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Painfully Energetic

A tale of two proteins potentially connected

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Painfully Energetic

A tale of two proteins potentially connected

BIOCHEMISTRY AND STRUCTURAL BIOLOGY | LUND UNIVERSITY
EVA SPERLING



Painfully energetic

A tale of two proteins potentially connected

Eva Sperling



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DOCTORAL DISSERTATION

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Abstract <p>NADH:quinone oxidoreductase (Complex I) is the first enzyme of the respiratory chain and is involved in energy conservation generating an electro-chemical gradient across a membrane. The enzyme can be divided into a membrane spanning domain and a hydrophilic domain, which protrudes from the membrane. In the hydrophilic domain electrons from NADH oxidation are transported via a wire of iron-sulfur (Fe-S) clusters to quinone, which is reduced. While the membrane domain is responsible for proton translocation to maintain the proton motive force, which is important for ATP synthesis. Large protein complexes like complex I have evolved from an assembly of discrete functional building blocks of which there are extant homologs. Two very different protein families, the Mrp-antiporter and membrane bound [NiFe]-hydrogenases contain subunits which are homologous to complex I subunits. Part one of this work aimed to better understand the functional relationship between the related protein subunits of complex I, Mrp-antiporter and [NiFe]-hydrogenases. This knowledge will help us to elucidate the proton translocation pathway in complex I. First we compared the functional differentiation of complex I antiporter-like subunits with transporter subunits of the Hyc and Hyf hydrogenases and the 11-subunit complex I. For that we tested if the different subunits could rescue the growth of two salt sensitive <i>Bacillus subtilis</i> strain, which each lacked one of the two large Mrp-antiporter subunits (MrpA/MrpD). The 11-subunit complex I subunits could restore the growth in a similar manner as the complex I subunits, whereas the hydrogenase subunits could substitute equally well for the two MrpA and MrpD. We confirmed that 11-subunit complex I is a <i>bona fide</i> complex I. and that the hydrogenase subunits have intermediate forms of the antiporter-like subunits. Secondly we examined the functional relationship of the two homologous proteins MrpA from the Mrp-antiporter and NuoL from complex I. We located a stretch of amino acid residues which is conserved only in NuoL and MrpA, but not in the other complex I antiporter-like subunits or in MrpD. These residues were subjected to site directed mutagenesis and any resulting effects were examined in vivo by <i>B. subtilis</i> complementation studies and ^{23}Na-NMR. Only one mutation (M258I/M225I) showed differences in the efficiency of cell growth and sodium efflux in both subunits, the other mutants were all able to cope with high salt levels.</p> <p>Ion channels are important for many processes in the cell and critically depend on gradients over membranes to execute their functions. They are involved in the detection of changes in the environment, which is an important survival mechanism for every organism. One of these ion channels is TRPA1, which belongs to the TRP superfamily of non-selective cation channels. TRPA1 can be activated by changes in temperature and voltage, as well as by a wide range of electrophilic and non-electrophilic chemicals. As structural information is limited, the exact activation mechanism is still elusive. The aim of the second part was to study the structural and functional changes of TRPA1 upon activation by temperature and chemical activators. We studied the effect of increased temperature and ligands on the conformation of mosquito TRPA1 (AgTRPA1), using intrinsic tryptophan fluorescence, SRCD and nanoDSF. We showed that the electrophilic ligands tested were quenching the tryptophan fluorescence in the same way, suggesting a similar binding mechanism. We propose a putative model how temperature and ligand can activate AgTRPA1. Furthermore, we truncated the C-terminal region of human TRPA1, in an attempt to narrow down the minimal structural and functional unit of hTRPA1. This will facilitate future structural and functional studies of the activation mechanism.</p>		
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Painfully energetic

A tale of two proteins potentially connected

Eva Sperling



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Faculty of Science
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Paper I

Functional role of the MrpA- and MrpD-homologous protein subunits in enzyme complexes evolutionary related to respiratory chain complex I

Moparathi VK, Kumar B, Al-Eryani Y, **Sperling E**, Górecki K, Drakenberg T and Hägerhäll C*

BBA Bioenergetics (2014) 1837 178-185

Paper II

Functional Differentiation of Antiporter-Like Polypeptides in Complex I; a Site-Directed Mutagenesis Study of Residues Conserved in MrpA and NuoL but Not in MrpD, NuoM, and NuoN.

Sperling E*, Górecki K, Drakenberg T, Hägerhäll C

PLoS ONE (2016) 11(7): e0158972. doi:10.1371/journal.pone.0158972

Paper III

Temperature and ligand induced conformational changes in *Anopheles gambiae* TRPA1

Survery S, **Sperling E**, Haq SR, Dicko C and Johanson U

Manuscript

Paper IV

Truncations of the hTRPA1 C-terminal domain for structural and functional studies

Sperling E, Edwin A and Johanson U

Manuscript

* Corresponding author

Author Contributions

Paper I

I conducted the growth studies of the strains expressing the Hyf proteins (HyfB, D, F) and analyzed the data. I also participated in writing the manuscript and was involved in revising it after reviewer comments.

Paper II

I took part in design and planning the experiments. I prepared all the mutations and performed all growth study experiments and the data analysis. Furthermore I conducted some of the NMR experiments. I wrote the manuscript with input from the coauthors, submitted and revised it as the corresponding author.

Paper III

I purified mosquito TRPA1 and conducted one set of the DSF experiment. I was involved in preparing the first draft of the manuscript.

Paper IV

I helped to design the study. I cloned, expressed and purified the protein and wrote a first draft of the manuscript

Other publications not included in the thesis

Photo-electrochemical communication between cyanobacteria (*Leptolyngbia* sp.) and osmium redox polymer modified electrodes

Kamrul Hasan, Huseyin Bekir Yildiz, **Eva Sperling**, Peter O Conghaile, Michael A. Packer, Donal Leech, Cecilia Hägerhäll and Lo Gorton

Phys. Chem. Chem. Phys. 2014, 16, 24676-24680

Photoelectrochemical wiring of *Paulschulzia pseudovolvox* (Algae) to osmium polymer modified electrodes for harnessing solar energy

Kamrul Hasan, Emre Cevik, **Eva Sperling**, Michael A Packer, Donal Leech, Lo Gorton

Adv. Energy Mater, 2015, 1501100

Evaluation of photocurrent generation from different photosynthetic organisms

Kamrul Hasan, Valentina Grippo, **Eva Sperling**, Michael A. Packer, Dónal Leech, and Lo Gorton

ChemElectroChem 2017, 4, 1–7, DOI: 10.1002/celec.201600541

Abbreviations

AITC	Allyl isothiocyanate
ARD	Ankyrin repeat domain
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
CA	Cinnamaldehyde
DSF	Differential scanning fluorimetry
Ech	Energy converting hydrogenase
FAD	Flavin adenine dinucleotide (oxidized)
FADH ₂	Flavin adenine dinucleotide (reduced)
Fe-S	Iron-Sulfur
FhdF	Formate dehydrogenase
FHL	Formate hydrogenase lyase
FMN	Flavin mononucleotide (oxidized)
FMNH ₂	Flavin mononucleotide (reduced)
Hyc	Hydrogenase-3
Hyd	Hydrogenase
Hyf	Hydrogenase-4
MO	Mustard oil
Mrp	Multiple resistance and pH
NADH	Nicotinamide adenine dinucleotide (reduced form)
NAD ⁺	Nicotinamide adenine dinucleotide (oxidized form)
NDH-1	NADH dehydrogenase type 1
NiFe	Nickel-Iron

NMM	N-Methylmaleimide
NMR	Nuclear magnetic resonance
Nuo	NADH ubiquinone oxidoreductase
Pi	Inorganic phosphate
Q	Quinone
QH ₂	Quinol
UQ	Ubiquinone
ROS	Reactive oxygen species
TM	Transmembrane helix
Tm(DOTP) ⁵⁻	Thulium(III)1,4,7,10-tetraazocyclododecane-1,4,7,10-tetrakis(methylenephosphonate)
TRP	Transient receptor potential
TRPA1	Transient receptor potential ankyrin
TRPC	Transient receptor potential canonical
TRPM	Transient receptor potential melastatin
TRPML	Transient receptor potential mucolipin
TRPN	Transient receptor potential NOMPC
TRPP	Transient receptor potential polycystin
TRPV	Transient receptor potential vanilloid

Populärvetenskaplig sammanfattning

Varje cell har ett membran som skapar en gräns mot omvärlden och skyddar cellen. Men cellen måste alltjämt utbyta viktiga molekyler med omvärlden för att överleva. Därför finns specialiserade proteiner, som sitter i membranet och utgör portar i denna barriär. Denna avhandling fokuserar på två membranproteiner, en protonpump och en jonkanal.

Varje dag i våra liv använder vi hjärnan, muskler och hjärtat. Vart och ett av dessa organ behöver energi. Energivalutan i kroppen kallas ATP (adenosintrifosfat), en liten molekyl som deltar i många reaktioner i kroppen. ATP-produktion är mycket komplicerad och många olika enzymer är involverade. Nästan alla organismer utnyttjar flera stora proteinkomplex vilka är organiserade i en respirationskedja. I eukaryoter (t.ex. djur och växter) finns de i mitokondriernas membran, de organeller som brukar kallas cellens kraftverk. Många sjukdomar, däribland Alzheimers och Parkinsons, utvecklas på grund av defekter i mitokondrierna. Detta leder till energibrist i cellen. Därför är det viktigt att förstå funktion och struktur av energiproduktionsenzymerna. Det största och första enzymet i respirationskedjan heter komplex I. Komplex I består av en del som sitter i membranet och en annan vattenlöslig del som sticker ut från membranet. Man kan hitta andra proteiner i naturen som är väldigt lika enskilda delar av komplex I, och förmodligen har komplex I evoluerat från släktingar till dessa mindre proteiner som tillsammans har bildat ett stort proteinkomplex med nya funktioner. Vårt syfte med detta projekt var att karaktärisera dessa besläktade proteiner och testa om de fortfarande har liknande funktioner.

Den andra delen handlar om hur vi kan förnimma/känna av vår omvärld. Det är livsviktigt att vi kan detektera förändringar i omvärlden, t.ex. temperatur, tryck etc. Vår kropp genomsyras av nervceller som är kopplade till det centrala nervsystemet (hjärnan och ryggmärgen). Några nervceller har specialiserade jonkanaler, som kan aktiveras genom förändringar i temperatur, tryck eller elektrisk spänning. Dessutom kan olika molekyler binda till jonkanaler och aktivera dem. När en jonkanal aktiveras skickas en elektrisk impuls till centrala nervsystemet och vi kan reagera. En av de specialiserade jonkanalerna heter TRPA1 och utgör temat för den andra delen i avhandlingen. TRPA1 aktiveras av kalla och varma temperaturer och många olika molekyler (t.ex. från stark mat som wasabi och senap). Många människor lider av kronisk smärta, för vilket det saknas

läkemedel utan biverkningar. Därför är TRPA1 ett attraktivt mål i utvecklingen av nya läkemedel. Av den orsaken är det viktigt att lösa proteinstrukturen, så att man förstår hur TRPA1 aktiveras. Vårt syfte med denna del av projektet var att undersöka och minimera en liten strukturell enhet av TRPA1, som fortfarande fungerar, för att lättare urskilja vilka strukturförändringar som sker vid aktivering TRPA1.

Zusammenfassung

Jede Zelle besitzt eine Zellmembran, die das Zellinnere von der äußeren Umgebung abgrenzt. Somit ist die Zelle von möglichen äußeren Einflüssen geschützt und behält ihr Form. Vergleichen kann man das mit einem Gartenzaun, der das eigene Grundstück umzäunt. Allerdings müssen Zellen einige wichtige Stoffe entweder aus der Umgebung aufnehmen oder an sie abgeben um zu funktionieren. Je nachdem welche chemischen und physikalischen Eigenschaften diese Stoffe haben, können sie die Zellmembran nicht alleine passieren. Wie der Gartenzaun der nur einige kleine Tiere durchlässt, aber zum Beispiel keine Menschen. Um dennoch diese wichtigen Nährstoffe aufzunehmen, besitzen die Membranen spezialisierte Proteine, die als Kanäle für chemische Stoffe agieren. Vergleichbar zu der Gartentür die sich öffnen und schließen lässt, um Menschen passieren zu lassen. Zwei dieser Transporterproteine stehen im Mittelpunkt dieser Arbeit.

Das erste Membranprotein heißt, NADH:quinone oxidoreduktase oder einfacher gesagt Komplex 1. Von kleinen Zellreaktionen, wie z.B. die Verdopplung unserer DNA, über das Lesen eines Buches, bis hin zur Muskelbewegungen, benötigt unser Körper Energie. Die Quelle für unsere Energie liegt in unserer Nahrung, wohingegen für Pflanzen ist es das Sonnenlicht. Aber wie genau kommt die Energie aus unserem Essen in unsere Zellen und was für eine Energie benutzt unserer Körper eigentlich? Ich fange mit der zweiten Frage an, der Form der Energie. Die Energie unserer Nahrung wird auf ein Molekül übertragen das ATP (Adenosintriphosphat) heißt. ATP wird auch oft als die Energiewährung unseres Körpers bezeichnet, da es die Energie für alle Zellprozesse bereitstellt, es wird quasi „ausgeben“. Interessanterweise finden wir ATP nicht nur in Menschen, sondern auch in allen anderen Lebewesen. Die Energieübertragung von unserer Nahrung auf ATP ist nicht direkt und benötigt eine Reihe von spezialisierten Proteinen, die auch die Atmungskette genannt wird. Diese Atmungskette sitzt entweder in der inneren Membran von Bakterien oder in der inneren Membran von Mitochondrien in höheren Lebewesen. Mitochondrien werden deswegen auch oft als die Kraftwerke unseres Körpers bezeichnet. Es gibt verschiedene Krankheiten, die darauf zurückzuführen sind, dass die Proteine in der Atmungskette nicht richtig funktionieren und daher dem Körper weniger Energie zur Verfügung steht. Diese Krankheiten sind oft vererbbar. Um die Heilungschancen zu erhöhen und

um mögliche Medikamente zu entwickeln ist es wichtig, dass wir die einzelnen Proteine der Atmungskette bis ins kleinste Detail verstehen. Das erste und das am wenigsten charakterisierte Protein in der Atmungskette ist Komplex 1. Die Struktur von Komplex 1 sieht aus wie ein „L“ und kann in zwei Bereiche unterteilt werden. Ein Bereich sitzt in der Membran und ein Bereich ist wasserlöslich und ragt aus der Membran heraus. Unser Ziel ist es Komplex 1 besser zu verstehen, indem wir die Evolution dieses riesigen Proteins untersuchen. Proteinkomplexe entstehen oft indem sich kleinere Proteinmodule mit unterschiedlichen Funktionen über die Jahre zusammenfinden, um dann ein „neues“ Protein mit ähnlichen oder anderen Funktionen zu bilden. Komplex 1 hat verwandte Proteine in anderen Proteinfamilien, wie den Hydrogenasen und den Mrp-antiportern. In dieser Arbeit haben wir die Funktionen dieser zwei Proteinfamilien untersucht, um Rückschlüsse auf Komplex 1 Funktion abzuleiten.

Das zweite Membranprotein, welches in dieser Arbeit untersucht wurde heißt: TRPA1. Für alle Organismen ist es überlebenswichtig ihre Umwelt zu erfassen, um mögliche Veränderungen zu bemerken und auf sie zu reagieren. Diese Veränderungen können Unterschiede in der Temperatur oder dem Sauerstoffgehalt sein. Auch müssen potenziell schädliche Umwelteinflüsse (bestimmte Chemikalien) erkannt werden. Aber wie kann unser Körper unsere Umwelt messen? Unser Körper ist von Nervenzellen durchzogen, die an unser Zentralnervensystem gekoppelt sind (Gehirn und Rückenmark). Einige dieser Nervenzellen besitzen spezialisierte Proteinkanäle, die durch bestimmte Reize, wie Temperatur-, Druck- oder elektrische Spannungsveränderungen aktiviert werden. Auch können bestimmte Moleküle an diese Proteinkanäle binden um sie zu öffnen. Durch die Aktivierung dieser Kanäle wird eine Kaskade von chemischen Reaktionen ausgelöst, die dann im Gehirn verarbeitet werden und zu einer körperlichen Reaktion führen. Eines dieser spezialisierten Kanäle ist TRPA1 und gehört zu den Schmerzrezeptoren. TRPA1 kann durch Temperaturveränderungen (Kälte in Menschen und Hitze in Insekten) oder durch eine Fülle von chemischen Molekülen aktiviert werden, z.B. durch das Molekül was den Senf scharf macht. Viele Menschen leiden unter chronischen Schmerzen (Migräne oder nach Verletzungen) und bis jetzt gibt es keine Medikamente die helfen, ohne extreme Nebenwirkungen zu zeigen. TRPA1 ist ein vielversprechendes Ziel um neue Medikamente zu entwickeln. Allerdings müssen wir dafür die genaue Struktur und den Aktivitätsmechanismus verstehen. Im zweiten Teil meiner Arbeit haben wir die Strukturveränderungen, wenn der Kanal geöffnet wird, untersucht.

Background

Membranes are important structures in all living cells, as they protect the cell from outside harm and provide an essential structure for many chemical reactions. The lipid bilayers of membranes contain a hydrophobic interior and two hydrophilic surfaces and therefore act as a barrier for many molecules and ions. In theory any dissolved molecule given enough time will be able to diffuse over the lipid membrane down their concentration gradient, but the rate of passing depends on the molecule's biophysical properties. Hydrophobic molecules like CO₂ or O₂ and small polar uncharged molecules (H₂O or ethanol) are able to cross the phospholipid bilayer with relatively high diffusion rates. Large hydrophobic molecules are also able to diffuse through the membrane, but due to their poor solubility in water they often do not reach the membrane. In contrast large uncharged polar molecules like glucose, as well as any charged molecules, do not readily pass.

The impermeability of the hydrophobic phase of the lipid bilayer presents a problem for the cell, as important molecules like sugars or amino acids, and ions cannot enter the cell interior. To overcome this problem, cells produce membrane proteins that mediate the passage of different substrates. Some are very specific, transporting only one class of substrate or only one particular substrate, while others are less selective. This type of membrane proteins can be found in all domains of life, where they are involved in many different tasks, for example: transport of molecules or ions, cell signaling or energy conservation. These transport mediating membrane proteins can be divided traditionally into two different classes: channels and transporters (Fig. 1A). They can be further classified into families and subfamilies, compiled in the Transporter Classification Database (TCD) (<http://tcdb.org/>) [1].

The main difference between these two classes is the source of energy used for transport. Channels can mediate transport only in the direction of the (electro)-chemical gradient of the substrate, whereas transporters can also move the substrate against it. Channels are not always open and can be activated for example by different signals, changes in membrane potential or ligand-binding. They are also often highly selective for the substrate they transport. In their active state channels are open to both sides of the membrane and usually have a high flow rate of substrate. Transporters in contrast are not open to both sides of the

membrane at the same time. Often a cycle of alternating conformational changes can be observed, where one side of the transporter is open when the other is closed. Some transporters can move the substrates against their concentration gradient.

In some proteins the transport is coupled directly to metabolism, meaning that these transporters use the energy provided by ATP or redox energy to accumulate for example ions on one side of the membrane [2]. This type of transport is denoted primary active transport (Fig. 1B). Examples for this type of transport can be found in the Na^+/K^+ -ATPase, SERCA, complex I, III, IV of the respiratory chain and photosystem II. Another way to accumulate molecules is to couple the transport of a substrate against its (electro)-chemical gradient to the transport of another substrate down its gradient. This process is often referred to as secondary active transport [2].

Furthermore, transporters can be classified according to the number of different substrates that are coupled to each other. If only one substrate is involved the protein is called a uniporter. If two or more substrates are simultaneously transported, they are either called symporter (transport is in the same physical direction) or antiporter (transport is in opposite directions) (Fig. 1C) [2].

However, the classification of membrane transport proteins into channels and transporters is blurry, as we discover membrane proteins that have properties of both channels and transporters [3].

This work is about three different membrane proteins, NADH:quinone oxidoreductase (complex I), Mrp-antiporter and TRPA1. Two of them are transporters (Mrp-antiporter and complex I) and one of them is an ion channel (TRPA1). Next I will give brief introductions into the field of complex I and TRP-channels and describe my contributions.

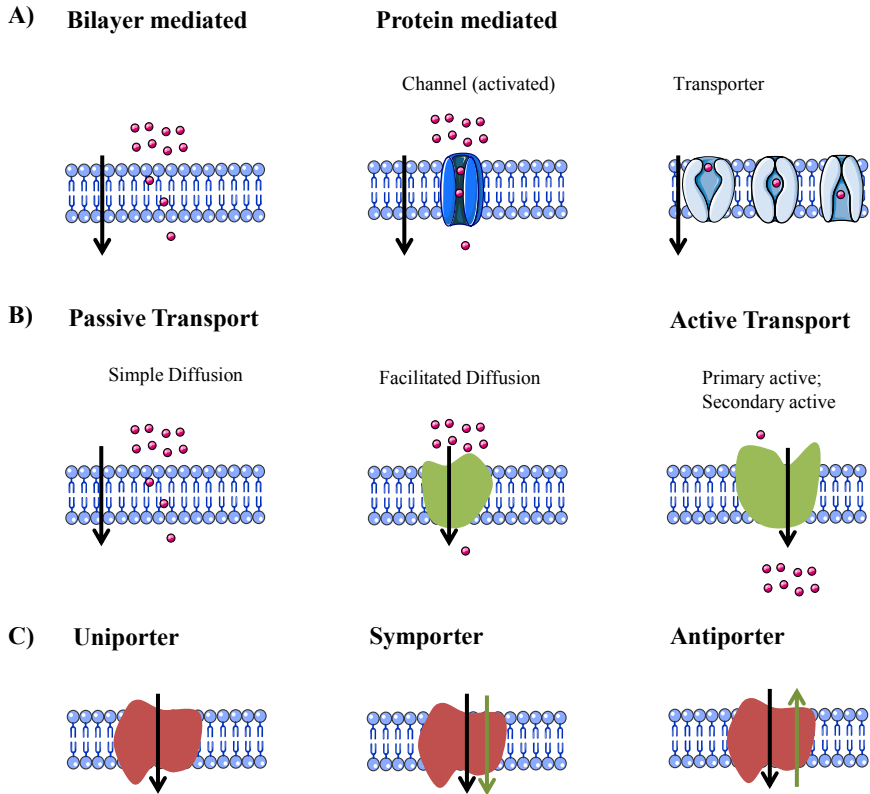


Figure 1 Overview of the classification of substrate translocation over the phospholipid bilayer. A) represents the passage of molecules or ions over the membrane either by simple diffusion or through proteins. Those specialised membrane proteins are divided into: Channels and Transporters. B) Translocation can be passive through simple diffusion or facilitated diffusion. In contrast to that is the active transport, where substrates can be transported against the concentration gradient. Either by the usage of cellular energy e.g. ATP or Redox energy (primary active) or by coupling of two or more substrates, whereas one is transported down its concentration gradient (secondary active). C) If only one substrate is transported the protein is called a uniporter. While the transport of two or more substrates coupled to each other is either defined as symporter (same direction) or antiporter (opposite directions).

Introduction to bioenergetics

I want to start this chapter with a fundamental question: why do we have to eat? All of us know the consequences if we do not eat, we will die from starvation. The reason why we need food is because we need energy to fuel vital reactions in our bodies, in the same way as our cars need fuel to drive. And we are not the only organisms in need of free (or available) energy; all living organisms depend on it. So where does the energy come from and how can we use it? These are a few questions I will try to answer in this chapter. But first we need to have a closer look into the fundamentals of thermodynamics and bioenergetics, starting with the two laws of thermodynamics.

The first law, the law of energy conservation, says that the total amount of energy in the universe remains constant, although the form of energy may change. This means that energy cannot be destroyed nor created, but it can flow from one place to another. The second law states that the universe always tends towards more entropy. Thus every cellular process (each chemical reaction) will increase the disorder of the universe.

Keeping this in mind we will take a closer look on what free energy is, which is so crucial for life. Heterotrophic organisms, like humans, other animals, fungi and bacteria obtain free energy from converting organic compounds into ATP (adenosine triphosphate), which provides the energy for biological work. Autotrophic organisms (plants, algae, some bacteria) on the other hand use sunlight to form ATP. The amount of energy (G) available to do work from a biochemical process can be calculated from the Gibbs-Helmholtz equation:

$$\Delta G = \Delta H - T\Delta S$$

ΔG is the change in Gibbs free energy, ΔH is the change in enthalpy (heat), T is the temperature and ΔS is the change in entropy [2]. This is true for constant temperature and pressure. The value of ΔG does not only tell us the amount of available energy, but also if a reaction occurs spontaneously ($\Delta G < 0$) and how far the reaction is from equilibrium. One important thing, which we should not ignore, is that ΔG does not tell us about the speed of a reaction. Even if a reaction has a negative ΔG and can occur spontaneously, if the kinetics of this reaction are non-favorable, the reaction might be very slow or not happening in a relevant time scale.

As mentioned earlier the energy provided for biological work is stored in a molecule called ATP, which is produced through conversion of organic compounds. It was first discovered in 1929 by Lohmann and has since then been called the energy currency of the cell, as it is used to pay the energetic costs for many reactions [4, 5]. It is also a universal energy currency, meaning it can be found in all organisms. The hydrolysis of ATP to ADP (adenosine diphosphate) and inorganic phosphate (P_i) is a highly exergonic reaction and can drive an unfavorable reaction ($\Delta G > 0$) forward if coupled to it [2].



One might ask now, what is so special about ATP?; because there are many other molecules which have the same type of phosphate bond. A common misunderstanding is that ATP has a high-energy phosphate bond, which is technically not true, as it is not so much about the bond. It is rather about the equilibrium constant of the hydrolysis of ATP and the conditions in the cell, which are kept far away from equilibrium [2].

Eukaryotic cells have specialized compartments (organelles) for ATP synthesis, called mitochondria, often also referred to as powerhouses of the cell. Prokaryotes on the other hand do not compartmentalize and build ATP in the cytoplasm. But how is ATP made? The process which converts the energy we take up with our food into ATP is called respiration it is built up of three key pathways: glycolysis, Krebs cycle and oxidative phosphorylation. Both in glycolysis and in Krebs cycle a few ATP molecules are generated, but the energy from the oxidation of glucose is mainly transferred to the electron carriers NADH and $FADH_2$. Most of the ATP is instead generated through oxidative phosphorylation, which comprises the electron transfer chain (respiratory chain) and the ATP synthase. The electron transfer chain includes three proton pumps (complex I, III, and IV) and succinate dehydrogenase (complex II), which is also part of the Krebs cycle. The electrons from NADH are transferred from complex I via quinone to complex III, which donates the electrons via cytochrome c to the final electron acceptor oxygen in complex IV. The oxygen is then reduced to water (Fig. 2). The electron transfer from the NADH/ NAD^+ redox couple to the final couple O_2/H_2O is mediated by several different redox couples (quinone, Fe-S, FMN etc.).

In which direction an electron is transferred depends on the ΔG of the reaction. The relationship of the redox potential and ΔG can be described as following:

$$\Delta G = -nF\Delta E$$

Where n is the number of electrons transferred between the donor/acceptor, F is the Faraday constant and ΔE is the redox potential difference between the donor and acceptor couple [2].

But how is the energy from electron transfer conserved? For that we need to have a look at another basic theory, the chemiosmotic theory, which was proposed by Peter Mitchell in 1961 [6]. This theory says that the driving force for ATP synthesis is a proton electrochemical gradient across the membrane. This electrochemical gradient is also called the proton-motive force Δp , which encloses two different parts. The first is the ΔpH , differences in proton concentrations across the membrane and second the membrane potential ($\Delta\psi$), distribution of electrical charges.

How is Δp generated? Proton translocation over the membrane is tightly coupled to the electron transfer, meaning that when the electrons are passed on, protons are transferred over the membrane (inner membrane of mitochondria/cytoplasmic membrane of bacteria) by complexes I, III and IV (primary proton pumps). As mentioned previously another (secondary) proton pump is located next to the electron transfer chain, the ATP synthase. When Δp is sufficiently large, the protons are forced back into the mitochondrial matrix via the ATP synthase, which in turn drives the synthesis of ATP from ADP + P_i [2].

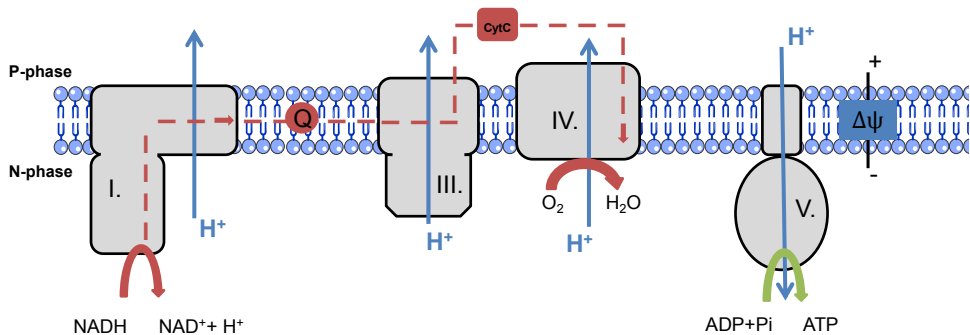


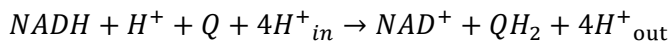
Figure 2 Electron transfer and the generation of a proton-motive force by the respiratory chain. The red dashed line represents the electron transfer from NADH through the different proteins to the final electron acceptor oxygen. H⁺ translocation from the negative (N)-phase to the positive (P)-phase leads to the generation of a sufficient proton-motive force (Δp), which drives the H⁺ back to the N-phase through the ATP synthase (complex V), which then produces ATP from ADP + P_i. Complex II is another entry point, besides complex I, of electrons into the respiratory chain, as it donates electrons to the quinone pool, but is not illustrated here because it does not pump protons.

As cellular respiration is vital for life, it can have devastating effects if some of the pathways are not functioning as they should. Therefore it is crucial to understand the proteins involved in detail. We do know the structure and function of most respiratory chain enzymes in detail and especially how the electron transfer is coupled to proton translocation. The only enzyme, which still leaves many

unanswered questions, is ironically the first enzyme, complex I, which stands in the focus of the first part of my thesis.

Complex I

As described in the previous chapter, complex I (NADH:quinone oxidoreductase) is the main entry point for electrons into the respiratory chain. It catalyzes the oxidation of NADH and the reduction of quinone, while protons are translocated over the membrane to the bacterial periplasm, corresponding to the intermembrane space in mitochondria.



These protons contribute to the overall proton-motive force, which is needed for ATP synthesis by the ATP synthase. The stoichiometry of the proton pumping is believed to be four protons per NADH oxidized ($4H^+/2e^-$), which was examined mainly in mitochondrial complex I [7-9]. While the mechanism and structure of the other respiratory chain enzymes could be determined fairly fast, complex I remains the least understood complex of the complexes. Especially the question how complex I couples proton translocation to electron transfer is still elusive with many researchers trying to find the right answers. In this chapter, I will present the knowledge which we have so far on this complicated and fascinating protein complex.

Biological relevance

Mitochondria are the powerhouses of eukaryotic cells as they provide energy in form of ATP which is needed for many important cell processes, e.g. DNA replication, muscle contractions etc. However, mitochondria are not only energy providers; they also play key roles in many cellular processes, for example in apoptosis (cell death). As they are so important for life, malfunctioning can have devastating effects for the cell and for the entire organism. A possible reason for mitochondrial dysfunction is the formation of reactive oxygen species (ROS). In the literature mitochondria are described as the major source of reactive oxygen species (ROS) in the cell [10]. One particular type of ROS is produced in mitochondria; the oxygen superoxide anion (O_2^-). Of the respiratory chain enzymes, especially complex I plays a significant role in the formation of superoxide [11-13]. Superoxide formation leads to oxidative stress in cells and can

negatively affect all macromolecules (DNA, proteins, lipids) and can even lead to apoptosis. Therefore oxidative stress has been connected to many diseases including cancer, inflammation, Alzheimer's disease, diabetes and even to aging [14]. However, some ROS are important for cell function, when they are used as signal molecules (hydrogen peroxide).

But not only reactive oxygen species lead to diseases, also numerous point mutations in complex I could be identified. The best characterized diseases connected to complex I mutations are: Lebers hereditary optic neuropathy (LHON), Leigh's syndrome and MELAS (mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes) [13, 15].

In order to find treatments and prevent these mitochondrial diseases in the future, it is important to fully understand complex I function and structure in detail.

The structure of complex I

Complex I has been shown to be the most challenging membrane protein complex in history so far. From the discovery of complex I [16], to the elucidation of part of a structure [17], to a complete X-ray crystal structure [18], it took 51 years of extensive research and development (Fig 3).

Overall complex I has an L-shaped structure and can be divided into two domains, the hydrophilic and the hydrophobic (membrane) domain [17, 19]. The 14 core subunits are conserved and can be found in almost all organisms [20]. However, some organisms contain an even smaller complex I, with only 11 or 12 subunits. The mitochondrial complex is significantly larger (molecular mass ~ 1 MDa) than the bacterial counterpart (~550 kDa), having around 30 accessory subunits in addition to the core subunits [20, 21]. The function of those accessory subunits is still not clear, but they are possibly involved in regulation or stability of the complex. Due to its size and lack of prosthetic groups in the membrane arm, it took until 2013 and 2015 to determine the structure of the bacterial complex I and the mitochondrial complex by X-ray crystallography [18, 22]. As the nomenclature of complex I for different organisms is not standardized, I will proceed using the nomenclature of *Escherichia coli* (NADH-ubiquinone oxidoreductase, Nuo).

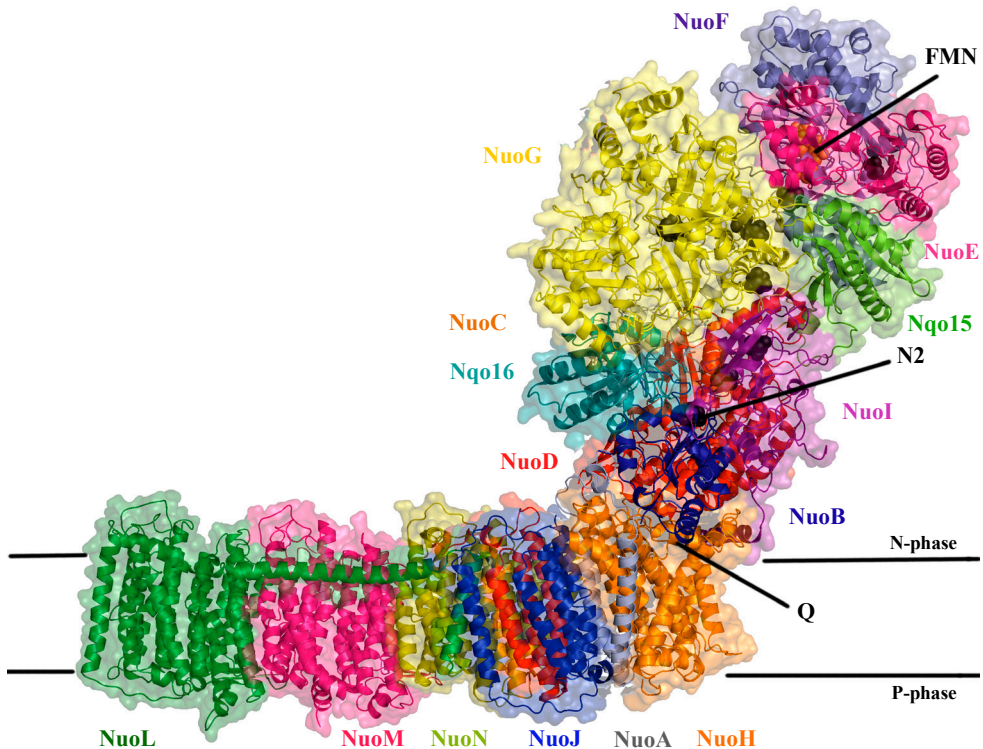


Figure 3 Crystal structure of the entire complex I from *Thermus thermophilus*, solved at 3.3 Å (PDB 4HEA). All 14 core subunits are visible, plus two extra subunits (Nqo15, Nqo16), which are specific for *T. thermophilus* and not found in *E. coli*. Besides the subunits, the FMN, N2 and quinone binding site are shown, by lines.

The hydrophilic domain

The first crystal structure of the hydrophilic (or peripheral) domain was determined in 2006 by Sazanov and Hinchliffe [23]. The hydrophilic domain constitutes half of the complex mass and contains seven subunits. This part of the complex is in charge of the electron transfer illustrated in Figure 4 from NADH via a stretch of Fe-S clusters to quinone, which passes the electrons to complex III. The electron donor NADH binds in the cavity close to the primary electron acceptor FMN (flavin mononucleotide) in subunit NuoF, which is then reduced to FMNH₂ [23-25]. In close by distance lies the Fe-S cluster N3, which receives the electrons from FMNH₂ in a step wise fashion [24]. NADH and FMNH₂ are two-electron carriers, but Fe-S clusters can only carry one electron at the time, therefore FMNH₂ can release electrons only individually. From Fe-S cluster N3 the electrons are transferred via Fe-S clusters N1b-N4-N5-N6a-N6b to N2. The total distance of the connecting redox chain from FMN to quinone by a stretch of seven Fe-S clusters is about 95 Å [26]. Depending on the species the hydrophilic domain

contains 8-9 Fe-S clusters situated in the subunits NuoB, E, F, G and I [23, 24, 27]. However, only seven are involved in the direct electron transfer. From the last Fe-S cluster N2 the electrons are transferred to quinone, which is reduced to QH₂. The quinone binding site is located in the subunits NuoD, B, A and H and is unusually large, being able to fit nearly an entire quinone molecule, instead of just the headgroup [18]. The quinone headgroup, guided by mainly hydrophilic residues, binds approximately 15 Å away from the surface of the membrane [18].

As mentioned only seven Fe-S clusters are involved in the direct electron transfer, but what is the purpose of the other two Fe-S clusters: Fe-S cluster N7 (only found in some bacterial species) and the fully conserved cluster N1a (Fig. 4). The cluster N7 is located far away from the rest of the other Fe-S clusters, hence electron transport via N7 is not possible [23]. This extra cluster is probably involved in stabilizing the fold of the protein [28]. Much has been speculated about the role of cluster N1a. This cluster is positioned close to the NADH-binding site and therefore close to FMN. Consequently it was speculated that the cluster N1a might be involved in the FMN or NADH chemistry. Hirst et al. 2013 investigated this hypothesis and could conclude that the cluster N1a is not reduced by NADH and therefore cannot act as storage for electrons [29]. They also proposed that cluster N1a does not affect the production of ROS, nor does it impede with the electron transfer [29]. Therefore the function of N1a still remains speculative.

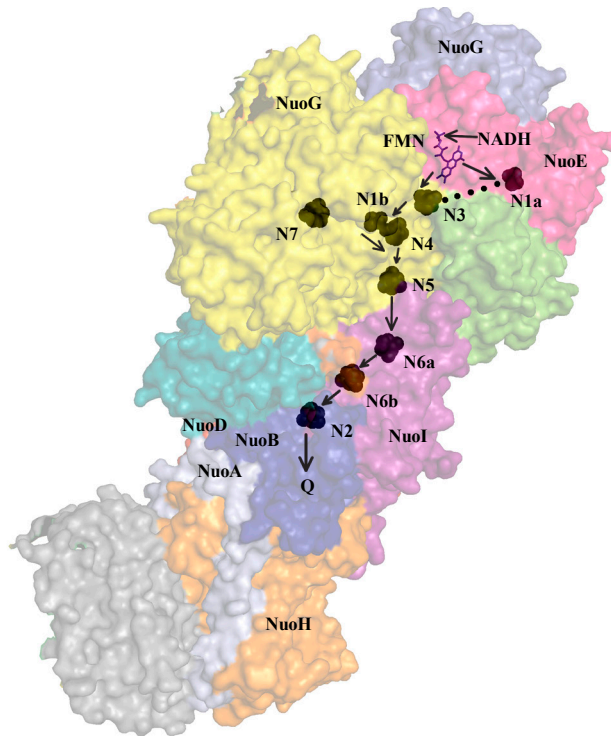


Figure 4 Electron pathway through the hydrophilic domain (PDB 4HEA). NADH donates electrons to FMN and then they are further transported via Fe-S clusters N3-N4-N5-N6a-N6b and N2 to the final electron acceptor quinone. The quinone binding site lies in the interface of the subunits NuoA/B/D and H.

The hydrophobic domain

The hydrophobic (membrane arm) consists of seven subunits: three large subunits (NuoL, M, and N), three small subunits (NuoA, J, K) and NuoH, which connects the hydrophilic arm to the hydrophobic. These seven proteins are encoded by mitochondrial DNA in eukaryotes. The first crystal structure of part of the membrane domain of *E. coli* was solved in 2010 to 3.9 Å, and just a year later it was improved to 3 Å [30, 31]. Both structures lacked the NuoH subunit and only in 2013 the entire structure of *T. thermophilus* complex I could be solved [18]. The hydrophobic domain of complex I is responsible for the H⁺ pumping, which contributes to the overall proton-motive force, which is in turn needed for ATP generation. In contrast to the well-established electron transfer mechanism of the hydrophilic arm, the proton translocation mechanism was for a long time a black box. Since the entire crystal structure of complex I became available, researchers are slowly discovering the pathways of proton translocation.

Already in 1992 the three largest subunits (NuoL, M and N) were considered prime candidates for proton translocation; as they are not only homologous to each other but also bear homology to Na^+/H^+ antiporters belonging to the Mrp-family [32, 33]. Hence, I will refer to them as antiporter-like subunits. Each of the antiporter-like subunits contains 14 transmembrane helices (TM), which are conserved. Interestingly each of the subunits harbors two helices at the same positions (TM7 and TM12), which are each discontinued through an extended loop. These types of ‘broken’ helices are implicated to be critical for the translocation of ions/protons in transporters [34]. TM1-3 and 14 are the least conserved helices and therefore are not considered part of the core helices (TM 4-13). The core helices are well conserved in sequence and structure and are organized with internal symmetry, meaning that TM4-8 share the same structure as TM9-13, only inverted. Each of the symmetry-related modules contains half a channel for proton translocation made up by conserved charged polar residues [18, 26]. Therefore the three antiporter-like subunits account for three proton channels.

One very peculiar feature, revealed in the crystal structure, is a long (110 Å) α -helix extension at the C-terminal of the distal NuoL subunit (HL). It is positioned along the cytoplasmic membrane surface until the interface of NuoN and the small subunits J, K, where it ends with an extra TM helix. The function of this long helix is still under debate, but it became evident through several studies that it is to be important for structural stability [35-37]. An involvement in energy transduction remains unclear [37, 38].

Looking back upon the agreed stoichiometry, which reports that four H^+ per NADH are pumped over the membrane, one channel is missing as only three antiporter-like subunits are present in the membrane arm. After the crystal structure of the entire complex I was solved, the missing fourth channel was identified to be situated in the small subunits NuoA, J, K and NuoH [18, 26].

The small subunits NuoK, NuoJ and NuoA contain in total 11 helices and form a bundle, connecting the antiporter-like subunits to NuoH. NuoA is involved in forming of the quinone binding cavity. Those three small subunits form together half a channel.

Subunit NuoH was the last subunit to be crystallized it was missing in the first structure in 2010. This subunit is unique to the family of complex I related proteins and is the most conserved in the hydrophobic domain [26]. Surprisingly as they share no sequence similarity, NuoH TM 2-6 showed the same symmetric fold as TM4-8 and TM9-13 of NuoL, M and N. These five helices also contain half a proton channel [18]. Furthermore, due to its position at the interface of the hydrophilic and hydrophobic domains, it is involved in quinone binding and is possibly important for the coupling mechanism [26].

Another characteristic which could be identified in the membrane part of complex I is a long continuous hydrophilic axis of charged and polar residues. This residue chain goes through the entire middle of the membrane domain, starting at the quinone binding site and ending at the tip of NuoL. Most of the residues are found close to the ‘broken’ helices and are either part of the half-channels or of the interfaces between two half-channels [18].

The mechanism

Having established the functions of the two different domains of complex I, one responsible for the electron transfer from NADH to quinone and the other for the proton translocating, one of the biggest mysteries in complex I research still remains elusive: How is the electron transfer coupled to proton translocation being so far apart from each other ($\geq 15 \text{ \AA}$), given that the maximum distance for electron transfer is 14 \AA . This question has been around for a long time, and we still do not have a definite answer for it. Here I will present some of coupling mechanisms that have been proposed.

After the first crystal structure of the membrane part was published, a “steam engine” mechanism was suggested. The electron transfer in the hydrophilic domain induces conformational changes in the small subunits in the membrane arm, which leads to a piston-like movement of the horizontal helix of NuoL. As the broken helices of the three antiporter-like subunits are connected to this helix, it will tilt them simultaneously and lead to proton pumping, similar to a steam engine [39]. After this proposal many research groups worked on the role of the horizontal helix and it was agreed that the helix is important for structural stability, but its putative role in proton pumping could not be confirmed [35-37]. Two recent studies by Steimle et al. [36] and Zhu et al. [37] proved through cysteine labeling and cross-linking that the long α -helix of NuoL does not undergo large conformational changes, nor did the complex I activity decrease. Thus complex I does not work like a steam engine.

The more likely explanation for the coupling is a series of small conformational changes in the antiporter subunits. When the electrons from N₂ are released to quinone, a conformational change in the fourth channel (NuoH, A, J, K) is triggered. Through interaction of certain charged residues the conformational changes are transferred to the antiporter-like subunits through the central axis of charged and polar residues, which runs through the entire membrane arm. This

leads to exposure to the solvent at both sides of the membrane and change in the chemistry of some key residues in the half channels, which promotes the proton translocation. But how these conformational changes look in detail, still remains unknown [26].

Mrp-antiporter and hydrogenases

The preceding chapter described the knowledge we have acquired so far about the structure and function of complex I. At several points it is mentioned that complex I shares homology to the Mrp-antiporter family. However, this is not the only protein family where homologous subunits could be identified. Another such protein family are the hydrogenases. In this chapter I will present these two different protein families.

Mrp-antiporter

The multiple resistance and pH-related antiporter, or Mrp-antiporter, belongs to the monovalent cation/proton antiporter-3 class (CPA-3) [40]. Mrp is a Na^+/H^+ antiporter, which uses the energy derived from the (electro)-chemical proton gradient (Δp) to power substrate transport, thus it belongs to the secondary active transporter group [41].

Mrp was discovered by Kudo [42] and Hamamoto [33], when they showed that the Mrp-antiporter was critical for cytoplasmic pH homeostasis in alkaliphilic *Bacillus* species. After its discovery more and more homologs of this protein could be identified in various bacteria and it became apparent that the occurrence of this antiporter was widely spread throughout the prokaryotic world.

This is also a reason why the Mrp-antiporter has so many different names, depending on the organism in which it was found. For example it is called Sha/Mrp in *Bacillus subtilis*, Pha in *Rhizobium meliloti*, Mnh *Staphylococcus aureus* and others [43-46]. Mrp does not only have many names, many cell processes are depending on its function. Malfunction or deletion of Mrp-antiporter was shown to result in errors in sporulation in *B. subtilis* [47], virulence [48], symbiotic nitrogen fixation [44] and photosynthesis [49]. Interestingly, in *B. subtilis* Mrp-transporter fulfills more than one function. Not only is it responsible for pH homeostasis [43, 50] and sodium resistance [43], but it also plays a role in sporulation [47] and cholate efflux [43, 51].

Initially, monovalent cation/proton antiporter was thought to be exclusively transcribed from single structural genes [50, 52, 53]. But after Hiramatsu et al.

discovered the Mrp-antiporter which is a multisubunit antiporter encoded on an operon this view had to be changed [45].

The Mrp-antiporter can be divided into two groups based on the structure of the operon. Group 1 has seven genes (*mrpABCDEFG*) and group 2 has six where the genes for *mrpA* and *mrpB* are fused together (*mrpA'CDEFG*) [41]. First, it was thought that all subunits are needed for function [45, 51, 54], but one *in vivo* study showed that MrpABCD was sufficient for Na⁺/H⁺ antiporter activity [55]. Consequently, the question if all subunits are needed for Na⁺/H⁺ antiporter activity is still under debate.

Hydrogenases

Next to the Mrp-antiporters, another family that contains proteins homologous to complex I subunits are hydrogenases (Hyd). Hydrogenases catalyze the reversible conversion of protons and electrons to H₂. They can be found in all three domains of life and the majority of bacterial and archaeal genomes encode one or more hydrogenases, thus they seem to play an important role in these microorganisms [56]. Based on the metal constitution of their catalytic centers, they can be subdivided into three evolutionary unrelated groups [56]: [NiFe]-hydrogenases [57], [FeFe] hydrogenases [58] and [Fe-only] hydrogenases [59]. As mainly the [NiFe]-hydrogenases share homology to complex I subunits, I will mostly focus on them.

[NiFe]-hydrogenases are widely spread taxonomically and can be found in around 27% of bacterial genomes [60]. All members of this group contain at least two core subunits, usually referred to as large and small. The large subunit harbors the catalytic [NiFe] center, and the small subunit has three conserved Fe-S clusters, which connect the catalytic center to the external electron carrier [61]. Depending on the function and phylogeny, [NiFe]-hydrogenases are further classified into one of four groups: membrane-bound H₂ uptake hydrogenases (group 1), uptake and sensory hydrogenases (group 2), NAD⁺-reducing hydrogenases (group 3) and energy-converting membrane associated hydrogenases (group 4) [56, 62]. This last group stands out from the others, as its members show little sequence similarity to the other three groups. Group-4 [NiFe]-hydrogenases are multimeric with at least 6 subunits [56]. In the following I will present representatives of Group-4 hydrogenases, which share homology to complex I.

The best characterized example of a group-4 hydrogenase is hydrogenase-3 (Hyd-3) of *E. coli*. Hyd-3 is encoded by the *hyc* operon (*hycABCDEFGH*) [63]. Together with formate dehydrogenase protein (FhdF) it forms a complex to

oxidize formate and produces CO₂ and H₂ [64]. This enzyme complex, called formate hydrogenylase-1 (FHL-1), is the key enzyme in hydrogen production in *E. coli* [65]. Six subunits share homology to subunits in complex I (HycBCDEFG) but only HycC belongs to the antiporter-like subunits.

Another representative of group-4 in *E. coli* is the hydrogenase-4 (Hyd-4), which also forms a complex with FhdF and is called FHL-2. This hydrogenase is larger than Hyd-3 and is encoded by the *hyf* (hydrogen four) operon (*hyfABCDEFGHIJ*) [66]. Three antiporter-like subunits could be assigned (Hyf B,C,D) and totally nine out of 10 subunits are homologous to complex I subunits.

The energy-converting-hydrogenase (Ech) is a six subunit hydrogenase (EchA-F), where four subunits are hydrophilic and two are membrane proteins [67, 68]. It was characterized in *Methanosarcina barkeri* and it uses ferredoxin as electron donor to reversibly produce H₂ [67]. All six subunits are homologous to complex I subunits.

Another hydrogenase belonging to the [NiFe]-hydrogenase is the CO-induced hydrogenase of *Rhodospirillum rubrum*, which is part of the CO-oxidizing system, which catalyses the oxidation of CO to CO₂ coupled to the production of H₂ to generate energy[69-71].

The evolution of complex I

As previously described complex I is a huge protein complex, with a core of 14 conserved subunits. Complex I can be divided into three modules, which can be isolated as subcomplexes [72]. The first is the NADH-oxidizing or N-module, which comprises the subunits NuoEFG and is the entry point of electrons from NADH. The Q-module (quinone) is the second module and is made up by the subunits NuoBCDI, containing the pathway for electrons to the quinone-binding site. The third module, the P-module (proton translocating) contains the membrane subunits (NuoLMNKAJH). From an evolutionary perspective, the structure of complex I might have evolved from independent pre-evolved modules (modular evolution hypothesis) with distinct functions [72-74]. In the previous chapter, I presented the two related protein families, Mrp-antiporters and [NiFe]-hydrogenases, in which we find homologs to various parts of complex I, and described their functions. In this chapter we will have a more detailed look on the relationship of these three different protein families and a theory on how they might have evolved.

The origin of the hydrophilic domain

The hydrophilic arm of complex I is formed by two functionally different modules, the N- and the Q-module, which share homology to two different protein families: soluble NAD⁺ reducing hydrogenases and membrane bound [NiFe]-hydrogenases. Table 1 shows a list of the homologous subunits in detail.

In some organisms we find complex I enzymes which are smaller than the ordinary 14 subunit complex I. Some of them lack the N-module (NuoE,F,G) completely (11-subunit) and some contain only NuoG (12-subunit) [75-77]. Therefore it is likely that the N-module was the last addition to complex I and occurred in a two-step fashion; first NuoG and then NuoEF. Two subunits of group-3 soluble NAD⁺ reducing hydrogenases resemble an almost complete N-module of complex I, found in some purple bacteria and cyanobacteria [78-81].

Table 1 Overview of the homologous proteins related to complex I

Complex I <i>E. coli</i>	Group-4 hydrogenases			Group-3 hydrogenases	Mrp- antiporter <i>B. subtilis</i>	Function
	FHL-1 <i>E. coli</i> (Hyc)	FHL-2 <i>E. coli</i> (Hyf)	Ech <i>M. barkerii</i>	NAD ⁺ -reducing hydrogenase <i>A. eutrophus</i>		
N-module						
NuoE				α-subunit (N- term)		Fe-S
NuoF				α-subunit (C-term)		NADH binding Fe-S
NuoG	FdhF (C- term)	FdhF (C- term)		γ-subunit (N-term)		Fe-S
Q-module						
NuoB	HycG	HyfI	EchC			Fe-S
NuoC	HycE (N- term)	HyfG (N- term)	EchD			
NuoD	HycE (C- term)	HyfG (C- term)	EchE			[NiFe] site
NuoI	HycF	HyfH	EchF			Fe-S
P-module						
NuoA						H ⁺
NuoH	HycD	HyfC	EchB			H ⁺
NuoJ					MrpA (C-term)	H ⁺
NuoK		HyfE			MrpC	H ⁺
NuoL	HycC	HyfB/D	EchA		MrpA	H ⁺
NuoM/N		HyfF			MrpD	H ⁺

In the β-purple bacterium *Alcaligenes eutrophus* the hydrogenase contains four subunits, which form two dimers (δ/β and α/γ) [78]. The genes are encoded in the *hox*-loci and are arranged in the same way as the corresponding complex I genes [73]. The α subunit resembles a fused NuoE and F, whereas the γ subunit is homologous to the N-terminal of NuoG. This dimer, which is responsible for NAD⁺ reduction, contains a non-covalently bound FMN as well as several Fe-S clusters [73]. Sequence comparison of the complex I subunits and the corresponding subunits in the NAD⁺-reducing hydrogenase and the derived evolutionary tree display that the distances between the species are similar. Therefore both protein families presumably have a common ancestor [73].

A second origin are the membrane bound [NiFe]-hydrogenases (group 4), in which we find homologs to the complex I subunits NuoB, C, D and I (Q-module). NuoD is structurally and sequence wise related to the C-terminal of the large subunits of FHL-1 and FHL-2 HycE and HyfG respectively. These subunits contain the [NiFe] binding site, which is partially conserved in complex I structure [20, 23]. NuoC also shares homology to HycE and HyfG, but only to their N-terminal regions. Consequently HycE and HyfG are both fusions of NuoC and NuoD, implying that these two complex I subunits have a common ancestor [82]. This is strengthened by the fact that in *E. coli* complex I NuoC and NuoD exist as a fusion protein [83].

NuoB on the other hand is homologous to the small subunits of FHL-1 and -2, HycG and HyfI [73]. Those subunits are involved in the electron transfer of [NiFe]-hydrogenases and contain one Fe-S cluster, which is similar to the Fe-S cluster in NuoB [23]. Last is NuoI, which is related to HycF (HyfH). HycF belongs to the ferredoxin family and the structure of NuoI shares similarities with the common structure of this protein family [23, 73].

NuoG (N-module) is not only similar to NAD⁺-reducing hydrogenases, but also to the formate dehydrogenase (FhdF), a representative of group 4 hydrogenases [23, 84]. Structural comparison showed that the C-terminal domain of NuoG and FhdF are structurally similar [83].

The origin of the hydrophobic domain

Already in the 90s it was discovered that the NuoL, M, N subunits from complex I are homologous to each other and to the large subunits (MrpA/D) of the Na⁺/H⁺ antiporter family Mrp [32, 33]. This led to the hypothesis that these antiporter-like subunits harbor the proton translocation machinery in complex I. Eventually the homology between MrpC and NuoK as well as NuoJ to the C-terminus of MrpA could be detected [85, 86].

Besides the homology to the Mrp-antiporter family, we can also find homologous proteins in the group-4 of [NiFe]-hydrogenases. As previously stated FHL-1 has one antiporter-like subunit, HycC which is related to NuoL, M and N. This is in contrast to FHL-2, where three antiporter-like subunits could be identified (HyfB, D and F).

NuoH and the corresponding subunits in FHL-1 and -2 (HycD, HyfF) share no detectable sequence homology to the three antiporter-like subunits in complex I and were often considered to be unique to complex I and hydrogenases. After the

entire structure of complex I was published it was seen that NuoH has the same fold as NuoL, M and N [18].

Neither FHL-1 nor FHL-2 have analogues of the small membrane subunits NuoA and J.

The evolutionary model of complex I

We know that complex I shares origins with hydrogenases and Mrp-antiporters. In the following section I will present a possible scenario to explain how the 14-subunit complex I and multisubunit hydrogenases might have evolved. A popular hypothesis is that a simple soluble [NiFe]-hydrogenase with two subunits (NuoB and NuoD/C homolog) merged with an antiporter protein (NuoL homolog) and NuoH, NuoC and NuoI homologs to form a complex. This membrane protein would then be the last common ancestor of complex I type proteins and [NiFe]-hydrogenases [81].

To get to a modern complex I with 14 subunits, several adjustments must have been made from the last common six subunit ancestor. As mentioned, in the complex I structure we still find remnants of the [NiFe] binding site (NuoD), which does not correspond to the position of the quinone binding site (surface of NuoB and NuoD) and therefore it is unlikely that the [NiFe] binding site turned into a quinone binding site over time [83]. One suggestion is that at one point in evolution this complex I ancestor was able to use both H₂ and ubiquinone as substrate [83], but this needs validation.

As we can find smaller complex I enzymes in nature, which either lack the N-module entirely or only have NuoG, it appears that the N-module was the latest addition to modern complex I. In 2011 Hägerhäll et al. conducted sequence alignment studies and they showed that 11-subunit complex I was widely distributed in the phylogenetic tree of life and that it was more closely related to complex I than to [NiFe]-hydrogenases [87]. Therefore they concluded that the last common ancestor of modern complex I like proteins is 11-subunit complex I [87].

But how and from where did the membrane arm appear? Focusing on the antiporter-like subunits NuoL, M and N there are now two proposed scenarios. The first one by Friedrich et al. 2000, suggests that the last common ancestor would have recruited only one antiporter protein [81]. This antiporter protein was then duplicated once or twice in the course of evolution and therefore the antiporter-like subunits are homologous to each other and to the two big subunits of the Mrp-antiporter.

The second proposal by, Mathiesen et al. 2003, suggested a slightly different way [85]. Instead of only one antiporter protein, a whole antiporter module was recruited to the last ancestor, as visualized in Figure 5. Through a sequence comparison of the antiporter-like subunits of complex I and the homologous subunits in Mrp it was shown that MrpA and NuoL group together whereas MrpD, NuoN and NuoM built another group [88]. Furthermore, not only the large antiporter-like subunits of complex I are homologous to Mrp subunits, but also NuoK to MrpC [85, 88]. These theoretical predictions strengthen the idea of a whole antiporter module (NuoL, N and K) being recruited to the ancient complex I progenitor. The same group tested their theoretical predictions *in vivo* studies, using a *B. subtilis* complementation experiment [55].

A *B. subtilis* wild type strain was genetically modified, so that either lacked the gene for *mrpA* or *mrpD*. Those two different deletion strains showed a salt sensitive phenotype, meaning that there was a growth deficiency in a high salt medium. This phenotype could be reverted when the proteins for MrpA or MrpD were expressed *in trans* from a low copy number plasmid. When exchanging the two proteins in the strains, meaning that MrpD would be expressed in the *mrpA* deficient strain and *vice versa*, the growth could not be restored, suggesting functional differentiation between the two Mrp proteins.

In a second experiment the different antiporter-like subunits (NuoL, M, N) were separately expressed in the two different strains to test if any of them could revert the growth effect. Interestingly some of the complex I subunits were able to restore the growth of the two strains under some conditions (pH 7.4, 80 mM NaCl). Furthermore the same differentiation as noticed in MrpA and MrpD could be seen for the antiporter-like subunits. NuoL was able to rescue the growth of the $\Delta mrpA$ strain, but not the *mrpD*, whereas NuoN could restore the growth of the $\Delta mrpD$ strain, but not of the $\Delta mrpA$. However, NuoM was not so easy to differentiate.

This type of arrangement was already seen in the theoretical predictions: NuoL and MrpA, NuoN and MrpD. All in all the results of the theoretical and practical experiments suggest that MrpA and MrpD are functionally different and that this difference can be seen in the corresponding complex I subunits [55, 88].

Coming back to the suggested scenario of the roots of the membrane arm of complex I, these results favor the idea that a whole multi-subunit antiporter module (NuoL, N, K) was recruited to the progenitor of modern complex I [89]. As the role of NuoM is not distinct, it is possible that NuoM was part of the antiporter module or that NuoM arose of a gene duplication of *nuoN*, as the sequence alignment groups NuoM and NuoN closer together than NuoM and NuoL.

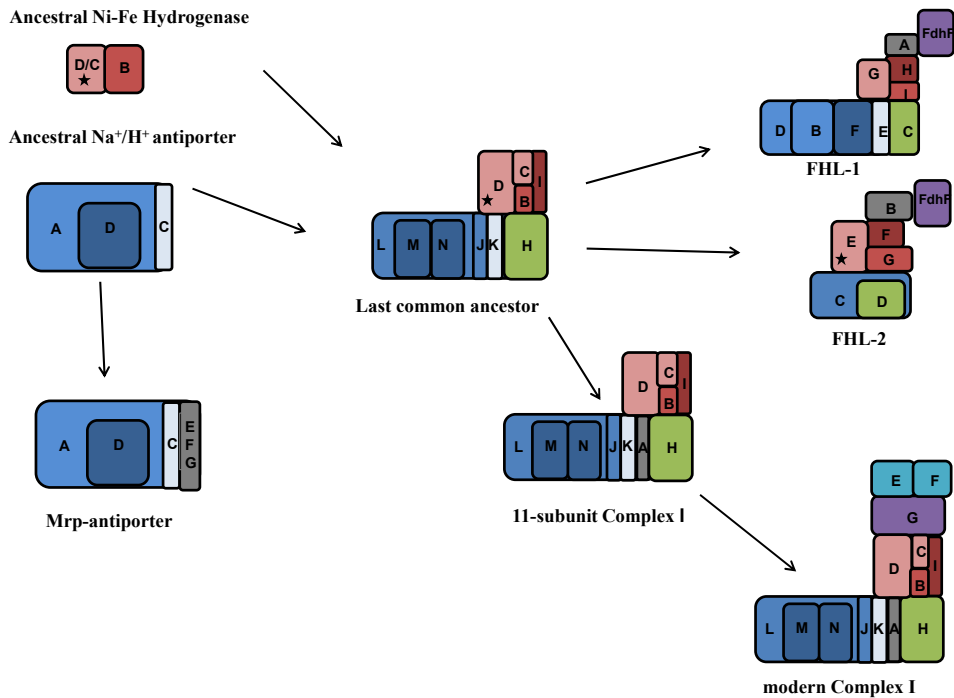


Figure 5 Hypothetical scheme of the evolution of modern complex I, Mrp-antiporter and membrane bound hydrogenases. The different protein complexes are color coded, to depict homology. The [NiFe] binding site is marked with a star. An ancestral [NiFe]-hydrogenase and an ancestral Na⁺/H⁺ antiporter, together with progenators of NuoH and I formed, the last common ancestor of a membrane bound [NiFe] hydrogenase. From this ancestral protein complex the modern day simpler membrane bound hydrogenases emerged, as well as the complex I protein family. The modern Mrp-antiporter might have evolved from the ancestral Na⁺/H⁺ antiporter, by addition of the subunits MrpE, F and G to the complex.

Aim of the work on complex I

The existence of complex I in many organisms has been known for a long time, but we still have more questions than answers on the working mechanism. Even though structural information has become available, we still struggle with the details, especially on the proton translocation pathways and the coupling between electron transfer and proton pumping. As it is evident by now, complex I has evolved through different building blocks, which came together at one point in evolution. Many homologous proteins from different protein families (antiporters and hydrogenases) have been identified and they seem to share an evolutionary connection.

The first part of my thesis focuses on the relationship of transporter subunits of different hydrogenases and Mrp-antiporter to the respective antiporter-like subunits of bacterial complex I. Deeper insight on the evolutionary history and functions of these subunits will hopefully help us understand modern day complex I and elucidate its mechanism.

In the first paper we compared the ion specificity of transporter subunits of different membrane bound [NiFe]-hydrogenases, the antiporter-like subunits in 11-subunit complex I, and the two big subunits of Mrp-antiporter. Theoretical predictions based on the phylogenetic tree of all the subunits were tested practically with two methods: a *B. subtilis* complementation assay and measuring internal Na⁺ concentrations by ²³Na-NMR.

In paper II our focus was entirely on the relationship between NuoL and the homologous subunit of the Mrp-antiporter, MrpA. As previously shown there seems to be a difference in ion specificity between MrpA (NuoL) and MrpD (NuoN, M) and in this work we wanted to explore and determine the structural origin for this difference[88]. For that purpose we constructed several mutants and tested their effect on Na⁺ transport.

Paper I: complex I and evolutionary related proteins

The first enzyme of the respiratory chain complex I is composed of three building blocks, the N-module (electron input), the Q-module (electron transfer to quinone) and the P-module (proton pumping). This structural feature agrees with the modular evolution hypothesis, which implies that complex I has evolved from separate building blocks, which linked together over the course of evolution to form the modern standard 14-subunit complex I. In the previous part I presented protein families which share homology to complex I: the Mrp-antiporter family, group-4 membrane-bound [NiFe]-hydrogenases and group-3 NAD⁺-reducing hydrogenases.

Although we describe the 14-subunit complex I as the minimal functional unit, this is not the entire truth, as we can find complex I-like proteins with fewer subunits in nature. One example is the 11-subunit complex I, which can be found in phylogenetically distant organisms [87]. It was first discovered in chloroplasts and cyanobacteria [75, 76], where it is involved in cyclic electron flow associated with photosystem I, chlororespiration and CO₂ acquisition [90, 91]. Interestingly this form of complex I lacks the entire N-module and so far no replacement for this module has been found, neither in plants nor in cyanobacteria [87, 90-93]. As the 11-subunit complex I is more closely related to modern complex I than to [NiFe]-hydrogenases, it was suggested that this complex I type is the last common ancestor of all present day complex I proteins [87].

As described in the previous chapter we can find many proteins in nature which are similar to complex I subunits, hinting at a common evolutionary relationship. In order to test the theoretical predictions regarding functionality we investigated experimentally if the subunits from [NiFe]-hydrogenases, a Mrp-antiporter, a 11-subunit complex I and modern complex I retained a functional relationship.

Update on the phylogenetic tree

In 2002 our group presented a phylogenetic tree which showed that MrpA and MrpD form two different clusters and that NuoL belongs to the MrpA cluster and NuoN/M belong to the MrpD cluster [88]. Due to lack of sequence data, it was not possible to group the antiporter-like subunits of the [NiFe] hydrogenase group-4 properly. Twelve years later with more sequence data available we present a revised phylogenetic tree (Paper 1, Fig 2), including the antiporter-like subunits from complex I (NuoL,M,N), Mrp-antiporter (MrpA,D) Hydrogenase-3 (HycC), Hydrogenase-4 (HyfB,D,F), Ech hydrogenase (EchA), and CO-induced hydrogenase (COOM1),(COOM2). The complex I subunit sequences from different organisms (including the 11-subunit complex I) clustered and two distinct domains could be observed, MrpA-like and MrpD-like proteins. Furthermore the hydrogenase subunits could be grouped.

Measuring intracellular sodium concentration by NMR

A new method had been established in our lab to test ion translocation *in vivo* [55]. Employing this method we showed that NuoL could rescue the growth of a $\Delta mrpA$ strain in high salt but not a $\Delta mrpD$ strain, whereas NuoN could save the $mrpD$ strain, but not the $\Delta mrpA$. To prove that the growth defects truly arise from the accumulation of Na^+ , we used a newly established method in our lab, measuring internal sodium in intact cells by ^{23}Na -NMR [94]. (details are in the method section of paper I and II). In brief, the deletion strains expressing one of the antiporter or antiporter-like subunits are challenged with 80 mM Na^+ for two hours. After that the internal Na^+ concentration is measured using a shift agent thulium(III)1,4,7,10-tetraazocyclododecane1,4,7,10-tetrakis (methylenephosphonate) ($\text{Tm}(\text{DOTP})^{5-}$) and ^{23}Na -NMR. Indeed the deletion strains had an elevated internal Na^+ concentration, but when complemented with the corresponding subunit, the internal Na^+ concentration decreased dramatically. As previously described MrpA could not rescue the growth of a $\Delta mrpD$ strain and vice versa. Given these points both antiporter subunits (MrpA and MrpD) seem to be necessary for Na^+ antiport.

Complementation studies

After assigning the different hydrogenase subunits in the phylogenetic tree and showing that the 11-complex I subunits group together with the corresponding complex I subunits, we tested if we could see any functional differentiation *in vivo*. Furthermore, we tested the behavior of the hydrogenase subunits (the details of the method can be found in (Method & Materials) in Paper I and II).

11-subunit complex I

As previously described 11-subunit complex I is considered a *bona fide* complex I enzyme. After the updated phylogenetic tree showed that the antiporter-like subunits from this compact complex I enzyme grouped in the same way as NuoL, NuoM and NuoN from 14-subunit complex I, we tested if they would also behave similarly in the complementation studies. As there is no 11-subunit complex I in *B. subtilis*, we used the genes from the close relative *Bacillus cereus* (*Bc*) in these experiments. The growth studies showed that NuoL_{Bc} was able to restore the growth at pH 7.5 of the salt sensitive $\Delta mrpA$ strain in a similar fashion as the previous studies on NuoL in *E. coli*., whereas NuoN_{Bc} and NuoM_{Bc} could rescue the $\Delta mrpD$ strain instead (Fig. 3, paper I). These results agreed with the theoretical predictions, supporting that 11-subunit complex I belongs to the complex I family and is likely an ancestor of the modern complex I

Hydrogenase-3 and hydrogenase-4 transporter subunits

We can find complex I proteins homologous to complex I subunits among membrane bound [NiFe]-hydrogenases. As they possibly share a common evolutionary ancestor, we wanted to investigate if these protein subunits still share functional similarities. Despite the fact that these hydrogenases did not follow the cluster observed for MrpA and MrpD-like proteins in the phylogenetic tree (they did not group consistently), we wanted to test their functionality *in vivo*. For that we cloned the genes encoding transporter subunit HycC from hydrogenase-3 and the transporter subunits HyfB, HyfD and HyfF from hydrogenase-4, all from *E. coli*. All of the four transporter subunits were able to restore both of the growth deficient $\Delta mrpA$ and $\Delta mrpD$ in similar fashion, suggesting no functional differentiation (Fig. 4 paper I).

The results of the complementation studies mirror the difficulties we had when we tried to group the different sequences of the hydrogenase subunits to MrpA-like or MrpD-like clusters.

Conclusions

In this paper we could strengthen several hypotheses which were published by our group previously. Moparathi et al. 2011 described an 11-subunit complex I, which was found in archaea and in eubacteria, and therefor assigned it as a *bona fide* complex I enzyme [87]. In this paper we examined if the NuoL, M and NuoN subunits from 11-subunit complex I would behave similarly to their counterparts from complex I. We showed that they indeed behaved in the same manner, supporting the previous conclusions that 11-subunit complex I is likely to be a true complex I enzyme, although missing the electron input module. Furthermore, we verified our previous observations regarding the assignment of NuoL/MrpA is a Na⁺ channel and MrpD/NuoN is the H⁺ channel, as shown by ²³Na-NMR experiments. We also demonstrated experimentally that the antiporter-like subunits from hydrogenase-3 and hydrogenase-4 did not show ion specificity, as they could equally substitute for MrpA and MrpD. This lack of specificity agrees well with the conducted theoretical predictions, that the transporter proteins of the two [NiFe]-hydrogenases are not easily divided into MrpA-like and MrpD-like proteins. It also accords with the observation that modern membrane bound [NiFe]-hydrogenases lack primary structural features found in the Mrp-antiporter and in the complex I antiporter-like subunits. Therefor the experimental data support the evolutionary theory that the last common ancestor was different in many ways from the modern [NiFe]-hydrogenases.

Paper II the relationship between MrpA and NuoL

One conclusion from the paper by Mathiesen et al. 2002 was that MrpA and MrpD form two different clusters in the phylogenetic analysis NuoL belongs to the MrpA cluster and NuoM, N to the MrpD cluster [88]. This was strengthened through a more detailed phylogenetic tree in Paper I. This hypothesis was put to the test when the antiporter-like subunits were expressed in a *mrpA* (or *mrpD*) deletion strain and grown in elevated salt concentrations. Both deletion strains exhibited salt sensitive phenotypes, but when NuoL was expressed in the *mrpA* deletion strain, the cells could recover. The same result was seen for NuoN/M expressed in the *mrpD* deletion strain. Therefore we confirmed that MrpA and MrpD have different functions, one being responsible for H⁺ and the other one for Na⁺ transport. To further test this hypothesis and to characterize what distinguishes MrpA (NuoL) and MrpD (NuoM,N), we focused on two of the proteins: MrpA and NuoL in paper II. We performed a mutagenesis study on both proteins and studied the effects of the mutations using complementation studies and ²³Na-NMR.

Sequence alignment

As we wanted to focus entirely on NuoL and MrpA we conducted a sequence alignment between *E. coli* NuoL,M,N and *B. subtilis* MrpA,D and looked specifically for amino acids only conserved in NuoL and MrpA but not in the other polypeptides (MrpD, NuoM and NuoN). One of the positions we identified was stretch of 4 amino acids in helix VIII. In close proximity to helix VIII lies helix VII, which is one of the broken helices in the antiporter-like subunits of complex I [18]. These discontinuous helices are often important for ion-translocation, as they are a source of flexibility and charge in the structures [34, 95]. Therefore we decided to mutate the four residues in helix VIII and study their effect on sodium extrusion. The changes made are: NuoL H254A, T257A, M258I, V259L and MrpA H221A, T224A, M225I, V226L (Fig. 2, Paper II). Moreover, we mutated two more positions, which are highly conserved in all proteins of

interest. These two positions (NuoL: E144Q, K229A and MrpA: E113Q, and K196A) have been previously reported to affect complex I activity negatively.

B. subtilis complementation studies

First we tested the different mutants in the complementation studies, using the $\Delta mrpA$ strain. As previously described the different *B. subtilis* strains were plated on low salt media. After 8 hours the cells were transferred to liquid media containing 80 mM NaCl and grown at 37°C, 200 rpm. The OD₆₀₀ was measured every hour to follow the growth. For more details see Method & Materials in Paper I and II.

All mutants of MrpA and NuoL were able to grow in media containing 80 mM NaCl, but the main differences were observed in the lag-phase. The NuoL mutants M258I and V259L showed a significant extended lag phase (3-4 hours), whereas the other mutations grew almost like wild type NuoL. In MrpA the growth differences were not so distinct in 80 mM NaCl, so we increased the salt concentration to 200 mM NaCl. Both M225I and H221A showed a prolonged lag phase compared to wild type MrpA.

²³Na-NMR

To further investigate the phenotypes seen in the complementation studies and to test if the reason for the slowed growth was accumulation of internal Na⁺, we studied the mutants by ²³Na-NMR. Contrary to the complementation studies, the cells were not grown in high sodium for a long time. The cells were first grown in liquid media without NaCl and then challenged with high NaCl (80 mM) for 1 hour. Using a shift reagent Tm(DOTP), we measured internal Na⁺ by NMR. The mutations which showed a prolonged lag-phase in the complementation studies, showed higher intracellular Na⁺ in comparison to the wildtype NuoL and MrpA respectively.

Conclusions

In previous work we showed that MrpD and MrpA although homologous, show differences in ion specificity. Their homologous counterparts in complex I can be grouped to either MrpA-like or MrpD-like. In this study we focused entirely on MrpA and NuoL to characterize these two proteins further. We mutated a stretch of amino acids which have a promising location within the proteins and are only conserved in NuoL and MrpA but not in any other polypeptide. One of the mutations (NuoL: M258I, MrpA: M225I) showed a severe effect on growth and sodium extrusion in both proteins, hence we concluded that this particular amino acid is important for function in both MrpA and NuoL. Several mutations did not have any visible effects on growth or internal Na⁺ concentrations and some had a phenotype in one of the proteins but not in the other (e.g. V259L).

The theoretical predictions suggest that NuoL and MrpA are very similar to each other, however, all things considered we conclude that NuoL and MrpA differ on the functional level. None of the mutations showed a severe phenotype, concluding that they are not involved in ion specificity.

The two methods we used (²³Na-NMR and *B. subtilis* complementation studies) are suitable tools to describe sodium sensitive phenotypes *in vivo*.

Future outcome

It is remarkable how our knowledge about complex I has advanced since the first structural information became available. But even with the availability of structures, many questions remain unanswered. One of focuses of the initial research group was the evolution of complex I. If we better understand about the separate building blocks of complex I, we will be able to gain a better understanding of modern complex I. It will also advance the information on not well characterized proteins, the [NiFe]-hydrogenase family and the Mrp-antiporter family.

One part of my project was to examine the relationship between MrpA and NuoL. We tested different mutants on their ability to extrude Na^+ using complementation studies and $^{23}\text{Na}^+$ -NMR. All mutations were analyzed in the Δmrp background in *B. subtilis*. It would be interesting to see how the mutations in NuoL affect the proton translocation capability in complex I background, especially the methionine mutation (NuoL: M258I, MrpA: M225I), which had a drastic effect in both MrpA and NuoL. Furthermore, some of the proteins with different mutations, which showed a phenotype, can be purified to characterize their function further. As mentioned in the introduction, helix VIII was not the only location where we could find residues which were conserved only in MrpA and NuoL. One can mutate these residues as well, in order to find the region which is important for ion specificity.

Furthermore obtaining structural information of the Mrp-antiporter would benefit not only the complex I field, but also the field of Na^+/H^+ -antiporter.

The second part of my project was to examine and compare antiporter-like subunits from evolutionarily related proteins. We tested subunits from hydrogenase-3, hydrogenase-4 and 11-subunit complex I. A next step would be to study the subunits from other hydrogenases, like the Ech and CO-induced hydrogenases to complete the picture.

Introduction to pain

Fire is something which has fascinated humans for a long time and especially children are magically attracted to the burning candle. As a child we dared each other who can put the hand closer to the flame and we all remember the burning pain when we got too close to it. Next time we saw a fire we remembered the pain and were more cautious. Pain taught us a lesson. But what is pain and why do we need it? Nearly everyone in the world has experienced pain in their life and knows the unpleasant feeling of it. One could imagine that the world would be a much better place if we would not feel the sharp pain, when hitting the bed frame with our toe or when suffering from a bad headache. Especially as we treat pain with medication to escape it. But pain is crucial for survival; it is a way how our body protects itself from harm. For example, after a foot injury we feel pain, so we stop, and rest to let our foot heal. Without the pain we would have continued using the foot and it might not have healed properly.

To revisit the example of the candle, pain also changes behavior towards potentially dangerous situations, if we have encountered them before. The child is more careful with the candle, after a previous burn. Generally speaking pain is a very complicated and important protective mechanism of our body. But how does our body know that we are in pain? For that we need to have a closer look on the molecular mechanism of pain sensation. The somatosensory system senses differences to the surface of the body and to internal organs. The sensory nerves responsible for somatosensation can be divided into four different groups: mechanosensation (touch), proprioception (mechanical displacement of muscles and joints), thermosensation (heat and cold) and nociception (pain). Nociception is the ability to recognize noxious (harmful) chemicals, temperature and mechanical stimuli [96, 97]. The responsible primary sensory neurons are called nociceptors and are often polymodal, meaning that they can recognize more than one of the stimuli. There are three different pain stimuli: The first pain, which is a rapidly transmitting signal and the pain feels like a sting. Then there is the second pain (burning), which is signaled much slower and is harder to locate. The last pain signal is the deep pain, which comes often from joints, muscles or inner organs and is often chronic. The cell bodies of nociceptors are clustered in the dorsal root ganglia (DRG) or the trigeminal ganglia (TG), which are located in the body or the

head respectively. Moreover there are two different kinds of axons, which transfer the nociceptive signal: A δ -fibers and C-fibers.

So how are the environmental stimuli (chemical, temperature or mechanical) converted into an electrochemical signal? Those specialized nociceptors contain special ion channels. These ion channels are activated by one or several of these stimuli and cations, mainly Ca²⁺ enter the cell. This inward flow of positive currents, changes the membrane potential and the membrane depolarizes and an action potential is formed. This leads to the opening of different voltage-gated ion channels, which propagate the electrochemical signal to the spinal cord and then to the brain. The brain then interprets the signal as pain. An overview of the pain transmission pathway is shown in Figure 6. One of these specialized ion channels is called TRPA1 and is part of a larger ion channel family the (TRP-superfamily), which play a role in pain transduction pathways. TRPA1 is the subject of the second part of my thesis.

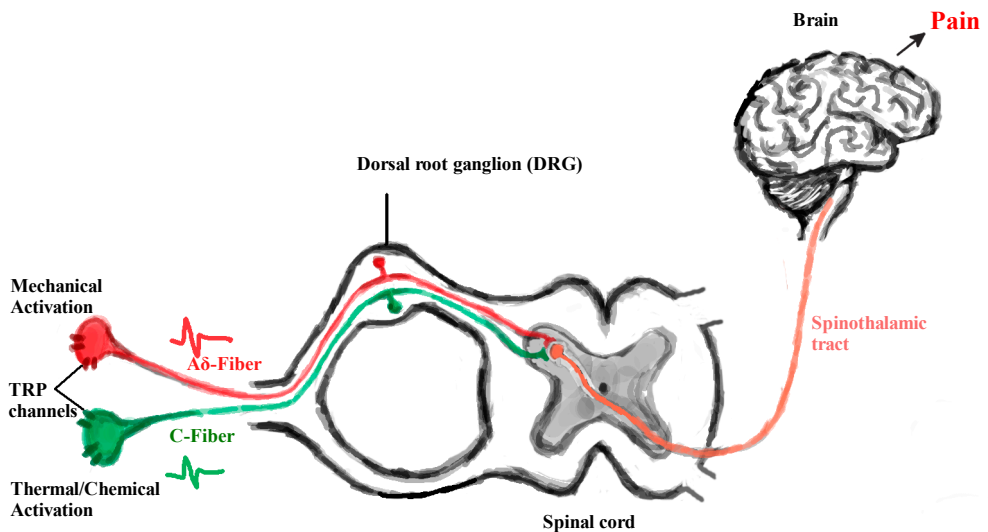


Figure 6 Schematic diagram of the pain transmission pathway. Ion channels (e.g. TRP-channels) are activated by noxious stimuli (mechanical, temperature or chemical) which leads to the propagation of an action potential along the fibers (C- and A δ -Fiber) up to the Dorsal root ganglion to the spinal cord. The spinal cord transmits the signal to the brain via the spinothalamic tract and the brain interprets the signal as pain.

The TRP-superfamily

The story of the TRP family starts in 1969, with the discovery of a *Drosophila melanogaster* mutant. Cosens and Manning worked on the identification of genes which are involved in signal transduction [98]. They tested the response of *Drosophila* flies to light and recorded electroretinograms (ERG), to see the potential change from extracellular current flow in the eye, when exposed to light. One mutant behaved as if blind under low light exposure, whereas the wild type would adapt to the light signals. Furthermore the ERG showed a different phenotype in comparison to the wild type. Instead of a steady response, the mutant displayed a transient response to bright light. For this reason the gene was called: *transient receptor potential, trp* [99].

From that time point on the *trp* mutation was analyzed to a great deal [100]. However, it took twenty years after the discovery of the *trp* gene locus, to clone and characterize the first TRP protein [101]. Already by then it was proposed that TRP is an integral membrane protein found in the plasma membrane, which shares structural features with known receptor/transport proteins [101]. This was later confirmed when Hardie and Minke demonstrated that TRP is a calcium channel, which is activated by light [102]. Phillips et al. came to the same conclusion, while studying a homologous TRP protein, called TRP1 (*trp*-like), and comparing it with TRP [103]. From then on homologous *Drosophila* TRP proteins could be identified in mammals [104], other insects and even fungi. Interestingly no TRP homologs could be found in land plants, although present in chlorophyte algae [105-107]. This could be an indication that the *trp* gene was lost during the evolution of land plants [108].

Since the discovery of TRP in *Drosophila* many more TRPs could be identified in various animals. These were divided into 7 subgroups: TRPC (canonical or classic), TRPV (vanilloid), TRPM (melastatin-like), TRPA (ankyrin-rich), TRPML (mucolipin), TRPP (polycystin) and TRPN [109-111]. Additionally another group of TRPs can be found in fungi, TRPY, which is only distantly related to the other groups [112, 113]. Mammal TRPs do not include TRPN and therefore can only be divided into six subgroups [114]. Until now 28 mammalian (27 human) TRP homologs have been identified [115]. Characterization of the different TRP members showed that TRPs are cation channels, which show a great diversity in ion selection, modes of action and physiological function [109].

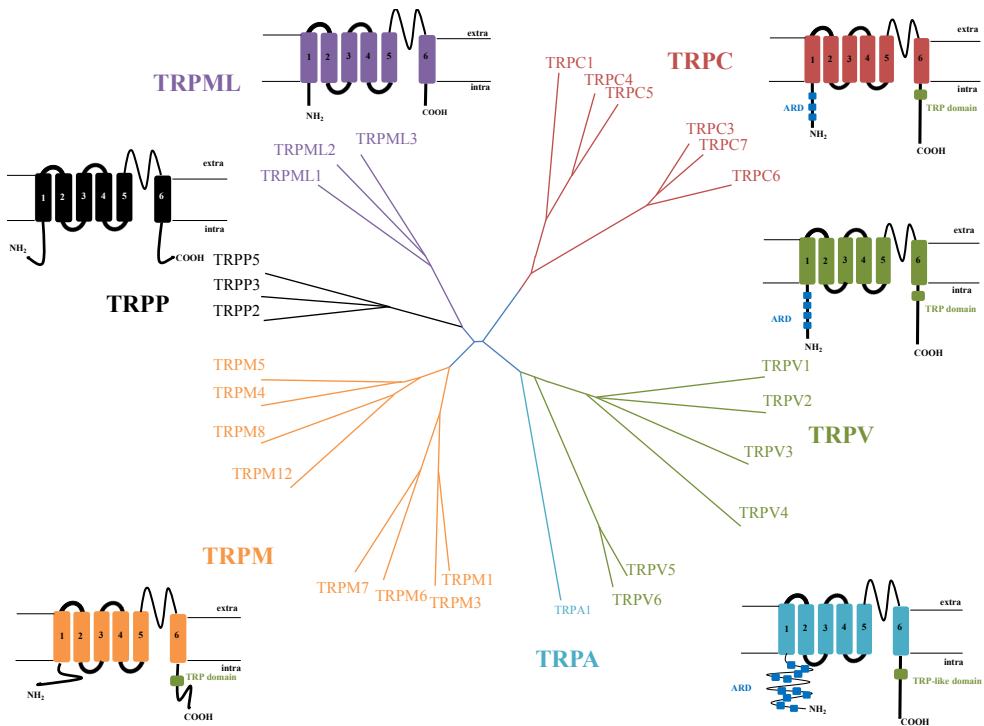


Figure 7 Phylogenetic tree of the human TRP channel superfamily (adapted from Nilius et al. 2007 [116]) including the basic topology. TRPML stands for mucolipin, TRPC for canonical or classic, TRPV for vanilloid, TRPA for ankyrin repeat, TRPM for for melastatin-like and TRPP for polycystin.

Structure & localization

Identification of the different TRP channels has given us some insights into their diverse function, but one of the biggest obstacles in the field is gaining structural information. It was suggested that TRP channels have a similar structure as voltage gated K⁺ or Na⁺ channels. Up till now there is only one X-ray crystal structure available: TRPV6 [117]. TRPV6 is one of the channels which has presumably a less diverse activation pattern than the other channels, as it is not activated by temperature nor by tastants or odors. With new improved technology in the field of single-particle electron cryomicroscopy, the first near-atomic resolution of a TRP channel (ratTRPV1) was determined in 2013 [118]. After this break-through more structural information became available, solving the structures for human TRPA1, rabbit TRPV2 and human TRPP2 [119-121]. Nevertheless, we

are still in the beginnings of the structural era of TRP channels and we need more information to deduct mechanistic insights.

Generally TRP channels comprise of six transmembrane helices, denoted S1-S6 [122, 123] and form mostly homotetramers [103, 122, 123]. The ion conducting pore loop is situated between the S5 and S6 helix. The N- and C-termini are intracellular and differ widely in size and function between the different TRP channels. The N-terminus contains an ankyrin repeat domain (ARD) (except TRPM, TRPML and TRPP), with different numbers of repeats depending on the channel [103, 123]. The ankyrin repeat is one of the most common protein motif found in bacterial, archaeal and eukaryotic proteins [124]. The structure contains two α -helices which are connected by a β -turn and are often arranged in tandem to form a long ankyrin repeat domain (ARD). ARDs are linked to protein recognition and protein-protein interactions [125]. So far the structure of the ARDs of rat and human TRPV2, rat TRPV1, mouse TRPV6 and human TRPA1 have been solved [119, 126-129]. The role of the ARD in TRP channels is still not fully determined; however, an involvement in channel modulation seems likely. The C-terminus can comprise a coiled coil domain and in some cases holds an enzyme activity, for example a 2-kinase domain in TRPM6 and TRPM7 [130, 131]. Another common feature in some of the TRPs (TRPC, TRPM, TRPV and TRPN) is the so called TRP domain or helix. This is a region of 23-25 amino acid residues conserved in TRPC, TRPM, TRPV and TRPN. The TRP domain contains a highly conserved stretch of amino acids denoted box I(EWK FAR) and a proline rich motif called box II [115, 132]. The TRP helix follows directly after the S6 domain and is proposedly involved in tetramerisation and/or allosteric regulation of the channel [115, 133, 134].

TRP channels are expressed in nearly all cell types and they are located in all cellular membranes (except in the nuclear envelope and mitochondria) [135].

Biological Importance

TRP channels fulfill very diverse tasks in the cells; they can detect external or endogenous stimuli, maintain different types of homeostasis and even support the regulation of crucial cell functions. This diversity has unfortunately a catch, making TRP channels prime candidates for channelopathies. Channelopathies is the umbrella term for diseases which are caused by non-functional channels. These

damages can arise from mutations and can lead to devastating diseases. For example mutations in TRPP can lead to polycystic kidney disease [136]. Furthermore, some of the TRPs are connected to chronic inflammation, cardiovascular diseases and even cancer [136, 137]. Recently a connection between migraines and TRP channels has been proposed, as many known migraine triggers are TRPA1 channel activators [138]. This might be the final puzzle piece to reveal the complicated mechanism of migraine. Being associated to so many health issues and diseases, TRP channels are a new and important target for drugs, particularly for the development of analgesics to treat/manage chronic pain [139]. In order to do so it is crucial to understand the structure and mechanisms of the different TRP channels.

TRPs and temperature sensing

For every organism (from bacteria to humans) it is crucial for survival to detect changes in the environment and react to them in an appropriate manner. One of the environmental cues can be the change of temperature inside or outside of the organism. When we go out for a long walk in the middle of winter, our body senses the change of temperature and urges us to go back to the warmth of our homes. Another example would be the mosquito that flies in the darkness and records our body temperature to know where the prey is located. How are we or the mosquito able to detect temperature? Mammals possess specialized neurons in the peripheral nervous system which can detect temperature changes. But how does it work at a molecular level? At the end of these specialized neurons one can find ion channel receptors and so far all confirmed temperature activated channel receptors belong to the TRP family. These special TRP channels are also called thermoTRPs. The first cloned and identified member was TRPV1[140]. TRPV1 belongs to the vanilloid TRP channel family and is activated by many pungent compounds, like capsaicin from chili, which leads to a burning sensation. Furthermore, TRPV1 can be activated by noxious heat starting from 43 °C [140]. The nociception was also confirmed by mice knock out phenotypes as well as by the observation that the channel is expressed in relevant tissue. This channel seems to be particularly important for noxious heat detection during inflammation [141-143]. TRPM8 on the other hand was found to be a cold activated channel (26 °C), also activated by menthol and spearmint which translates to a cool sensation [144-146]. Moreover, the channel is expressed in sensory nerve endings [147]. TRPM8 is described as a channel for innocuous cold detection (coldness), as the knock-out mouse model showed only a temperature deficiency in the cool range, but not to

noxious (painful) cold [146, 148, 149]. Another member of the TRPM family is also confirmed to be a temperature activated channel, TRPM3. Interestingly opposite to TRPM8, TRPM3 is activated by noxious heat and plays a role in inflammatory sensitivity to pain [150]. The only member of the TRPA family in mammals, TRPA1 is also a thermosensor and is described in more detail in the next chapter.

TRPs in chemical sensing

Another important survival mechanism is to recognize noxious chemicals, from environmental irritants, plants, food or cosmetics. The process of sensing chemical stimuli is called chemosensation. In 1997 an archetypal TRPV protein was the first protein of the TRP family identified to be involved in chemosensation [151]. After this discovery it became obvious that more TRP channels were involved. Many TRP channels (TRPA1 and TRPV1) are activated by hundreds of different chemical compounds, which have been heavily reviewed over the time [152-154]. Some are also involved in gustatory perception (taste) (TRPM5) or pheromone sensing (TRPC2). Interestingly the capability and the activators of chemosensation are very species dependent. Therefore it is important to be careful when extrapolating channel properties between different species. The mechanisms of how those chemicals activate the different channels are not fully understood. But it has become apparent that there are many different mechanisms underlying chemosensation.

TRPA1

In the previous chapter I introduced the TRP superfamily and gave an overview over some general features. In this chapter I will focus on one of the TRPs, TRPA1. TRPA1 is the sole member of the TRPA family in mammals and was the main research subject of the second part of my thesis. In the following I will introduce TRPA1 in more detail, concentrating on the structure and the thermal and chemical activation properties.

The TRPA1 (Ankyrin repeat channel one) ion channel was discovered in 1999, after the cloning of its gene from lung fibroblasts [155]. Jaquemar et al. first named it ANKTM1 (Ankyrin-like with transmembrane domains protein-1) which was later changed to TRPA1 [155]. Both names highlight the most characteristic property of this channel, the multiple (up to 18 putative) ankyrin repeats in the N-terminal part [156]. TRPA1 can be found in humans, mouse, chicken, zebra fish and in many other vertebrates and invertebrates. However, only one copy of the *trpa1* gene is found in mammals whereas other species can possess multiple copies. Furthermore, TRPA1 expression could be mainly allocated to nociceptive neurons in the dorsal root ganglion, trigeminal ganglion and nodose ganglion [156, 157].

The TRPA1 channel is a non-selective cation channel (permeable to Na^+ , K^+ , Ca^{2+}) and is activated by a wide range of stimuli, such as chemical irritants (for example mustard oil, cinnamaldehyde), temperature and mechanosensation [158, 159]. Moreover, TRPA1 can be activated and deactivated by intracellular Ca^{2+} , working as an internal feedback loop [158, 160, 161]. In addition TRPA1 shows a weak voltage dependency, although much less than TRPV1 and TRPM8 [162]. Upon activation of the channel calcium is released into the cytoplasm, depolarizing the membrane, activating the voltage-gated ion channels, which results into action potential propagation. This eventually triggers the feeling of pain and avoidance behavior, but also long term reactions like inflammation [159].

The activation by electrophilic compounds is evolutionary preserved throughout the TRPA1 channels from different species, thus having evolved from a common ancestor [163]. Contrary to the thermosensitivity, which has diverged much later [164]. Since TRPA1 is connected to pain, particularly to neuropathic and inflammatory pain, it is an attractive target for novel analgesic drugs. Especially for patients suffering from chronic pain, as the currently available treatments show

only a low efficacy, but severe side effects. Till now a handful of the TRPA1 agonists that have been discovered are in pre-clinical trials [165].

TRPA1 structure

Structural information about the channel has been limited, which complicates efforts in understanding the structural and functional relationship of TRPA1. Until writing this book there is only one structure of a TRP channel (TRPV6) determined by X-ray crystallography available, possibly a reflection of the dynamic and nature of this ion channel family. In 2011 Cvetkov et al. published the first mouse TRPA1 structure, obtained by single particle electron microscopy, resolved to 16 Å [166]. However, due to the low resolution no firm conclusions could be drawn [166]. After the first structure it took four more years until a first low resolution (≈ 4 Å) structure of the human TRPA1 was published and opened up real insights into the regulatory mechanisms of TRPA1 [119]. Paulsen et al. were able to determine the structure of hTRPA1 in the presence of an activator (AITC) and an inhibitors (HC-0300031 alone or together with A-967079) using the recent advancements in single-particle electron cryo-microscopy. At first glance hTRPA1 structure is similar to the structures of TRPV1 and the distantly related voltage-gated potassium (K_v) channels. As predicted, hTRPA1 consists of six transmembrane α -helices (S1-S6) with N-and C-termini located at the intracellular side. Four subunits come together to form a functional homotetramer with a channel at the central axis (Fig. 8C).

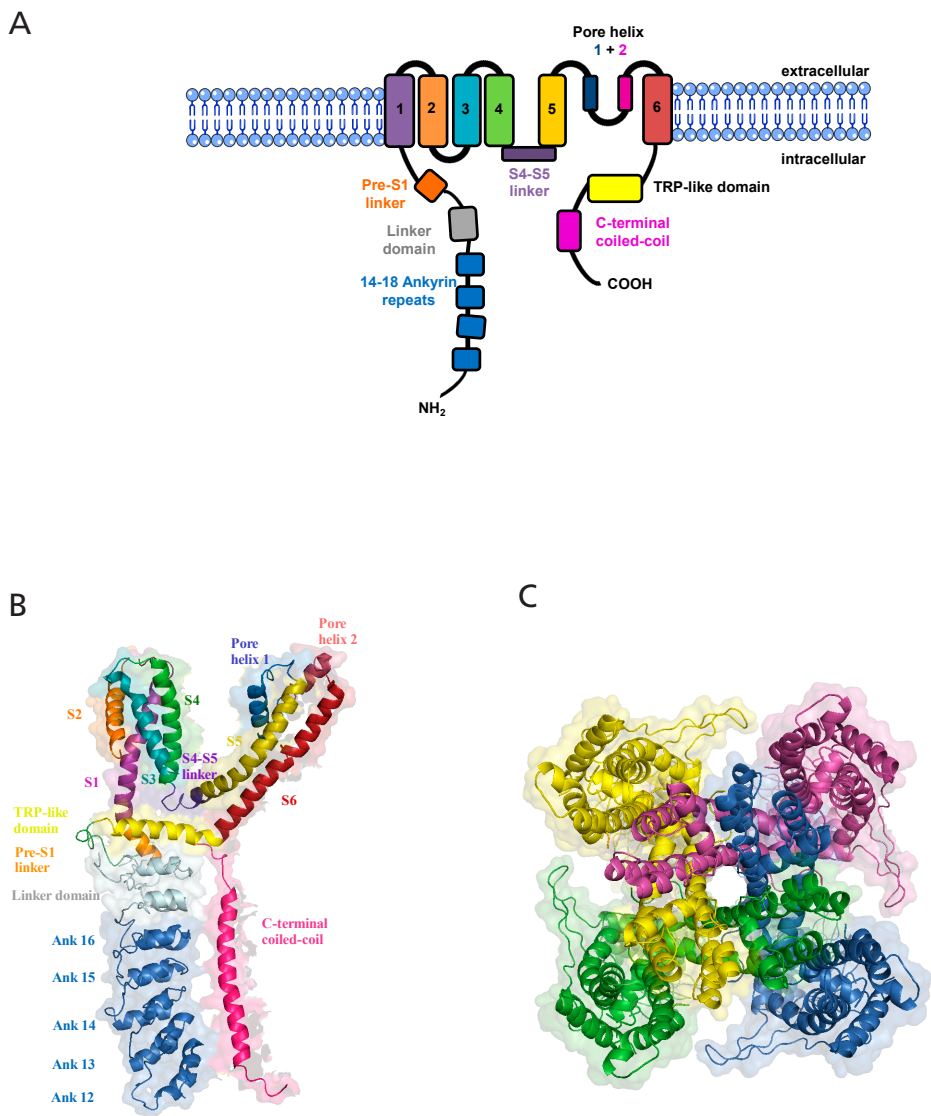


Figure 8 Structural overview of the human TRPA1 channel (based on PDB 3J9P [119]). Pictured are the topology (A) and the structure (B) from the side of a single hTRPA1 subunit and (C) the top view of a hTRPA1 tetramer, the single subunits are depicted in four different colors. The main structural domains are color coded in both graphics. The connection between the third β -strand and the coiled coil, the ankyrin repeats beyond Ank12 and the C-terminus after the coiled coil, as well as the linkers between S1-S2, S2-S3 and S3-S4 could not be resolved in the structure by Paulsen et al. 2015.

The pore and selectivity filter

As hTRPA1 forms tetramers the ion conducting core is assembled by the S5 and S6 of each of the four subunits. Two gates could be identified, which control the the ion conductivity [119]. The lower gate, assumed to be closed in the structure, is formed by two residues: I957, V961 (Fig. 9). Whereas the upper gate is formed only by one residue D915, which has been previously characterized as a key residue for calcium selectivity (Fig. 9) [167]. This is in contrast to the TRPV1 upper gate, which comprises two residues (G643, M644). A new study predicts that the side chain of D915 faces the pore and can therefore interact directly with the permeating ion and not through wide range electrostatic interactions, as proposed earlier [167, 168]. The size of the restriction site at the upper gate was predicted to be around 7 Å [119]. This was disputed by the study from Christensen et al., which based on functional assays predicts a restriction of at least 8.2 Å at the narrowest point [168]. The agonist treated channel is not entirely opened nor completely closed in the cryo EM structure, rather a mixture of both, which could be an explanation for the two different results. Then again these are only estimations for now, as it was shown that TRPA1, as well as TRPV1, is permeable for larger organic cations as well [169, 170]. Interestingly, two pore helices between S5 and S6 could be identified, which form the pore entrance of hTRPA1 (extracellular facing). This is in contrast to TRPV1 or K_v channels, which contain only one pore helix. D915 is located in a loop between the two pore helices. A stretch of acidic residues (E920, E924 and E930) located in the second pore helix was suggested to be responsible for attracting ions from the extracellular surface (Fig. 9). However, a site directed mutagenesis study concluded that mainly E920 (less E924) is involved ion attraction at the outer mouth of the pore [168].

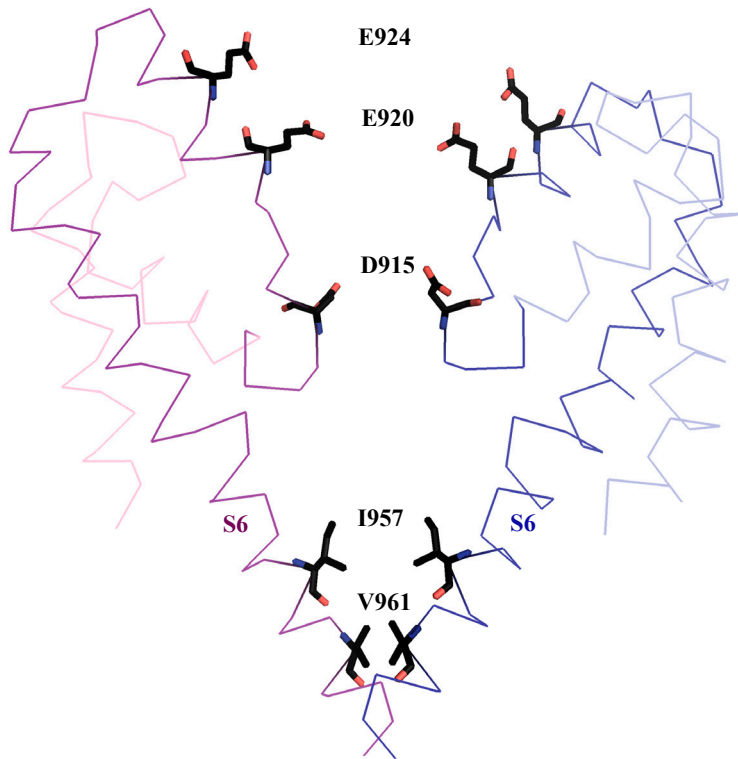


Figure 9 Pore of hTRPA1 (adapted from PDB3J9P), showing only selected residues of two of the subunits, for clarity.

The intracellular domains

Paulsen et al. could resolve some of the vast intracellular domains of the N- and C-terminal, which make up approx. 80% of the protein [119]. The N-terminal consists of a very long stretch of ankyrin repeats, which gave the channel its name. In this structure only five of 14-18 ankyrin repeats could be resolved. But it was possible to divide the N-terminal into two parts, based on the visible differences in densities. A convex stem, which contains the resolved Ankyrin repeat, and a crescent shaped.

The C-terminal domain revealed an unexpected coiled-coil domain below the ion permeation pore (Fig. 8A/B). This coiled-coil is a connection point for all four subunits of TRPA1 and might be important for tetrameric stability and subunit interactions.

The TRP domain

One of the biggest surprises of the human TRPA1 structure was the identification of the TRP-like domain. Several of the TRP channels have a structural domain, which is called the TRP domain. This TRP domain is located directly after S6 and contains 23-25 highly conserved amino acids [115]. The function of this domain is not fully understood yet, but research suggests it is involved in tetramerisation and regulation of channel activity. However, TRPA1 was predicted to not contain such a domain. Surprisingly an α -helix after S6 resembles structurally and topologically a TRP domain. This TRP-like domain is in contact with several other structural domains in TRPA1, like the C-terminal domain and the pre-S1. This suggests that it is involved in allosteric modulation [171]. Altogether the human TRPA1 structure shares similarities to the TRPV1 and voltage gated potassium channels. Nonetheless the structure showed new features, which functional roles need to be unraveled.

TRPA1, a sensor for temperature?

As previously described, some of the TRP channels mediate signals in response to changes in temperature. In mammals the responsible heat channel was quickly identified, unlike the cold response channel which remained a mystery [140]. After the discovery of TRPM8 and its role in innocuous cold mediation, as well as the menthol activation, the search for the noxious cold receptor started [144-146, 148, 149]. Henceforth TRPA1 moved into the focus of the researchers. Cold activation and TRPA1 was for a long time (and possibly still is) the biggest controversy of the TRP field. It all started when TRPA1 was cloned and characterized by Story et al. in 2003 [156]. They showed that heterologously expressed TRPA1 was not only activated by cold, but also showed an insensitivity to menthol and could be activated by icillin. Therefor mammal TRPA1 was denoted a cold-activated channel [156].

This was strengthened by studies examining the involvement of TRPA1 in hyperalgesia [172, 173]. Hyperalgesia is a symptom often found in patients who suffer from inflammation or neuropathic pain. These patients have an increased sensitivity to pain by cold stimulation. Contrary to the findings of Story et al., other groups expressed TRPA1 and could not see cold activation [157, 174]. After the heterologously expression of TRPA1 gave inconclusive results, two TRPA1 knock out mice studies were conducted. However, the results were ambiguous as well. Bautista et al. could not detect any increased cold sensitivity [175], whereas

Kwan et al. came to the conclusion that TRPA1 must be a noxious cold sensor [176]. Moreover the researchers saw differences between male and female mice, wherein the female mice were in general more sensitive [176]. The varied findings were explained by differences in the cellular environments of the studies, especially the calcium levels. Two studies presented that the cold activation of TRPA1 is only indirect and calcium depending [160, 177]. However, a different study by Karashima et al. disagreed with these findings [162]. They expressed TRPA1 heterologously and conducted behavioral studies of knock-out mice. They concluded that cold activation of mammalian TRPA1 is not calcium dependent and that TRPA1 is a noxious cold sensor [162].

Furthermore, a study by Chen et al. presented a new hypothesis, why cold activation by TRPA1 was so highly inconclusive [178]. In their work they compared the temperature sensitivity of four mammalian TRPA1: mouse, human, rat and rhesus monkey. The mouse and rat TRPA1 could be activated by low temperature, whereas the human and rhesus monkey TRPA1 could not. They concluded that the discrepancy between all the studies was due to the species differences of mammalian TRPA1 [178]. This was questioned only one year later when Moparthy et al. 2014 published their studies on purified human TRPA1 [179]. Here they reconstituted the purified protein into lipid bilayers and studied the channel activity by patch-clamping. The purified protein showed cold activation (in the absence of calcium), concluding that human TRPA1 is intrinsically cold sensitive. In another study by Moparthy et al. in 2016 they showed that the cold response was depending on the redox state and ligands [180]. These results might provide an explanation for different study outcomes in the past. However, until now the question if mammalian TRPA1 is cold sensitive or not is still highly under debate. We know now that the purified protein has the capability to get activated by low temperatures and agonists, but this might be highly regulated in the cells. As it is for now we need more evidence to understand the cold activation by TRPA1 in mammals.

Interestingly the picture is much clearer in invertebrates. After mammalian TRPA1 was described as a cold sensor for the first time, researchers started to look into its role in invertebrates, like the fruit fly, mosquitos, snakes etc. To their surprise the *Drosophila* TRPA1 orthologue showed heat sensitivity and a TRPA1 mutant lacked the ability to move towards or away from heat (thermotaxis) [181, 182]. After this discovery more studies were conducted on invertebrate TRPA1 channels. It became clear that TRPA1 was involved in the host seeking behavior of the main malaria mosquito *Anopheles gambiae* [183], as well as in infrared detection by snakes to seek prey [164, 184]. These findings show that TRPA1 is involved in many important physiological processes involving heat sensing in invertebrates.

TRPA1 activation by ligand binding

To detect and avoid tissue damage caused by certain chemicals is an important survival mechanism for many organisms. One of the chemosensors that has evolved is TRPA1, which is activated by a vast number of compounds from plants, food, cosmetics and pollutants, recently reviewed by Zygmunt and Högestätt [153]. In general these agonists can be divided into thiol-reactive electrophiles/oxidants, non-electrophilic compounds, Ca^{2+} activation and G-protein coupled receptor activation. Having this vast amount of activating compounds, TRPA1 must have several different overlapping modes of activation, which are not fully understood yet. We will have a closer look on how electrophilic and non-electrophilic compounds can activate TRPA1.

Electrophilic activation

Many irritants belong to the group of reactive electrophiles, which is a class of noxious compounds. Those irritants usually elicit an avoidance behavior in many organisms. Humans for example experience a pungent and irritating feeling. The compound found in mustard oil, wasabi or horseradish, allyl isothiocyanate (AITC), produces a feeling of pain by activating TRPA1 when applied to the skin [174].

Cinnamon oil contains cinnamaldehyde (CA) which is used as a flavoring agent and can produce a burning and tingling sensation [185]. Interestingly at certain concentrations CA can also inhibit the channel, having therefor a bimodal effect [186].

Garlic has been used in food and medicine for hundreds of years and can also cause a pungent feeling, when consumed in a concentrated form. The compound behind the feeling of pain is allicin, which has a similar chemical structure as AITC [158, 187].

Each of these kind of irritants can cause acute pain and neurogenic inflammation by activating TRPA1. Henceforth it would be of interest to understand how these chemicals can activate the channel. A site-directed mutagenesis study by Hinman et al. 2006 showed that these irritants bind covalently but reversibly to cysteines [188]. Particularly mutations in the positions C621, C641 and C665 located in the N-terminal of the human channel lead to a loss of activation. Therefor they concluded that the N-terminus is crucial for chemosensation [188].

Another study identified three more important cysteines (C414, C421, C621) by Mass spectrometry, also located in the N-terminus [189]. The role of cysteines is still not fully understood, especially if they are involved in binding and/or channel activation, as most intracellular cysteines can be covalently modified [190].

A recent paper by Bahia 2016 et al. characterized the cysteines in position C621 and C665 further under more physiological relevant conditions [191]. They showed that electrophilic binding is necessary for activation of the channel and that C621 is crucial for binding and C665 for activation of the human channel.

Interestingly in a study from Moparthi et al. 2014 [179] and Survery 2016 [192] a purified N-terminal truncated human TRPA1 (Δ 1-688 hTRPA1) and mosquito TRPA1 (Δ 1-776 AgTRPA1) showed activation by electrophiles, when reconstituted into lipid bilayers. These results question the importance of cysteines in the N-terminal. This is also strengthened by the fact that rattlesnake TRPA1 has all the reported cysteine key sites, but is not activated by electrophilic compounds [193].

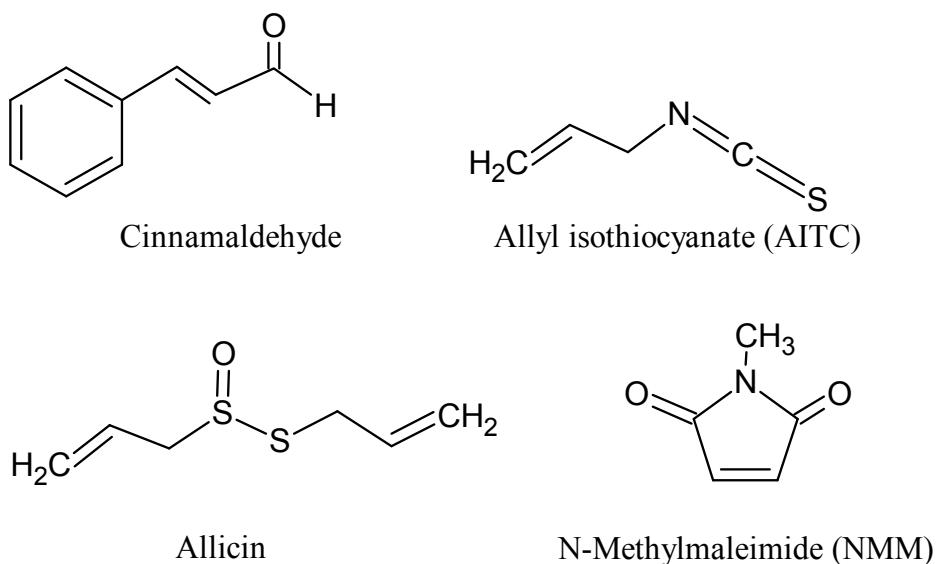


Figure 10 Representatives of TRPA1 agonists belonging to the group of reactive electrophiles

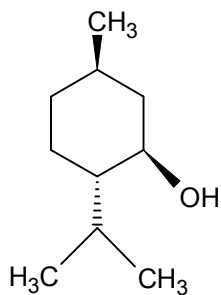
Non-electrophilic activation

TRPA1 is not only activated by compounds which contain a reactive electrophilic group, but also by non-electrophilic substances. The activation mechanisms are less studied than for electrophilic interactions, but a few chemicals could be identified.

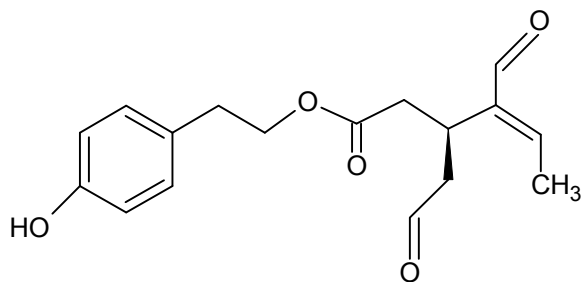
One of these compounds is menthol, which triggers a cooling sensation in humans [194]. Originally it was believed that it only activates TRPM8, the menthol receptor [144, 145]. But studies showed that it can also activate hTRPA1. This is not an uncommon phenomenon, as many agonists are able to activate several different TRPs. Menthol activation exhibits a strong species-specific dependency. Mammalian TRPA1s can be activated by menthol, whereas non-mammalian TRPA1 is insensitive to menthol [195]. Moreover, even between the mammalian TRPA1s differences could be identified. Mouse TRPA1 (mTRPA1) presents a bimodal effect, meaning that in high concentrations menthol can inhibit the channel, but this was not seen for the human channel [162, 195]. A study by Xiao et al. revealed that S5 is the key structural element for menthol sensitivity [195]. The serine and threonine residues in S5 were shown to be important for menthol sensitivity in both hTRPA1 and mTRPA1. The authors propose that upon menthol binding these residues change the structure of the protein in helix 5 and these conformational changes lead to gating of the channel. The reason for species-specific differences was pinpointed to nine key residues in the pore. In a chimeric experiment exchanging these residues between human and mouse TRPA1, they could inverse the differences.

Another non-electrophilic chemical is eudesmol, which is important for flavoring beer [196]. A mutational study determined three important residues (T813,Y840,S873) in transmembrane helices S3,S4 and S5 for eudesmol activation [197]. It is possible that eudesmol has several different binding sites in TRPA1 and that eudesmol could form hydrogen bonds with any of these three amino acids.

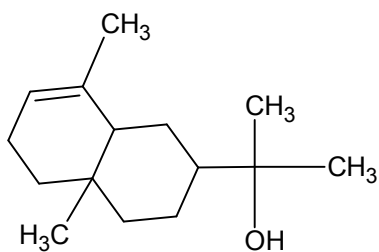
Many other non-electrophiles like nicotine, Δ^9 -tetrahydrocannabinol (THC) [179, 198, 199] or oleocanthal (from extra virgin oil) can activate TRPA1 [200], but their mode of action is not known yet.



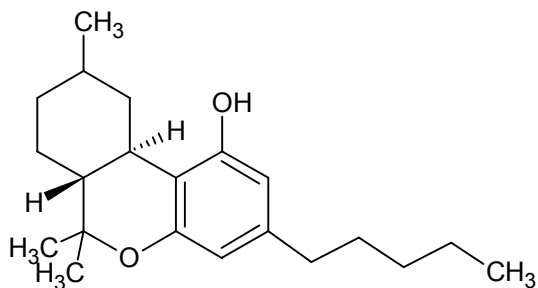
Menthol



Oleocanthal



α -Eudesmol



Δ^9 -tetrahydrocannabinol (THC)

Figure 11 Representatives of TRPA1 agonists belonging to the group of non electrophiles

Aim of the study

TRPA1 is a polymodal ion channel, which can be amongst other things be activated by temperature and many different chemicals. In previous work my group established a way to express human TRPA1 (hTRPA1) and mosquito TRPA1 (AgTRPA1) heterologously in *Pichia pastoris*. Furthermore, the two proteins could be successfully purified to conduct functionally and structural studies. One interest was the role of the N-terminal in temperature and ligand activation. As mentioned in previous chapter, many cysteines located in the N-terminus were identified to be important for ligand binding. Furthermore, cold activation of human TRPA1 is still controversial and the N-terminus has been described as being essential for temperature sensing. Both human and mosquito TRPA1 were truncated, so only the linker would be left of the N-terminal. Both the wildtype and truncated TRPA1 were reconstituted into lipid membranes and single ion channel measurements were conducted under different conditions. The results showed that both the truncated and wildtype hTRPA1 could be activated by cold, as well as by the tested ligands (AITC, NMM, MO). Similar results could be obtained for the AgTRPA1, the ligand activation was still possible and the channel could be activated by heat. Hence the N-terminal seems not to be crucial for ligand and temperature activation and probably plays more a role in fine tuning the channel.

Being able to produce structurally and functionally stable N-terminal truncated TRPA1, we wondered if we can truncate the C-terminal as well. Such a construct would possibly aid attempts to gain detailed structural information which are still not available. Furthermore this minimal structural unit might give us more information of the activation mechanism.

Another project was to monitor possible conformational changes in mosquito TRPA1 upon binding different ligands and when activated by temperature to elucidate the activation mechanism.

Paper III Temperature and ligand induced conformational changes in *Anopheles gambiae* TRPA1

Every year thousands of people die from Malaria and other tropical diseases like Dengue fever, Yellow fever and Zika virus (WHO, fact sheets 2016). There are attempts to develop vaccines against these diseases and although some of these have proven successful, they depend on mass vaccination. One thing these diseases have in common is their mode of transmission through mosquitos. Repellents against mosquitos is therefore another option to control the spread of these diseases. The most common repellent against mosquitos, flies and other insects is the synthetic compound DEET (N,N-diethyl-3-methylbensamide) which was developed in 1954 [201]. However, there are many health issues connected to the use of DEET. Another repellent is citronellal, which can be isolated from lemon grass [202].

The main vector for malaria in sub-Saharan Africa is *Anopheles gambiae* and this mosquito is therefore an attractive target for the development of repellents [203]. *An. gambiae* possesses TRPA1 ion channels, which are crucial for host seeking, larval thermal-induced locomotion and avoidance behavior [183, 204]. TRPA1 belongs to the TRP-family of non-selective cation channels and is activated by heat in invertebrates. Besides being activated by heat, the channel also exhibits activation by electrophiles.

The focus of this work was to monitor the conformational changes upon temperature, ligand activation (AITC, CA, DEET and Citronellal) and a combination of both, using three methods: intrinsic tryptophan fluorescence, synchrotron radiation circular dichroism (SRCD) and nano differential scanning fluorimetry (nanoDSF). We studied both the full length AgTRPA1 and a previously constructed N-terminal truncated version, $\Delta 1-776$ AgTRPA1 to compare them to each other.

Tryptophan fluorescence

Intrinsic tryptophan fluorescence is a common method to study changes in the structure of proteins when binding ligands or other proteins. Conformational changes can alter the environment tryptophan residues are exposed to (change to polar or nonpolar) and can lead to quenching or shift in wavelength shift of the intrinsic fluorescence of the tryptophan which can be easily monitored [205]. Based on the results of Survery et al. [192], we studied the fluorescence changes in the two proteins, when exposed to either of the four electrophilic ligands and determined their EC_{50} values (Table 1, Paper III). Of all the four ligands CA had the lowest EC_{50} value, meaning it is the most potent. Furthermore, the EC_{50} s between AgTRPA1 and $\Delta 1-776$ AgTRPA1 did not differ much from each other, suggesting that the binding sites of these ligands are located on the transmembrane or C-terminal domain. All four ligands quenched the signal in a similar way, which is an indication that the mode of activation/binding is similar.

SRCD

In a previous study the overall secondary structure and the changes due to increasing temperature were determined for both AgTRPA1 and $\Delta 1-776$ AgTRPA1 [192]. Here we reanalyzed the data using a convex constrained algorithm and showed that AgTRPA1 had one transition temperature (53°C) and that $\Delta 1-776$ AgTRPA1 had two (36°C and 58°C). Furthermore, $\Delta 1-776$ AgTRPA1 exhibits a stable intermediate state, its first transition is close to the activation temperature of the protein (30°C).

nanoDSF

NanoDSF is a new method which provides a way to measure protein stability by intrinsic tryptophan or tyrosine fluorescence at ultra-high resolution. We used this method to monitor structural changes on the proteins when exposed to temperature alone or in combinations with various ligands. Unfortunately we could only obtain usable data for the $\Delta 1-776$ AgTRPA1, but not for the AgTRPA1. This might be due to impure samples or a lack of homogeneity of the protein. As was shown

already in the SRCD there are multiple transition states, and by nanoDSF we detected three transition states for all samples. These three transition states were differently affected by the presence of ligands showing minor, but statistically significant changes mainly in the third transition.

Conclusions

In this work we investigated conformational changes putatively connected to thermo and chemical activation of TRPA1 from *A. gambiae* using three techniques. We conclude from our results, that all four ligands have binding sites outside the N-terminal ARD. Furthermore, all tested ligands quenched the tryptophan signal fully, suggesting that they have a similar binding/activation mechanism, although citronellal might be an exception based on the nanoDSF results.

We also showed for the first time to our knowledge that DEET binds to AgTRPA1. Given the polymodal activation of this receptor, it is likely that it is also directly activated by the synthetic mosquito repellent DEET. Previous studies suggested that DEET functions through binding to odorant receptors and Orco and/or through gustatory receptors. Our results indicate that at least *A. gambiae* has an alternative way besides the odorant/gustatory pathways to detect DEET. However, further investigation have to be conducted using electrophysiological methods to confirm our proposal.

The SRCD findings of a stable intermediate close to the activation temperature of the channel were supported by the results of the nanoDSF which showed three different transition temperatures. The transition temperatures were differently affected by the ligands in which the second transition (closest to the temperature activation of the channel) was not modified at all. This leads to the conclusion that the thermal activation may not be modulated by ligands, or alternatively that it is indirectly modulated by ligands at the neighboring transition states.

Paper IV Purification of a C-terminal truncated hTRPA1

The transient receptor Ankyrin repeat 1 channel (TRPA1) belongs to the functional diverse TRP-superfamily. TRPA1 is a non-selective cation channel which plays a role in pain transmission and is accordingly expressed in nociceptive neurons. Similar to the other TRP channels, TRPA1 contains six transmembrane helices (S1-S6). The pore is formed by the S5 and S6 domain of four TRPA1 subunits assembling into a homotetramer. TRPA1 is activated by a wide range of different stimuli, for example cold temperature and electrophilic/non-electrophilic agonists [153]. But exactly how and where in the protein the activation takes place is still unclear. Interestingly, the large N- and C-terminal regions constitute 80% of the protein. Many studies have been conducted to elucidate the role of the large ARD in thermal and agonist activation, resulting in somewhat contradicting conclusions. Two recent papers by Moparthy et al. 2014 and Survery et al. 2016 concluded that the N-terminal region has a regulatory function in activation of the channel, but it is not an essential part of the mechanism [179, 192].

In paper IV we focused on the other end of the channel, the C-terminal. The cryo EM structure, which was recently published, could resolve some of the C-terminal domains. Most surprisingly was a domain, subsequently of S6, which structurally resembled a TRP-domain. The TRP-domain is conserved in many TRP-channels and might be important for gating of the channel. Furthermore the structure showed a coiled-coil domain, which is formed by all four subunits and might be involved in stabilizing the tetrameric form. Previous mutational studies on the C-terminal suggest that this domain is involved in chemical and voltage-gated activation [206] and might provide the binding site for Ca^{2+} [207]. Our goal is not to mutate, but rather to probe if it is possible to truncate most or some of the C-terminal region without compromising the structure and function of TRPA1.

Truncating the C-terminal domain

In a previous paper by Sura et al. they truncated the C-terminal region of hTRPA1 by 20 and 26 residues, expressed the two truncated versions in HEK cells and conducted electrophysiological measurements [207]. Interestingly, only the 20 residue truncation lead to functional channels, as the researchers were not able to detect currents in the 26 residue version. Possible explanations might be that the truncation was in the middle of a C-terminal domain, which destabilized the protein or made it a target for degradation.

As the EM structure was not available at the time, we conducted multiple sequence alignments to predict potentially conserved structural features on the C-terminal. The truncation positions were picked based on this alignment to avoid destabilizing the C-terminal by disrupting structural elements. We picked three positions, all of them leaving the TRP-like domain intact ($\Delta 87$, $\Delta 110$, $\Delta 129$). The positions are shown in Figure 1, paper IV. Furthermore, all three positions would remove the coiled-coil assembly domain seen in the hTRPA1 structure. As our goal is to identify the minimum structural and functional unit of TRPA1, we used the previously constructed N-terminal truncated version of hTRPA1 ($\Delta 1-688$ hTRPA1) as template for the C-terminal truncations. Moreover in a paper published by Moparathi et al. it was shown that $\Delta 1-688$ hTRPA1 had a higher expression as its full length counterpart, which we might be able to use to our advantage [179]. We decided to express the different proteins in *P. Pastoris*, as our group has successfully overexpressed different versions of TRPA1 (full length *Anopheles gambiae* and *human* TRPA1 and N-terminal truncated versions of both) using *P. Pastoris* as expression host. After screening for expression clones in small scale (Materials & Methods, paper IV), we could only detect expression for the shortest truncation, $\Delta 87$, but not for the other two. We then expressed that clone in large scale in a table top fermenter and started with the purification. Unfortunately the protein was not very highly expressed which compromised the purification. After pooling different fractions, where the protein could be detected through western blot, we loaded the sample on a gel filtration column to purify it further. Unfortunately the protein concentration was too low, to detect any peaks. Rather than repeating the transformation into *Pichia* and screening for another clone, which might have a higher expression, we decided to change our truncation strategy.

New truncating strategy

As the low expression of the C-terminal truncated protein is difficult to optimize, we decided to use a different approach of truncating the protein. Instead of adding a stop codon, we added a TEV protease cleavage site shortly after the TRP- like domain. As we know that the $\Delta 1-688$ hTRPA1 is highly expressed in Pichia, we hope that the extra TEV site will not disturb the expression levels. Furthermore we have a His₁₀-tag at the N-terminus, which we can use to wash off the C-terminal from the rest of the protein through affinity chromatography. Using this way of truncating, we can potentially also isolate and conduct some structural studies on the C-terminus. These experiments are work in progress.

Future outcome

One of the biggest goals in the TRP field is to understand the activation mechanism in detail. For that it is essential to identify the structural elements involved and their conformational changes upon activation. Furthermore, the causes of observed species dependent differences need to be clarified.

In this work we focused on gaining a better understanding of the structural details of human and mosquito TRPA1. Removal of the N- and C-terminal domains of the hTRPA1 will give us several possibilities to proceed further. We will try to crystallize this construct with and without ligands and hopefully gain more detailed structural information on the membrane domain. Furthermore, studies of the isolated C-terminal domain might extend our knowledge on the structural role of this part of the protein and help to complete the picture of TRPA1.

To elucidate the mechanism of activation it is important to supplement structural studies with functional data. Thus it will be interesting to see if the N- and C-terminal truncated construct can still be activated by various ligands and how it behaves at different temperatures. This will give us an idea about putative functions of the C-terminal domain in activation. In order to facilitate studies of channel activation it would be beneficial to develop new methods. Single ion channel measurements using patch clamp is a powerful method to monitor channel activity. However, it is a very challenging and time consuming method to monitor the individual activation of reconstituted ion channels. One potential assay is based on the paper by Reubish et al., in which they monitored the activation of TRPV1 expressing HEK293-TREx cells by heat, using a Ca^{2+} -sensitive fluorescent dye (Fluo-4) and a real time PCR cyler [208]. Instead of expressing TRPA1 in HEK-cells, we want to use the purified protein reconstituted into proteoliposomes to easier correlate functional and structural changes. As Ca^{2+} can activate the channel even in low concentrations, Ca^{2+} -sensitive dyes cannot be used in the assay, instead we have to rely on a Na^+ or K^+ -sensitive dye. However, pilot experiments using a K^+ -sensitive dye (Asante Potassium Green) did not lead into reproducible results, as the dye exhibited a non-linear response at the different K^+ concentrations used. Hence, we need to screen for a more suitable dye.

Furthermore we showed that intrinsic fluorescence, SRCD and nanoDSF are powerful tools to study conformational studies in AgTRPA1 upon ligand binding and temperature activation. Mutational studies can identify the residues

responsible for the intrinsic fluorescence, and clarify which are quenched by temperature activation and which are quenched by agonists. Furthermore, it would be interesting to monitor the effect of antagonists by these methods. An obvious step would be to do similar experiments on the human TRPA1 and to compare the results.

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