenblot (left) and Western Blot (right)

1: Negative control for his- tag; 2: Positive control; 3: his- trx- HAE1ct cut with TEV; 4 and 5: Concentrated fractions of gelfiltration

It seems as if it does not contain a his- tag. Therefore the origin of the 12kDa band is still unclear.

Even more striking is the fact that the 4kDa HAE1ct peptide is not detectable in the fractions with should contain the cleaved complex.

3.4.2 TEV- cleavage followed by complex formation

A small volume (0.5-1ml) of concentrated his- trx- HAE1ct (12- 16mg/ml) in Gelfiltration-Buffer (See Buffer list) was cleaved for 2h on RT with TEV. More TEV was added and cleavage was continued over night on 4°C.

To separate the cleaved from the uncleaved part a gravity Nickel-column with 1ml resin was used. The cleaved fractions will be in the flow through while the uncleaved his- tag containing fractions and TEV will bind to the resin.

The sample was loaded and up to 4ml Gelfiltration- Buffer were used to wash out the nonbinding part. With Gelfiltration- Buffer + 400mM Imidiazol the Nickel- binding fractions were eluted.

The Flow- trough fraction was freeze dried because HAE1ct (4kDa) is too small to concentrate it with a Centripore device. Therefore the volume should be kept is small as possible because the buffer components will also be concentrated by freeze drying.

Aliquotes of the different steps were loaded on a 12% SDS Gel (Figure 41).

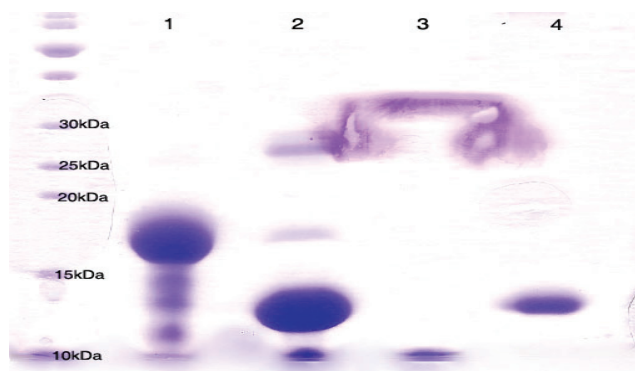


Figure 41: Cleavage of his- trx-HAE1ct and purification of HAE1ct

1: his- trx-HAE1ct; 2: his- trx- HAE1ct cleaved with TEV; 3: Flow through fraction on Nickelcolumn; 4: Eluted fraction of Nickelcolumn

The flow through fraction contains a band at 10kDa. This is due to the low percentage of the gel. On a 18% SDS- PAGE the fraction runs at 4kDa (Data not shown) and therefore can be identified as HAE1ct region. The separation of cleaved and uncleaved parts seems to be sufficient.

Finally the freeze dried fraction was diluted with water to the desired concentration. It has to be taken into account that the solution contains the amount of 6ml of Gelfiltration- Buffer. Therefore high volume of buffer before freeze drying can impede the complexing or even make it impossible. The complex with HCAII was formed on 4°C for 24h.

3.5 Crosslinking of the Complex

3.5.1 Crosslinking with BS²HG

To identify the interaction site between HCAII and HAE1ct the complex had to be with BS²G and afterward analyzed by mass spectrometry (Rappsilber, Siniosoglou et al. 2000; Sinz, Kalkhof et al. 2005). The complex was formed for this purpose with different ratios of HAE1ct (1-5parts). As a negative control HCAII was crosslinked. α - actinin was crosslinked to serve as a positive control because it is forming a homodimer and therefore the probability that Lysins or the Amino-termini come close enough to be crosslinked is rather high. Aliquots were taken and loaded on 10%Gels (Figure 42).

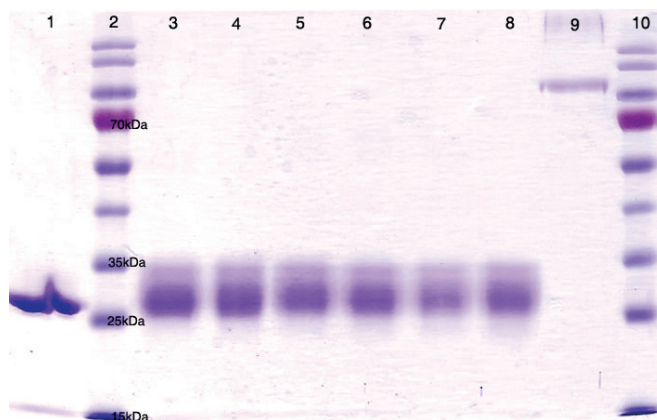


Figure 42: Crosslinking with BS²G;

1: Complex without crosslinker; 2: Marker; 3: HCAII crosslinked; 4: Complex (1:1) crosslinked; 5: Complex (1:2) crosslinked; 6: Complex (1:3) crosslinked; 7: Complex (1:4) crosslinked; 8: Complex (1:5) crosslinked; 9: Positive control α -actinin; 10: Marker

It is easy to see that the uncrosslinked complex (1) contains the peptide in an sufficient amount. As mentioned before it runs higher size than 4kDa on Gels lower then 18%. α -actinin was partially crosslinked by BS²G and therefore the reaction seems to work.

Unfortunately the negative control of HCAII crosslinked (3) looks exactly the same like the aliquots taken from complexes crosslinked (4-8).

All of these fractions show a smear from circa 27- 35kDa. HCAII is containing 24 Lysins to which the crosslinker could bind without crosslinking and therefore produce this smear.

It could be possible that the band of the crosslinked complex HCAII –HAE1ct is overlaid by this smear and therefore no difference to the negative control is detectable.

This was tested by a series of RESOURCE Q runs.

HCAII has an pI of 6.5 while the complex HCAII – HAE1ct is more acidic with a pI of 5,64. In pH = 6 HCAII carries positive charges. In contrast the complex is negatively loaded. That means that these fractions can be easily separated by an anion exchanger. The flow through will contain HCAII while the complex will bind to the column and will be eluted by salt.

The RESOURCE Q column has lower capacity but higher resolution than HiTrap Q 1ml column and was therefore chosen for this purpose.

In the first two runs HCAII and Complex native were loaded (Figure 43). For both a peak after 5ml (1 column volume) was detectable. This was expected because the HCAII should be positive charged. HAE1ct should be separated from the complex by the positive groups in the column because its pH alone is 3.82 and should be eluted later. No peak is detected because HAE1ct only contains two aromates and therefore will not show a high peak of absorption in the chromatogram.

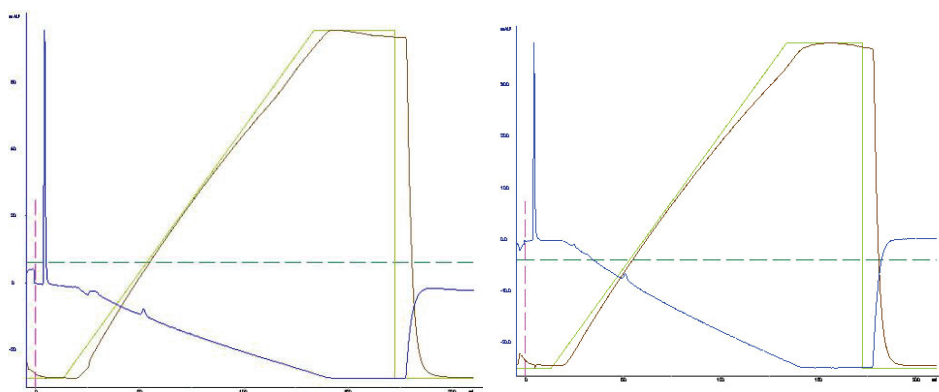


Figure 43: HCAII not crosslinked (left) and Complex not crosslinked (right)

(x- axis = volume in ml; y- axis= absorption at 280nm in mAu)

The next two sample loaded were HCAII and Complex, both crosslinked with BS²G (Figure 42).

Their chromatograms show a peak at 25ml at the beginning of the salt gradient. The crosslinker has masked some Lysins of the HCAII therefore it is not as positive charged as the native form. For successful crosslinking a second peak at a higher percentage of salt would be expected.

The chromatograms show clearly that no crosslinking of the complex has taken place.

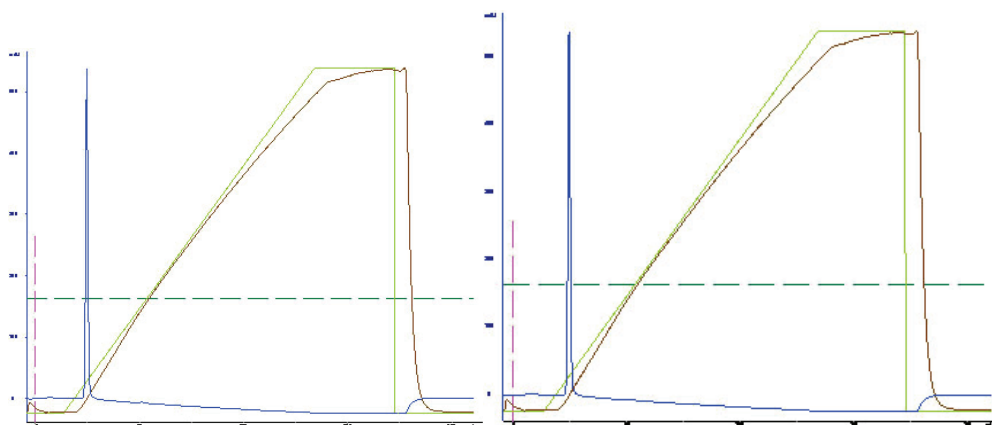


Figure 44: HCAII crosslinked with BS²G (left) and Complex crosslinked with BS²G (right)

(x- axis = volume in ml; y- axis= absorption at 280nm in mAu)

The different fractions were also crosslinked with formaldehyde to compare the methods and the results.

3.5.2 Crosslinking with Formaldehyd

The complex in ratio 1:2 (HCAII : HAE1ct) was crosslinked with 0.5-1% Formaldehyd. α - actinin was acting again as a positive control. Here 1% Formaldehyd was used. For the negative control HCAII was crosslinked with 2% Formaldehyd. The samples were run on a 10% SDS PAGE (Figure 45).

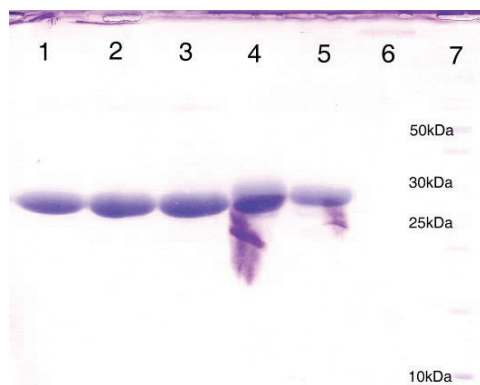


Figure 45: Crosslinking with Formaldehyd

Slot 1- 6: Complex crosslinked with 0.5%, 1%, 1.5%, 2% and 10% Formaldehyd; Slot 6: α - Actinin crosslinked with 1% Formaldehyd (positive control)

The positive control is completely crosslinked while the negative control does not show any crosslinking. Also the complex does not show any crosslinking.

This could be due to the high pH of the buffer which could destroy the complex which was formed in the Gelfiltration-buffer in high concentration. The complex should be rather stable when once locked on but the dilution in a buffer with pH = 8 might have destabilized it to a high extent.

3.6 Crystallography

As mentioned before two orders of TEV- cleavage and complex formation were tried. Crystallization was tried with both forms.

3.6.1 Crystallization of complex formed by complex generation followed by TEV- cleavage

Different initial crystal screens of the complex were done with the crystallization nano-robot:

- JCSG (Quiagen)
- PACT (Molecular Dimensions Limited)
- HAMPTON Research Index Screen (HAMPTON)
- HAMPTON Research Grid Screen (HAMPTON)

They were kept at 22°C. After 24h crystal plates were found in conditions D5, D6, D7, D8, D10, D11, D12 and G11 of the Grid screen. The Index Screen gave also rise to hits in condition B8 after one day.

By analysis of the crystallization condition three possible refinement strategies were designed:

- A. 0.1 M buffer (Tris, HEPES, Bicine) with pH from 7 to 9 containing 2.4 or 3.2 M ammonium sulphate
- B. 0.1 M citric Acid pH 4 or 5 containing 3.2M ammonium sulphate
- C. 0.1 M HEPES pH= 7.5 containing 1.4 M Na citrate

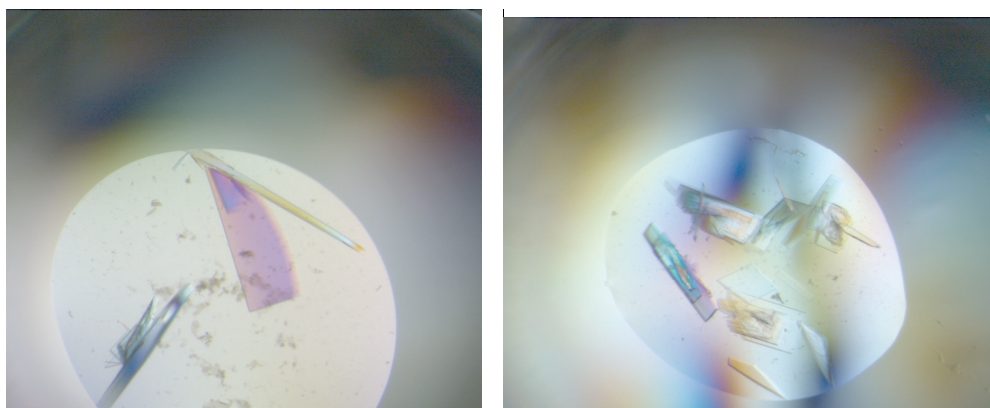
Three refinement Screens were done manually by hanging drop vapour diffusion set-up in Nextal plates.

1. 0.1 M Citric acid pH 4 up to 5 against 2.4- 3 M ammonium sulphate
2. 0.1 M bicine buffer pH 8 – 9 against 2.4- 3M ammonium sulphate
3. 0.8 – 1.8 M Na- citate against 0.1 M Buffer pH 5- 8 (MES and bicine)

The whole second plate gave rise to crystals (Figure 46). In third plate crystals grew in the conditions marked with a cross (Table 4).

Table 4: Refinement screen

	0.8M Na- Citrat	1M	1.2M	1.4M	1.6M	1.8M
pH= 5						
6						×
7					×	×
8				×	×	×

**Figure 46: Crystals in 0.1M bicine pH = 8.6 2.6 M (NH₄)SO₂ (left) and 0.1M bicine pH=7 1.8 M Na- Citrate**

For citric acid with ammonium sulphate (Plate 1) the crystals could not be reproduced.

Diffraction experiments:

Diffraction data on the two crystal grown in conditions 0.1 M Bicine pH= 8.2; 2.4M ammonium sulphate and 0.1 M bicine pH = 8; 1.4 M Na- citrate were collected on our home facility (Table 5 and 6).

Table 5: Data collection statistics of 0.1 M Bicine pH= 8.2; 2.4M ammonium sulphate

Data collection

Beamline	Bruker proteum X8
Wavelength (Å)	1.54
Space group	P2 ₁
Unit cell parameters (Å)	
<i>a</i>	42
<i>b</i>	41.3
<i>c</i>	71.9
Resolution range (Å)	69.84 – 1.75 (1.85 – 1.75)
Observed reflections	118089
Unique reflections	44371
<i>R</i> _{merge}	0.0211 (0.1914)
<i>< I > / < σ I ></i>	21.55 (5.1)
Completeness (%)	98.9 (96.3)

Redundancy	4.67 (2.25)
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Table 6: Data collection statistics of 0.1 M bicine pH = 8; 1.4 M Na- citrate crystal

Beamline	Bruker proteum X8
Wavelength (Å)	1.54
Space group	P2 ₁
Unit cell parameters (Å)	
<i>a</i>	42
<i>b</i>	41.3
<i>c</i>	71.9
Resolution range (Å)	41.44 – 1.70 (1.79 – 1.70)
Observed reflections	122079
Unique reflections	50620
<i>R</i> _{merge}	0.0271 (0.1626)
<i><I>/<σI></i>	16.9 (4.38)
Completeness (%)	99.8 (98.7)
Redundancy	4.5 (2.63)

Both were monoclinic and were members of the space group P2₁. The unit cell had in both cases a size of 70×41×41 Å³.

The phase problem was solved with MR using the structure of HCAII alone (pdb entry code 1ca2).

Inspection of difference electron density maps showed no additional electron density for the presence of the bound peptide, indicating the crystals contained HCAII only.

Analysis of crystal packing revealed that it will be difficult to soak crystals of this space group because the N- terminus which is supposed to interact with HAE1 C-terminal region makes crystal contacts, and might therefore in this crystal lattice not be available for additional intermolecular interactions.

3.6.2 Crystallization of complex formed by TEV- cleavage followed by complex formation

12 - 19 mg /ml complex were used for crystallization set-ups.

The following crystallization screens were set up with the nano-robot:

- JCSG (Quiagen)
- PACT (Molecular Dimensions Limited)

They were kept at 22°C. After 2 days in conditions 5,7,12, 15, 23, 24, 34, 49, 50, 52, 57, 59, 60, 67, 68, 69, 70, 76, 78, 79 and 80 of JCSG- screen small crystals mixed with a lot of precipitate were visible. In condition 71 (1 M Succinic acid pH= 7; 0.1 M HEPES pH 7; 0.5% v/v Jeffamine ED- 2001 pH 7) really regular but very small pyramids were found.

25% of the conditions that yielded positive hits contained malic acid or malonate.

In the PACT screen only amorphous brownish precipitate and phase separation were found.

Two methods for refinement were chosen:

- A. In Nextal plates for the hanging drop 1µl condition 59 was mixed with 1µl complex and different concentrations of NaCl (0.1 – 1M) or different percentages of PEG 8000 (15- 30%) were put in the reservoir to push the drop in the region of crystal growth
- B. For refinement 50- 600 mM Na- Malonate pH 5.5 - 6.5 were used with 20% PEG 4000 because malonate and malic acid seem to stabilize the complex

For method A the crystals could be reproduced and the best results were obtained with reservoir containing 0.5 M NaCl. They contained pyramids slightly bigger than those obtained from the initial screen (Figure 47) and in addition slightly irregular crystals with cubic morphology.

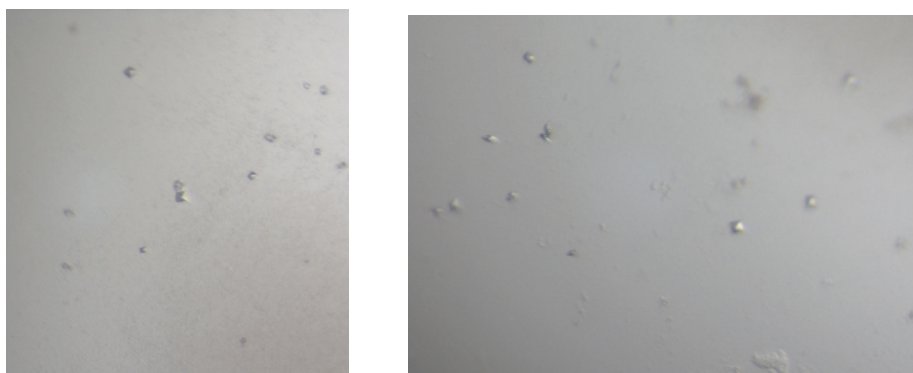


Figure 47: Complex in E11 (JCSG- screen) over reservoir with 0.5 M NaCl

Micro-seeding was tried in hanging drops with 1 μ l condition 59 and 1 μ l complex over 0.5M NaCl or 15- 30% PEG 8000 equilibrated over night. The seeding was successful.

To control that the obtained crystals are not made of salt control drops with 1 μ l gelfiltration – buffer and 1 μ l Condition 59 were set over a reservoir of 0.5 M NaCl. No crystals grew in two weeks.

Refinement strategy B only gave precipitate and phase- separation. Therefore higher concentrations of Na malonate (2- 3 M) pH 6 without PEG were used.

Spherulites were found (Figure 48).

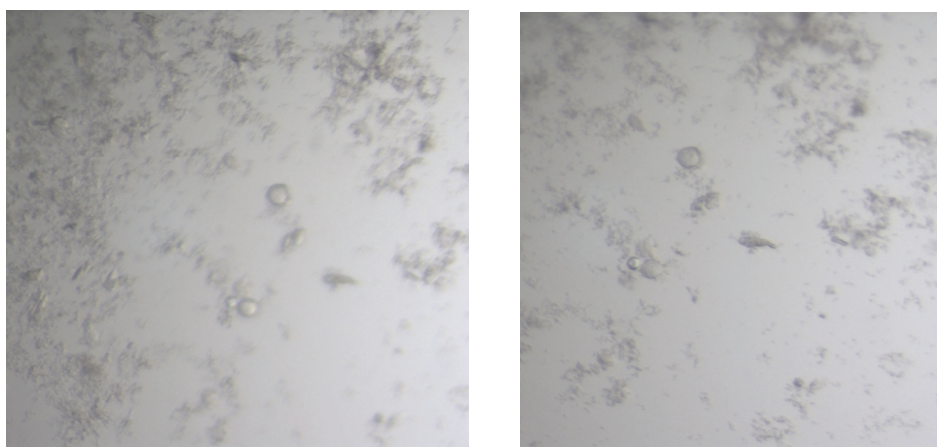


Figure 48: Complex in condition 2.8 M malonate pH = 6

Crystals at Synchrotron Grenoble:

Therefore some crystals of both types were sent to the European Synchrotron Facility in Grenoble because the crystals were too small to test them with the home facility.

From diffraction experiments it became clear that all of them consisted of salt.

3.6.3 Soaking

As the co- crystallization did not work it was tried to soak HCAII crystals with HAE1 C-terminal region. Crystals of HCAII were grown from condition 0.1 M tricine pH 7.5 2.8 M ammonium sulphate at 22°C and 14°C in 24 well plates. After one day a lot of small crystals were visible in all drops at 14°C. The crystals in the trays on 22°C grew after one week but were single per drop and much bigger but they did not grow in all drops.

Crystallization experiments with 0.1 M Tris pH 8.5 2.4 M ammonium sulphate were also set up and put on 14°C. Again a lot of small crystals grew after one day in all drops.

3.6.3.1 Soaking with HAE1 C-terminal region

The small crystals obtained from Tris and Tricine – condition (14°C) were soaked with 2µl HAE1 (5 mg/ml) which corresponds to a molar ratio of 1:4. The crystals partly cracked and dissolved (Figure 49) but some of them seemed to be quite stable so that diffraction data was collected with our home X- ray facility (Table 7).

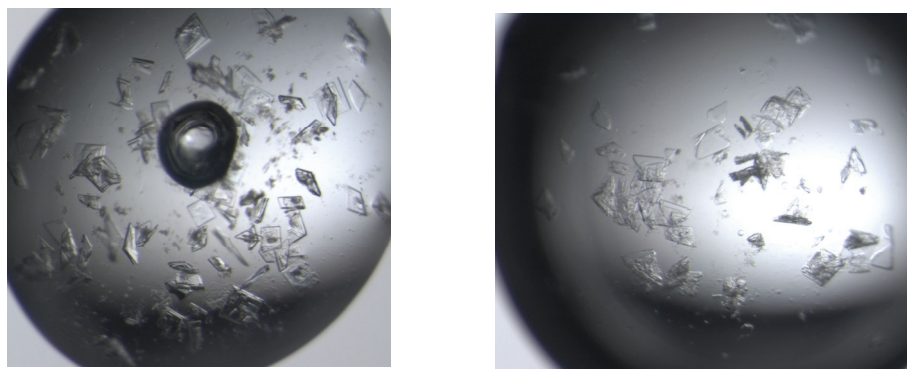


Figure 49: Crystals in condition 0.1M Tris pH=8.5 2.4M ammonium sulphate before soaking (left) and after soaking (right)

They were monoclinic and were members of the space group $P2_1$. The unit cell had a size of 70×41×41nm. The phase problem was again solved with Molecular replacement.

Table 7 Data collection statistics of HAE1 C- terminal region soaked crystals

<u>Data collection</u>	
Beamline	Bruker proteum X8
Wavelength (Å)	1.54
Space group	$P2_1$
Unit cell parameters (Å)	
<i>a</i>	41.9
<i>b</i>	41.4
<i>c</i>	72.2
Resolution range (Å)	70 - 2.08 (2.18 - 2.08)
Observed reflections	160896
Unique reflections	27498
<i>R</i> _{merge}	0.0134 (0.0585)

$\langle I \rangle / \langle \sigma I \rangle$	36.97 (11.07)
Completeness (%)	99.8 (99)
Redundancy	10.97 (3.93)

Unfortunately again no additional electron density was detected. That means HAE1 C-terminal region composed of 33 amino acid residues is maybe too big to be soaked into the crystals.

3.6.3.2 Soaking with LDADD

There seems to be a consensus sequence LDADD for the interaction with HCAII (Vince, Carlsson et al. 2000) which is highly conserved. It can also be found in HAE1ct. Therefore this small peptide was ordered from Novagen because it should be easier to soak this small peptide in without breaking the crystals.

A small amount of peptide powder was put into the drop with the HCAII crystal grown in 0.1M Tricine pH 7.5 2.8M (NH₄)SO₂.

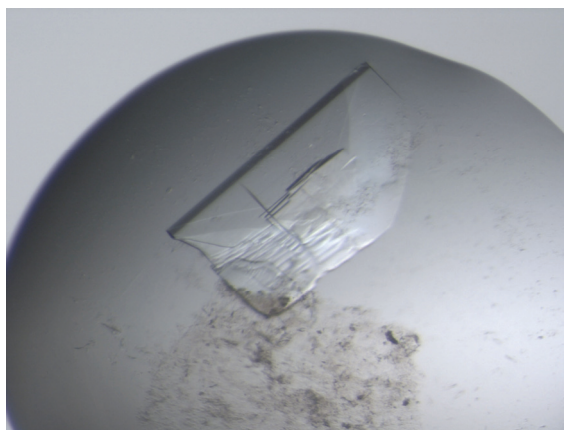


Figure 50: HCAII Crystal grown in 0.1M Tricine pH= 7.5 2.8M (NH₄) SO₂ soaked with LDADD

The crystal dissolved and cracked a bit on one side (Figure 50) but data was collected with our home facility (Table 8).

Table 8 Data collection statistics of LDADD soaked crystals

Beamline	Bruker proteum X8
Wavelength (Å)	1.54
Space group	P2 ₁
Unit cell parameters (Å)	
<i>a</i>	73.8
<i>b</i>	41.2

c	83.8
Resolution range (Å)	69.7 – 1.43 (1.53 – 1.43)
Observed reflections	755065
Unique reflections	143103
R_{merge}	0.0095 (0.1312)
$\langle I \rangle / \langle \sigma I \rangle$	27.45 (4.01)
Completeness (%)	90.3 (47.3)
Redundancy	8.56 (0.79)

The crystals were monoclinic and were members of the space group $P2_1$. But according to other experiments (soaking with HAE1ct and crystallisation of complex formed by complex formation followed by TEV- cleavage) the dimensions of the unit cell have changed from $70 \times 41 \times 41 \text{ nm}$ to $70 \times 41 \times 80 \text{ nm}$.

This indicates that something has happened in the crystals due to soaking.

The phases were solved by molecular replacement. However no additional electron density was found.

3.7 NMR Experiments

3.7.1 Focus on HAE1ct

3.7.1.1 3D Measurements of HAE1ct

The nitrogen and hydrogen shifts of HAE1ct peptide N¹⁵ C¹³ labeled were detected in a 2D HSQC spectrum.

To assign the amino acid residues of the peptide to the peaks in the HSQC spectrum different 3D measurements were performed (HNCA, HNCOCa, CBCACONH, HNCOCACB):

The combination of those spectra makes it possible to identify the connectivity between amino acid residues (peaks) in the HSQC (2.4.2.2 Three dimensional NMR-experiments).

As an example the HNCOCa and HNCA spectra of residue 26- 28 are shown (Figure 51).

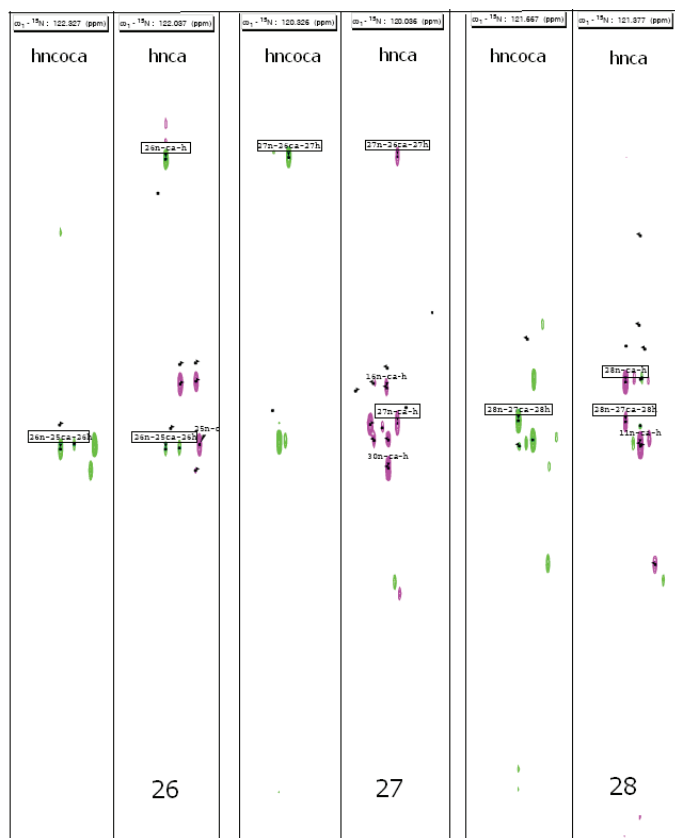


Figure 51: Assignment HAE1ct; HNCOCa (left) and HNCA (right) of residue 26- 28

The HNCOCA spectrum shows in the C- dimension of the HSQC spectrum the c_α - peaks of residue i-1 while the HNCA spectrum shows both residue i-1 and residue i. The same can be done with HNCACB and CBCACONH spectra which deliver important additional information because the chemical shift of c_β is very dependent on the amino acid residue.

Nearly the whole primary sequence was assigned to peaks in the HSQC spectrum (Figure 52). But still the identity of some peaks stays unclear. A? and D? show c_α -shifts of alanine and aspartate but they could not be assigned to the primary sequence with unique identity.

The peak assigned with Dar? n-h has the shifts of an aspartate whose precursor has a c_α shift of an aromatic residue (58ppm). This would match with residue 22 or 31 but could not be assigned with certainty.

Also for residue 18 two possibilities are existing. Therefore one residue is named 18 and one with K18_2.

E24 and E25 could not be distinguished from each other because they have both a glutamate as residue i-1.

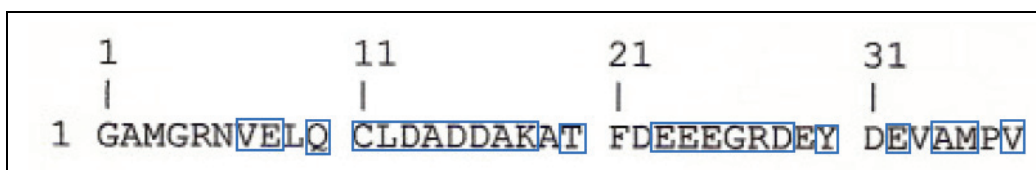


Figure 52: Assigned residues of HAE1 C- terminal (blue frame)

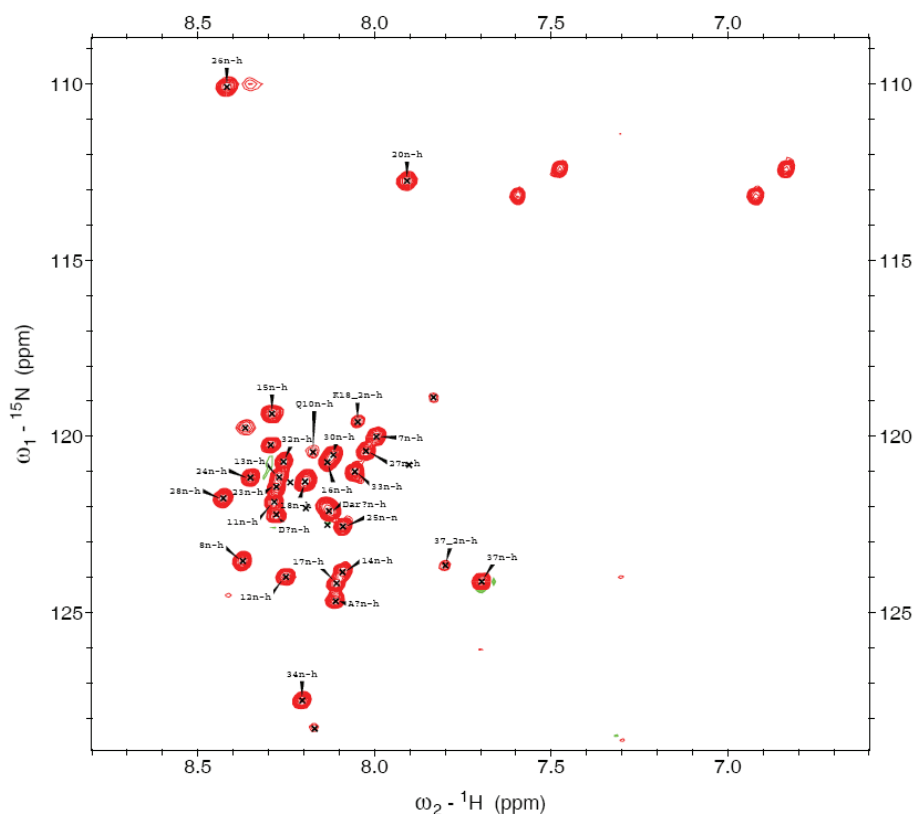


Figure 53: HSQC spectrum of HAE1ct native (below)

For residue yaline 37 two peaks were assigned because proline 36 can exist in the cis or trans form which is changing the environment of residue 37 (Figure 53). For prolin 36 no peak was detected because prolines are not visible in HSQC spectras.

3.7.1.2 Titration of labeled HAE1ct with HCAII

To map the interaction site C13 and N15 labeled HAE1ct (only N15 labeled would be necessary) and unlabeled HCAII were mixed in the following ratios (HCAII: HAE1ct):

2:1 1:1 1:2 1:3

The complex was each time incubated 24h on 4°C and HSQC- spectra were recorded respectively.

The spectra were aligned to each other by residue 14. To identify the most shifted residues the concept of the total shift was calculated, using the differences between spectra (Figure 54).

$$\text{totalshift} = \sqrt{(5 \Delta H)^2 + (\Delta N)^2}$$

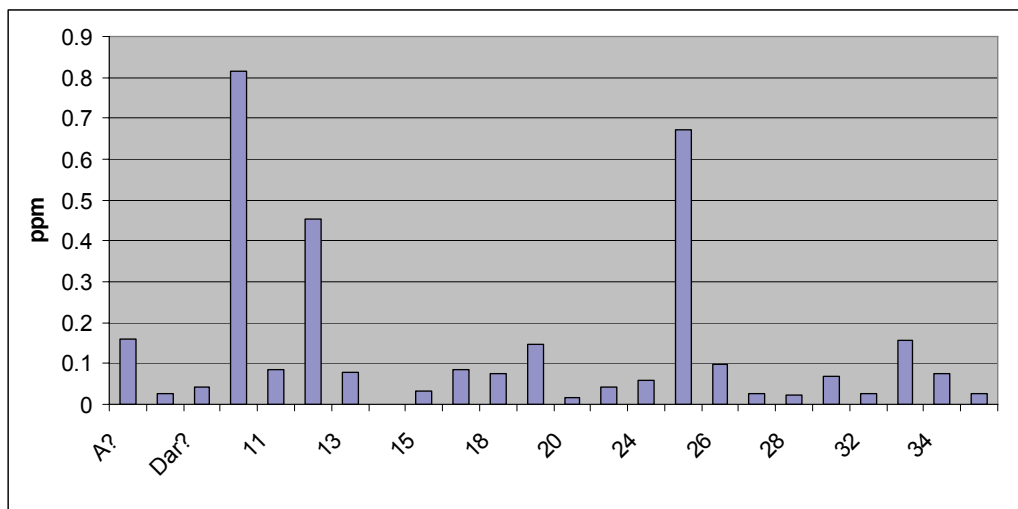


Figure 54: Diagram of amino acid residues of HAE1ct peptide and their total shift

Clearly visible three residues E8, L12 and E25 (or E24 because this residues could not be distinguished) seem to be extremely important for the interaction with HCAII. Therefore, a closer look was taken at these residues.

3.7.1.3 Following the saturation of HAE1ct with HCAII by residues E8, L12 and E25

Following E8, L12 and E25 during the respective titration experiments it is easy to see that these residues were extremely shifted downfield by the addition of HCAII up to a ratio of 1:1 (Figure 55).

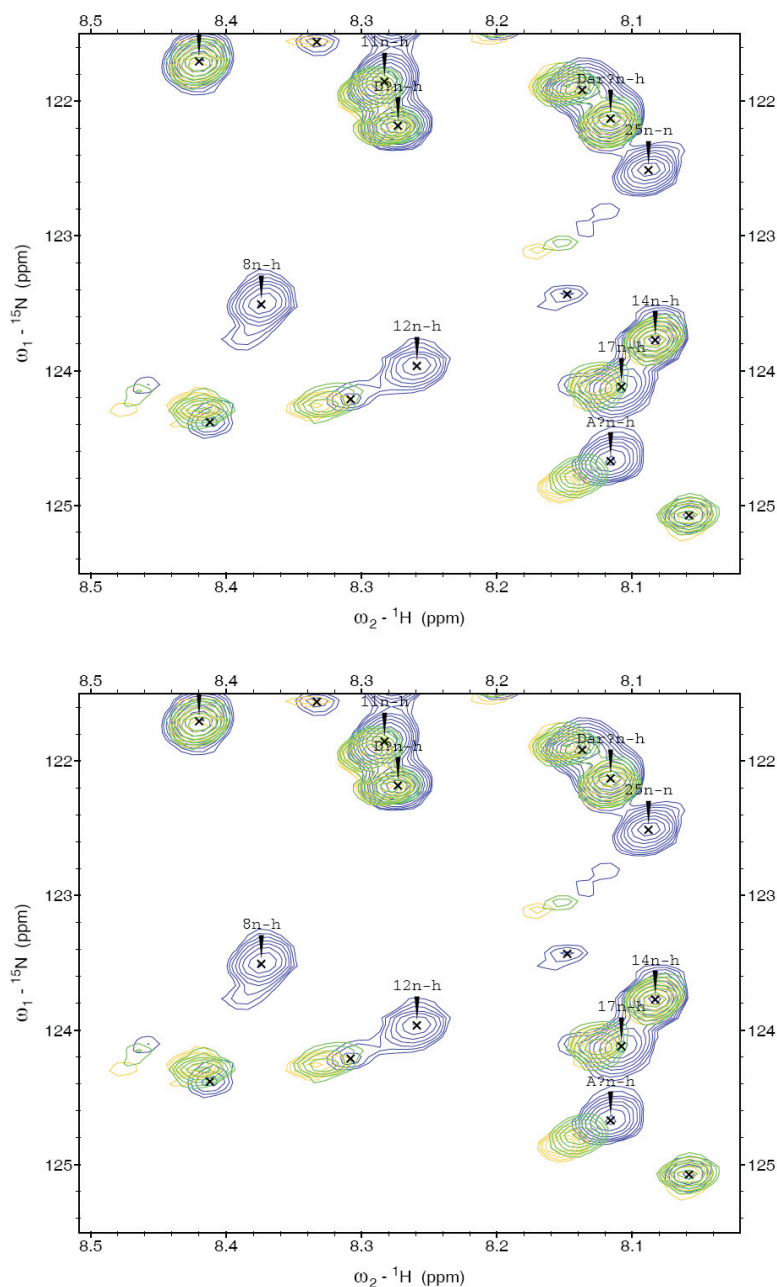


Figure 55: Overlay of different HSQC- Spectra of HAE1C- terminal

HAE1ct without HCAII (blue), HCAII and HAE1ct in ratio 2:1 (yellow); ratio 1:1 (green)

If more peptide is added two different patterns evolve (Figure 56): beside the shifted peaks for E8, L12 and E25, also the native shifts of HAE1ct can be detected. Results indicate that the complex HCAII - HAE1ct is already saturated which ratio 1:1, meaning that the affinity constant is very high. Furthermore, results prove that the underlying interaction dynamics are slow with respect to the chemical shift time scale.

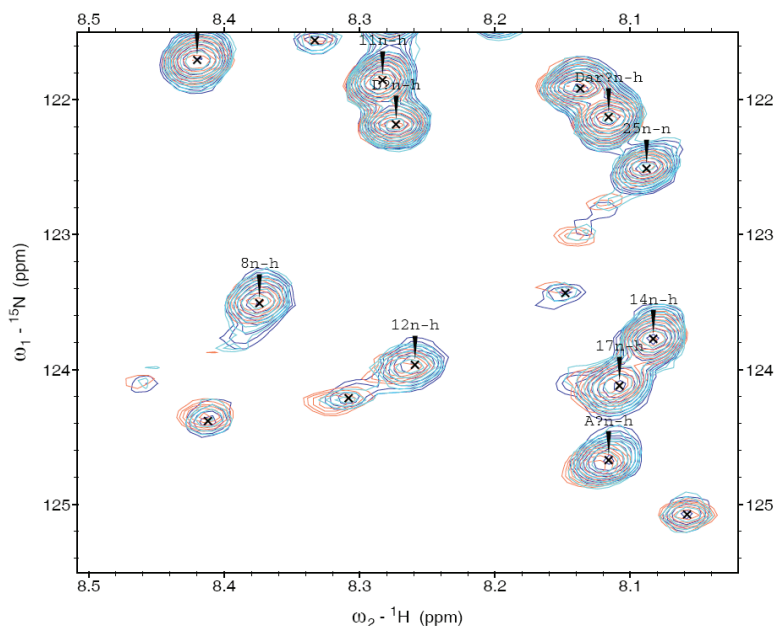


Figure 56: Overlay of different HSQC- Spectra: HAE1ct without HCAII (blue), HCAII and HAE1ct in ratio 1:2 (maroon); ratio 1:3 (turquoise)

Slow exchanges ($>\mu\text{s} - \text{ms}$) normally show this separated patterns. One reason for this slow exchange could be that HCAII can only interact either with the cis- or the trans-proline form of HAE1ct. Residue V37 shows a peak for the trans or cis form of its preceding proline residue without HCAII.

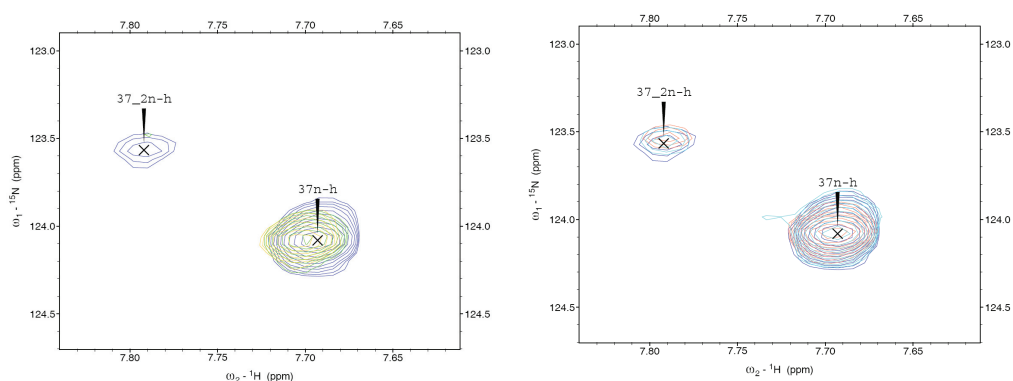


Figure 57: Detail of overlaid HSQC Spectra: Residue V37 of HAE1 without HCAII (blue); in ratio (HCAII: HAE1ct) 2:1 (green); 1:1 (yellow); 1:2 (brown); 1:3 (turquoise)

In the left overlaid spectrum (native; 2:1; 1:1) it is visible that peak 37_2n-h disappears when the binding is saturated (left) (Figure 57). This means that the peptide only binds with proline either the cis- or the trans- form. By the addition of more peptide again the peak reappears which shows that the preceding proline of V37

exists again in the cis- and trans- form because the binding is oversaturated by the abundant peptide.

3.7.2 Focus on HCAII

3.7.2.1 3D measurements of HCAII

3D measurements were done with double labeled HCAII. Because of problems with the spectrometer only c_{α} - shifts were measured in HNCA and HNCOCA spectra. Without the more characteristic c_{β} - shifts the assignment gets much more difficult. Moreover HCAII consists of 260 amino acids therefore many combinations have to be tested during assignment which is rather time consuming. Therefore we were looking for alternative ways to full manual assignment. Nevertheless we continued with titration (Figure 58).

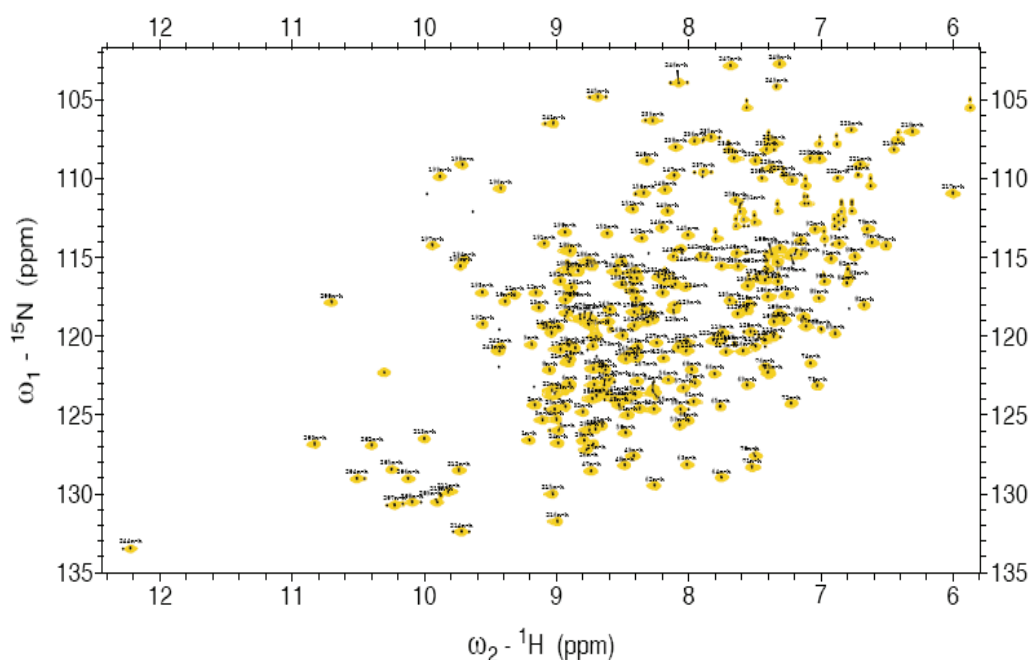


Figure 58: HSQC – Spectrum of HCAII

3.7.2.2 Titration of labeled HCAII with his- trx- HAE1ct

N^{15} labeled HCAII was mixed in different ratios with his- trx- HAE1ct and HSQC – spectra were measured. HAE1ct with tag was used because the measurements were made before we figured out the right order of tag- cleavage and complexing. As negative control his- trx : HCAII in ratio 2:1 was used. (Data not shown)

The following ratios (his-trx- HAE1ct: HCAII) were used:

3:1 2:1 1:1 1:2

The complex was formed 24h on 4°C.

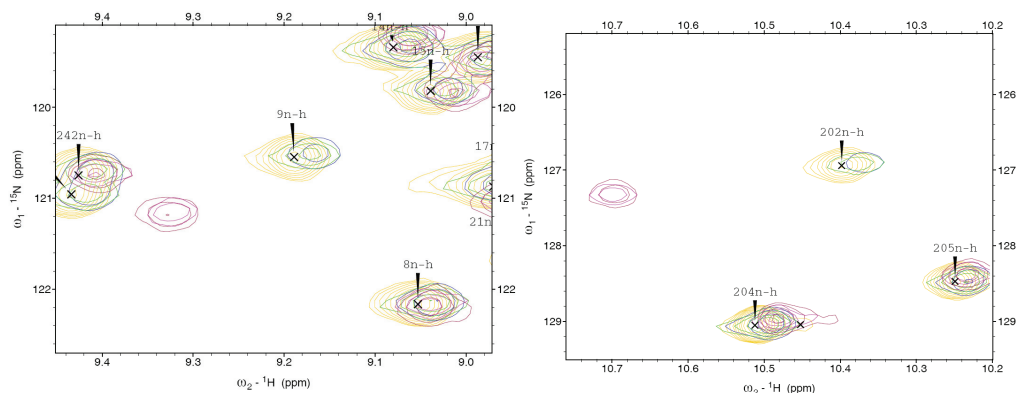


Figure 59: Detail in overlay of HCAII spectra; HCAII native (yellow), his-trx- HAE1ct : HCAII in ratio 2:1 (turquoise), 1:1 (blue), 1:2 (green) and 1:3 (magenta) ; Residue 9 (left) and Residue 202 (right)

It is easy to see that the peaks were shifting most at ratios 3:1 and 2:1 (HAE1ct: HCAII) (Figure 59). At the other ratios the peaks move back to the native state. That is a contradiction to the results we got from the titration of labeled HAE1ct with HCAII. Here the saturation of the binding was reached at the ratio of 1:2 (HAE1ct: HCAII). That means that in titrations of labeled HCAII with his- trx- HAE1ct much more peptide is needed to get sufficient interaction. This aberration could be due to the fused his- trx- tag on HAE1ct. It could make the peptide more bulky and therefore change the binding behaviour. Results indicate that the affinity constant increases due to the his-trx- tag. And therefore the dynamic seems to change from slow to fast exchange. For this reason higher ratios of peptide with fused tag are needed to show interaction.

The spectra were assigned with working numbers and the total shift was calculated by the difference of native HCAII to his- trx- HAE1ct: HCAII in ratio 3:1.

$$\text{totalshift} = \sqrt{(5 \Delta H)^2 + (\Delta N)^2}$$

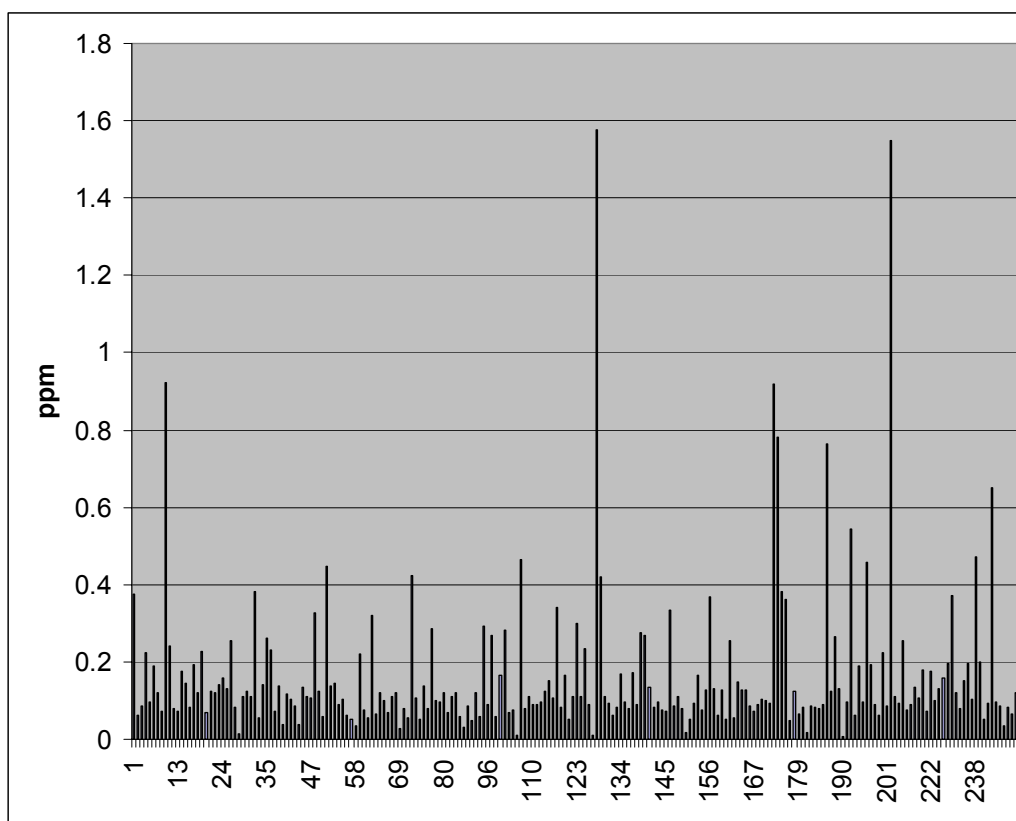


Figure 60: Diagram of HCAII residues (working numbers) and their total shift

Only a few peaks were highly shifted (Figure 60). But without an assignment we were not able to identify them. We focused on searching for the assignment of the shifted peaks. Different ways were tried.

3.7.2.3 Assignment by Homology

HCAII has 60% sequence homology to human carbonic anhydrase I (Liljas, Kannan et al. 1972).

This isoform is already assigned and this information can be found in the Biological Magnetic Resonance Data Bank (BMRB) on internet.

Therefore artificial peaks in HSQC spectrum based on the HCAI assignment were created and overlaid with the HCAII HSQC spectrum (Figure 61).

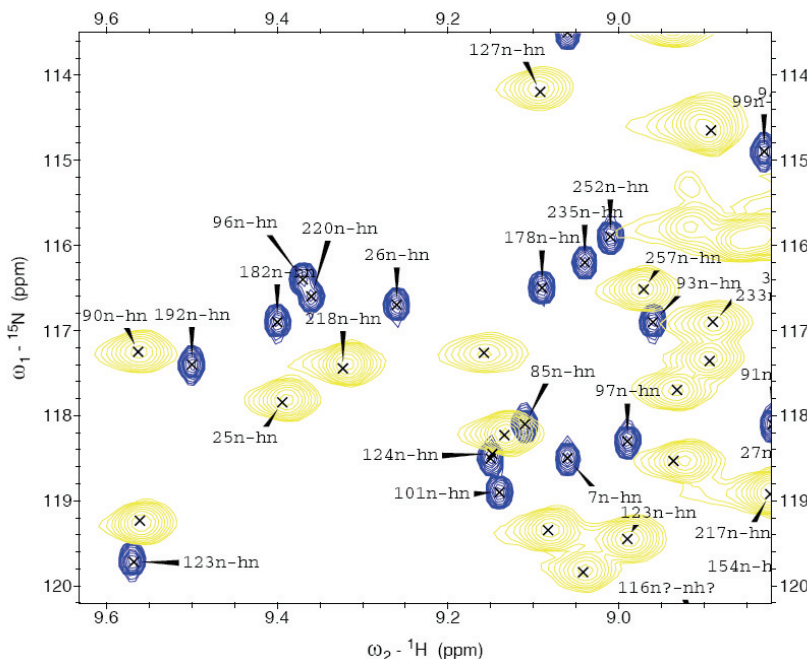


Figure 61: Detail spectrum of artificial HCAI peaks overlaid on unassigned HSQC spectrum

But the homology of the isoforms is not big enough to use it as a help for assignment. Only some peaks showed a correlation in the spectra.

3.7.2.4 Partial assignment of the shifted peaks

The idea was that it is rather probable that shifted peaks have neighbors in primary sequence which are also shifted because they are also near the peptide binding site. That means that it should be possible to correlate at least some of the shifted peaks to each other by their c_{α} -shifts. Therefore the strategy was to connect shifted peaks by their c_{α} -shifts and to identify patches of interaction. This reduces the possibilities for assignment dramatically.

To do this some borders had to be established to judge, which shifted peaks would be in the “pool” and how they were connected. The total shift had to be $> 0.2\text{ppm}$ and for connectivity the difference of the c_{α} -shifts had to be $\leq 0.3\text{ppm}$. Based on these borders a list of residues i and their possible preceding residue $i-1$ was made (Table 9).

Table 9: Possible correlation of residues (in working numbers)

i-1	i	i-1	i
1	174	125	35 212
9	19 128	128	32 129
10	4 128	129	19 128 186
19	4 139	139	32 107 129 188 231
32	107 117 129 140 146 175 188 231	140	32 107 129 188 231
35	4 139	146	1 32 107 129 188 231
51	62	156	19 128
59	36	161	59 238
62	26 95 97 200 238	172	161
72	48	173	26 59 97 200 238
77	72 202	174	161
95	36	175	19 128
97	192	188	192
100	9	192	59 200 238
107	51 123 196	200	48
117	1 107 129 140 146 175 188 231	231	62
123	4 139	243	1 36 140 146 175

The c_{α} - shifts (characteristic for an specific aa or a group of them) of these neighbors were crosschecked with the primary sequence to find matching residues compatible with the shifts. Some residues could be assigned with high certainty. If for residues with more uncharacteristic c_{α} - shifts two or more possibilities were detected, the closest one (in 3D structure) to residues assigned with high certainty, was chosen. By doing so ~ 50% of those peaks could be assigned. Three peaks with working number 100, 125 and 202 had such characteristic c_{α} - shifts that this was enough to identify them in the primary sequence of HCAII (Figure 62 and 63).

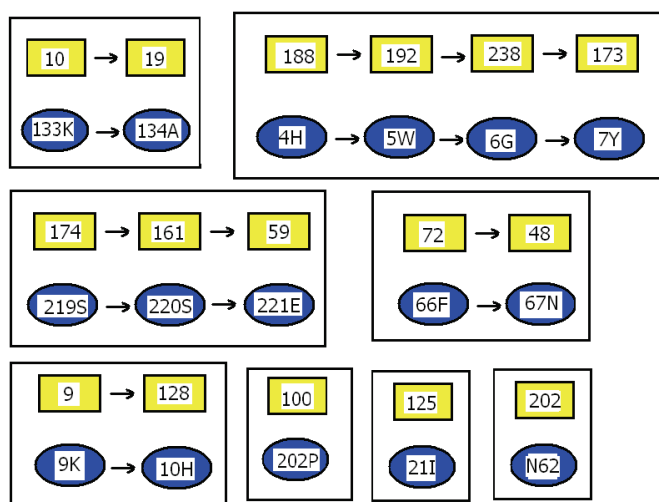


Figure 62: Correlations by partial assignment of working numbers (blue) and amino acids (orange)

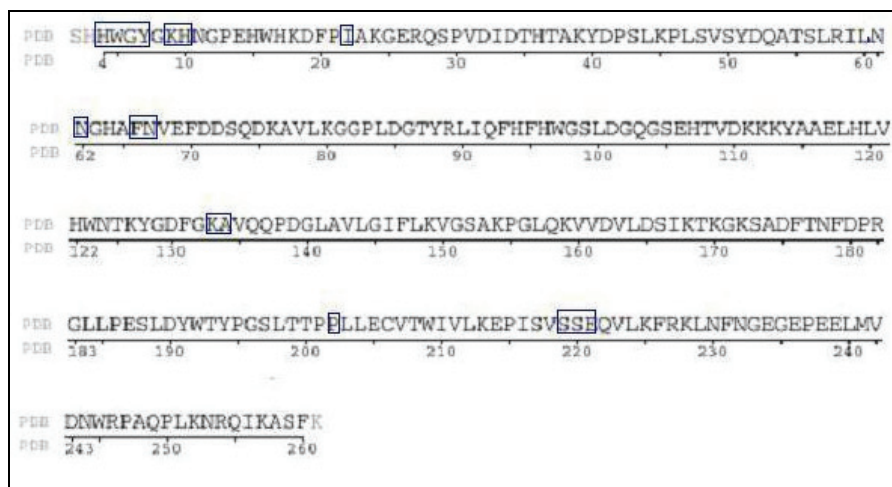


Figure 63: Primary Sequence of HCAII with residues mapped to binding site (blue squares) by partial assignment

With this information the binding site of his- trx- HAE1ct on HCAII could be marked with pymol (Figure 64 and 65).

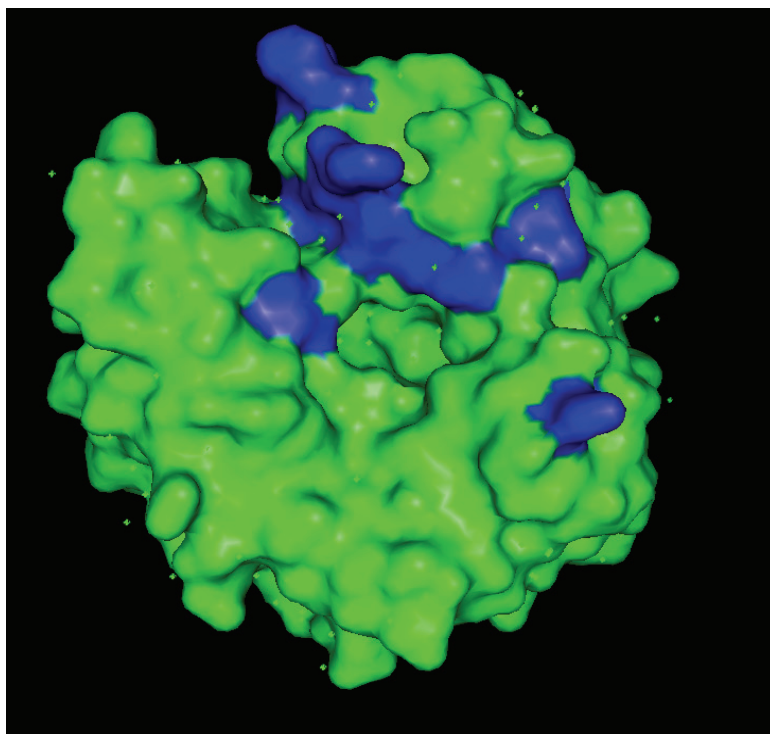


Figure 64: HCAII (green) with marked Bindingsite (Magenta) and entry to active site front view

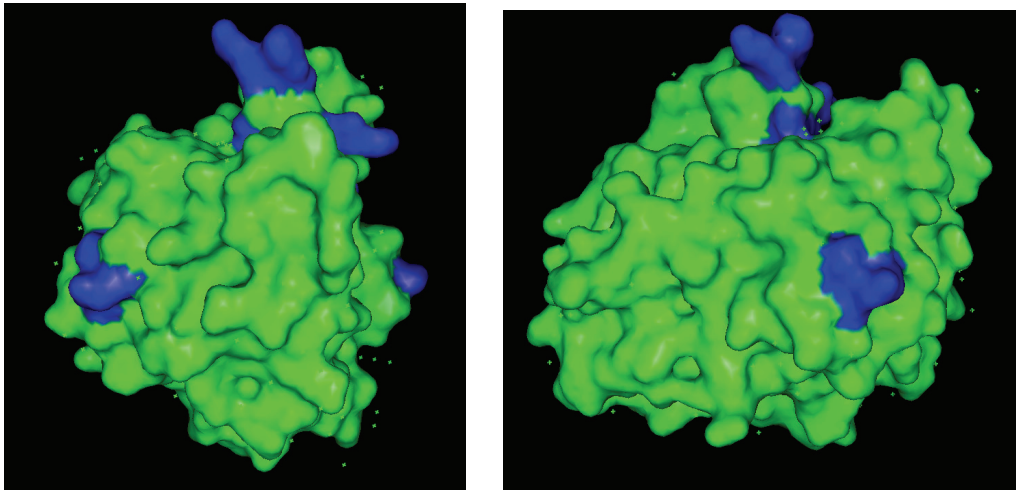


Figure 65: HCAII (green) with marked Bindingsite (magenta) side view (left) and back view (right).

The Binding site is situated near the entry to the active site of HCAII. The patch of shifted peaks which is separated from the binding site seems to be an unspecific interaction of his- trx- tag with HCAII.

4 Discussion

Due to problems cloning a co-expression vector, HCAII and his- trx- HAE1 C-terminal region were expressed separately in BL21 pLys strain. The expression and purification yielded sufficient amounts of unlabeled proteins.

The requirement for NMR experiments was to express the binding partners also in the labeled form by growing cells in M9 minimal media. The expression of HCAII was comparable to that in LB but not for the the fusion- protein his-trx- HAE1 C-terminal region. Eventually an expression screen, in which 7 bacterial strains were tested, revealed strain C41 pROS gave expression levels necessary to get reasonable amounts of protein. This strain is normally used for toxic proteins. The HAE1 C-terminal region part of the protein is believed to be unfolded unless bound to HCAII. Therefore under higher pressure like in minimal media the fusion protein could be encountered as toxic. The switch to expression in C41 pROS increased also the protein production in LB by around 20%.

Previous studies discovered that complex formation is impeded in buffer with high pH and high ionic strength (Vince and Reithmeier 1998). We therefore choose 20 mM MES buffer at pH 6, 20 mM NaCl for this task. TEV- cleavage of fusion protein his-trx- HAE1 C-terminal region (18 kDa) can be performed before or after complex formation. Both strategies were tried:

- i) Complex formation followed by TEV- cleavage produced bands at size of HCAII (29 kDa) and 12 kDa on SDS- PAGE while no band for HAE1 C-terminal region (4kDa) was detected. For a clear identification of the unknown 12 kDa band and its origin mass spectroscopy should be performed.
- ii) TEV- cleavage followed by complex formation - bands with size of HCAII (29 kDa) and HAE1ct (4 kDa) were detected on the SDS – PAGE. This strategy was therefore used to prepare the samples for NMR experiments while in the initial stages also samples derived from strategy (i) were used for co- crystallisation studies.

As crystals obtained from co- crystallization studies were identified to be salt or HCAII alone, soak of HCAII- crystals with HAE1 C-terminal region or with the consensus sequence LDADD peptide were performed. All used crystals were

belonged to the $P 2_1$ space group with of the unit cell dimensions of $41 \times 41 \times 70 \text{ \AA}^3$, in which the crystal contacts are formed by the N- terminal portion of the enzyme.

During soaking experiments with the HAE1 C-terminal region and the consensus sequence, the crystals partly dissolved, but nevertheless preserved sufficient diffraction capacity to give data to $2 \text{ \AA}$ resolution. The phase problem was solved by molecular replacement. Crystals soaked with HAE1 C-terminal region retained their unit cell dimensions upon soaking, but did not show additional electron density indicative of the presence of bound peptide.

Soaking of crystals with the consensus sequence LDADD gave the same results. No additional electron density was detected for the bound peptide, but the unit cell dimensions changed to $40 \times 80 \times 70 \text{ \AA}^3$. This could be due to a pH change induced by the high amount of LDADD containing amino residues with acidic character.

The unsuccessful soaking experiments with both peptides can be mostly attributed to the fact that the N-terminal region of HCAII is involved in the crystal contacts and therefore inaccessible for binding to the peptide. Moreover, high ionic strength (2.8 M ammonium sulfate) and slightly basic (pH 7.5) conditions from which the crystals used for soaking experiments were grown, were not appropriate for the complex formation. Moreover, the soaked peptide, in particular HAE1 C-terminal region might, destroy the crystal contacts and/or is too big and bulky to be soaked in this crystal lattice. It is also possible that the initial unfolded peptide folds upon interaction and is therefore destroying the crystal lattice.

Additionally, the consensus sequence peptide might not be sufficiently long to bind HCAII specifically. Binding studies of designed peptides would be necessary to address this question.

NMR interaction studies are based on shifts in HSQC- spectra were performed in parallel. The spectrum of a protein alone is collected and referenced to the spectrum of the same protein in interaction with a binding partner. Changes in the spectrum (up- or downfield shifts) are due to this interaction, meaning that the change of the environment of amino acid residues due to proximity of the peptide or the protein is affects their shielding in the magnetic field.

The shifted peaks can be assigned to amino acids involved in the interaction by 3D – NMR experiments. This was performed for labeled HAE1 C-terminal region in

interaction with HCAII. It was discovered that additional to L886 which was already known to play an important role in this interaction (Vince, Carlsson et al. 2000), also E882 and E899 are involved and are even more essential for the binding reflected in their values for the total shift (Figure 54).

The motif of leucine followed by a stretch of four amino acid residues containing at least two acidic residues like D or E can be found in the sequence of different HCO₃⁻ transporters. It is rather striking that nearly all of them have glutamate in position leucine-4 and a glutamate or aspartate in position leucine +13 (Figure 66). The E+4 is also found in the C- terminal part of aquaporine 1 (AQP 1). This reflects the universal importance of those residues for the interaction with HCAII.

The only exceptions to this observation is AE3 and pendrin (a iodide/ chloride transporter in thyroid and kidney) indicating that these transporters have other or additional interaction motifs or have a weaker binding constant to HCAII.

In agreement with this supposition, the transport capacity of AE3 does not enhance dramatically by the interaction with HCAII compared to AE1 and AE2 (Sterling, Reithmeier et al. 2001). The transporter DRA does not carry any consensus motif. But it is likely that a longer stretch of the C- terminus is exposed to the cytosol maybe including such a motif (Pushkin, Abuladze et al. 2004). Therefore it is not sure if this transporter also used a hydrophobic- acidic motif for the interaction with HCAII.

hAE1	RNV <u>E</u> LQCLD <u>ADD</u> AKATFD <u>E</u> EGRDEYDEVAMPV
hAE2	TDREMKCLDANAEPVFD <u>E</u> RGVDEYNEMPMPV
hAE3	QDRELQALDSEDAEPNFDGQDEYNELHMPV
rAE4a	SPQELWLDELMPEEERNVPEKGLPEGHSFSGSDSELMYQPKAPEINISVN
hNBC1b	SQHDL <u>S</u> FLDDVIPEKDKKKKE <u>E</u> DKKKKKKKGS <u>L</u> DSNDSDCPYSEKVPKIPMDIMEQQPFLSDSKPSDRERSPTFLERHTSC
hNBC3	TKREL <u>S</u> WLDDLMPESKKKKKE <u>E</u> DKKKKEEEAERMLQDDDDTVHLPEFGGSLQIPVKALKYSPDKPVSVKISFEDEPRKKYVDAETSL
hNDCBE1	SKREL <u>S</u> WLDDLTPESKKKKL <u>D</u> DAKKKAKEEEVIV
hPendrin	LEELDIPTKEIEIQVDWNSLPVKVNVKVPKPIHSLVLDCAISFLDVVGVRSLRVIVKEFQRIDVNVYFASLQDYVIEKLEQCGFFDDNIR KDTFFLTVDAILYLQNQVKSQEGQGSILETITLQDCKDTLELIETELTEEELDVQDEAMRTLAS
hDRA	TIHDAVLHILMKKDYSTSKFNPSQEKDGK

Figure 66: Primary C- terminal sequence of different HCO₃⁻ transporters; (h= human; r= rabbit); (Pushkin, Abuladze et al. 2004)

However, for the amino acid residues following leucine 886 in the consensus sequence L886DADD, no significant shifts in the HSQC- spectrum reflect their importance for the interaction were detected. These amino acids might not be

essential for the interaction but help to position leucine residue in the binding site. Namely, the acidic amino acids could increase the pressure on leucine to bind to a hydrophobic patch.

The same studies were performed for labeled HCAII in interaction with his- trx- HAE1 C- terminal region. Characteristic shifts were observed indicating an interaction. The difficulties of correlating the shifted peaks to amino acids in the primary sequence rise with the size of a protein. Therefore a different strategy for HCAII was used. By partial assignment approximately 50% of the shifted peaks could be assigned. With the program Pymol the binding site on the 3D structure of HCAII was visualized. The peptide binds near the entry to the active site (Figure 64 and 65).

Based on the value of total shift a row of importance for the interaction can be done for clearly shifted amino acids with a shift ≥ 0.47 ppm:

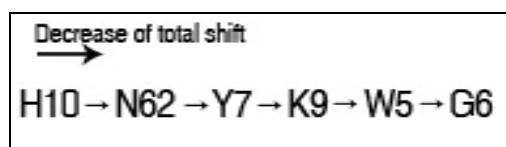


Figure 67: Residues with total shift ≥ 0.47 ppm

According to the value of the shift the residues 9 and 10 (Lysine- Histidine) as well as the stretch of the residues 4- 7 (histidine-tryptophan-glycine-tyrosine) seems to be essential for interaction. In previous studies the mutation of K9 and H10 did not go hand in hand with a significant decrease of binding to HCAII (Vince, Carlsson et al. 2000). However the binding site could consist of different binding motifs and therefore the mutation of one is not interfering dramatically with the binding.

It was surprising that not only residues in the N- terminal helical region are involved in the binding: given its significant shifts upon interaction N62 residue seems to have a key role in the interaction as well.

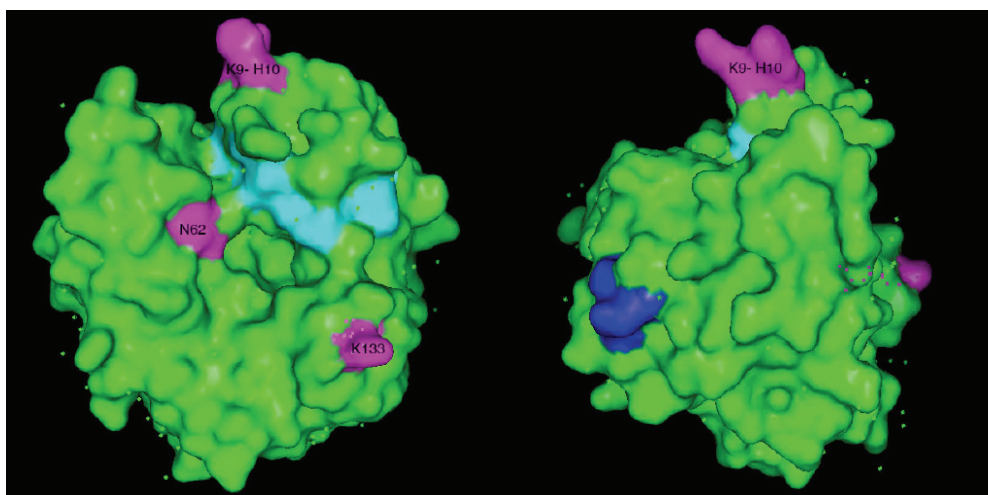


Figure 68: Front view HCAII with binding site (left) and side view (right) ; hydrophobic amino acids (cyan); amino acids with polar side chains (magenta); unspecific interaction site of his- trx – tag (blue)

The residues K9- H10, N62 or K133 could be involved in electrostatic interactions with glutamate residues in HAE1 C-terminal region. Due to the higher intensity of the shifts (≥ 0.47 ppm), induced by binding of HAE1 C-terminal region, it is more likely that K9- H10 and N202 are stabilizing E822 and E899.

The location of the unspecific binding of the his- trx- tag suggest an interaction of K9-H10 with E822 of HE1, while N202 interacts with E899. The distances would allow this interaction if the peptide folds into an α - helix.

Future studies should be based on the analysis of mutations in HAE1 C-terminal region and HCAII. The mutation of E882 and E899 in HAE1 C-terminal region should decrease or lead to a complete loss of binding to HCAII. Also the role of N62 and K9- H10 should be investigated.

Moreover the secondary structure of the bound HAE1 C- terminal region should be determined by comparison of the C_{α} shift of the unbound and bound state. An α - helical conformation would be reflected in a significant upfield shift in comparison with the unbound state, which was reported to be unfolded (Vince, Carlsson et al. 2000) .

5 Literature

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