Aquaporins - Novel approaches to old problems

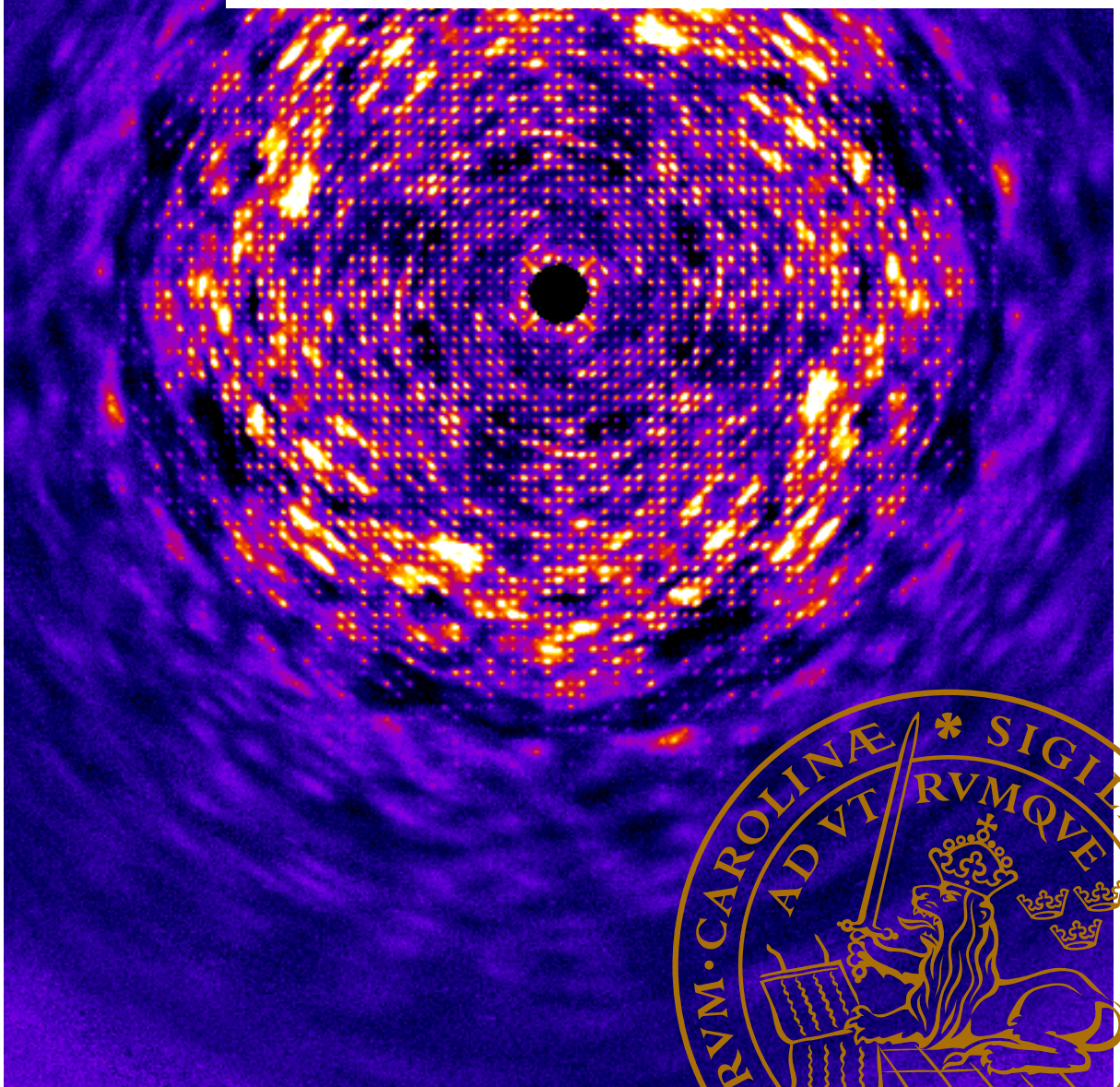
Hagströmer, Carl Johan

2023

Aquaporins

Novel approaches to old problems

CARL JOHAN HAGSTRÖMER
BIOCHEMISTRY AND STRUCTURAL BIOLOGY | FACULTY OF SCIENCE | LUND UNIVERSITY



Aquaporins

Novel approaches to old problems

Aquaporins

Novel approaches to old problems

Carl Johan Hagströmer

LUND
UNIVERSITY

Thesis for the degree of Doctor of Philosophy
Thesis advisor: Prof. Susanna Törnroth Horsefield
Faculty opponent: Prof. Martin Högbom

To be presented, with the permission of the Faculty of Scienceof Lund University, for public criticism in the Lecture hall A, Kemicentrumat the Department of Chemistryon Friday, 26th of May 2023 at 09:00.

Organization
LUND UNIVERSITY

Division of Biochemistry and Structural Biology

Document name
DOCTORAL DISSERTATION

Date of issue
2023-04-18

Sponsoring organization

Author(s)
Carl Johan Hagströmer

Title and subtitle

Aquaporins - Novel approaches to old problems

Abstract

As all life on this planet evolves around water, it is important for our bodies to be able to regulate it. This may be done passively, but just like with water in the ocean, if we were to rely solely on passive transport (such as evaporation), the flow of water would be too slow for biological functions to uphold the homeostasis that is so important for us to survive. All organisms have thus developed specialised water channels, aquaporins (AQPs), which, much like rivers, (usually) act as rapid transporters of water across land - or our lipid-rich cellular membranes.

When issues arise with AQPs, disease states such as nephrogenic diabetes insipidus, Sjögren's syndrome, and cataracts may occur. These diseases are all related to the malfunction in either the integrity or regulation of the AQP responsible, or proteins related to their regulation.

In this thesis, the following proteins are studied; AQP0, AQP2, and AQP5, along with their regulatory proteins calmodulin and ezrin. In the case of AQP2, the structural impact of mutations from patients are investigated in relation to their structure and function, as well as their role in causing the disease state.

The study of membrane proteins as a whole is a complicated matter, mainly due to the fact that they are situated in the cell membrane. Detergents are required to isolate them from their place in the membrane, and for structural studies using X-ray crystallography, this can be an impediment. Detergents may cause irregularities in the crystal lattice, resulting in lower resolution data. These irregularities may in some cases be possible to benefit from, if they cause continuous diffraction. We have studied the nature of this type of diffraction, what causes it, and if it can be manipulated to our advantage.

Key words: aquaporin, membrane proteins, calmodulin, ezrin, protein-protein interaction, x-ray crystallography, continuous diffraction

Classification system and/or index termes (if any):

Supplementary bibliographical information:

Language
English

ISSN and key title:

ISBN
978-91-7422-9608 (Print)
978-91-7422-961-5 (PDF)

Recipient's notes

Number of pages
57

Price

Security classification

Aquaporins
Novel approaches to old problems

Carl Johan Hagströmer

LUND
UNIVERSITY

Cover: Sliced image of merged 3D diffraction of aquaporin-2. Credit: Oleksandr Yefanov
Following page: Image generated by Midjournery with the prompt ”A small scientist in a white lab coat gazing out over a landscape of membrane protein structures, in the style of Dalí and Mucha”.

Faculty of Science, Department of Chemistry
ISBN: 978-91-7422-960-8 (print)
ISBN: 978-91-7422-961-5 (pdf)

Printed in Sweden by Media-Tryck, Lund University, Lund 2023

”I noticed even the crickets
Were actin’ weird up here
’N so I figured I might
Just drink a little beer”
- Frank Zappa

Contents

List of publications

This thesis is based on the following publications:

I **Ezrin is a novel protein partner of aquaporin-5 in human salivary glands and dhows altered expression and cellular localization in Sjögren's syndrome**

Chivasso, C., **Hagströmer, C.J.**, Rose, K.L., Lhotellerie, F., Leblanc, L., Wang, Z., Moscato, S., Chevalier, C., Zindy, E., Martin, M., Vanhollebeke, B., Gregoire, F., Bolaky, N., Perret, J., Baldini, C., Soyfoo, M.S., Mattii, L., Schey, K.L., Törnroth-Horsefield, S., Delporte, C. (2021)
International Journal of Molecular Sciences, 22, 9213.
https://doi.org/10.3390/ijms22179213

II **Structural basis for the interaction between the ezrin FERM-domain and human aquaporins.**

Hagströmer, C.J., Werin, B., Strandberg, H., Wendler, M., Franzén, R., Törnroth-Horsefield, S.
Manuscript

III **The role of phosphorylation in calmodulin-mediated gating of human AQP0**

Kreida, S., Roche, J.V., Missel, J.W., **Hagströmer, C.J.**, Wittenbecher, V., Gourdon, P., Törnroth-Horsefield, S.
Manuscript
Submitted to Biochemical Journal

IV **Structural and functional analysis of aquaporin-2 mutants involved in nephrogenic diabetes insipidus**

Hagströmer, C.J., Hyld-Steffen, J., Kreida, S., Al-Jubair, T., Frick, A., Gourdon, P., Törnroth-Horsefield (2023)
Manuscript
Submitted to Scientific Reports

V **Effect of growth conditions and detergents on continuous diffraction from membrane protein crystals**

Hagströmer, C.J.†, Yefanov, O.†, Glas, J, Kreida, S., Hedfalk, K., Chapman, H., Törnroth-Horsefield, S.
Manuscript

† Authors contributed equally.

Popular summary

All life evolves around water. In order to regulate the flow of water in rivers, we have developed dams. In order to regulate water within our bodies, one needs another kind of structure: aquaporins.

Aquaporins allow our bodies to either excrete water via saliva or tears, or to reabsorb excess water from the urine. If we had not been able to do this, the mucous membranes within our mouths or around our eyes would become very dry and cracked, and the likelihood of infection would increase. If we were unable to reabsorb water from the urine, we would need to urinate several litres per hour.

What happens if somethings happens to the genetic code, the DNA, which is the blueprint for all proteins? In the case of aquaporins and the proteins which take part in their regulation, diseases such as cataracts, Sjögren's syndrome, atopic dermatitis, and nephrogenic diabetes insipidus ensue.

In order to study proteins such as aquaporins, which are membrane proteins situated in the membranes of the cell, and investigate what happens to them on a structural level, it is necessary to first produce them in cells. Once the protein has been produced, it is possible to isolate the cell membranes from them, and in turn the proteins. This requires the use of detergents and various filtration methods.

We have studied three of these aforementioned aquaporins: aquaporin-0, aquaporin-2, and aquaporin-5.

The lens of the eye requires a certain level of circulation in order to function, which is performed by aquaporin-0 being present at the cell membrane of the cells. It also needs to be open to allow for passage of water molecules through it, which is regulated through phosphorylation and interaction with another protein, calmodulin.

Patients with nephrogenic diabetes insipidus may have it due to multiple reasons, and a subset of these are due to mutations in the gene encoding for aquaporin-2. These mutations cause the change of singular amino acids, which the protein

is composed of, to be replaced, thus changing the chemical properties. Our studies show that mutations from patients which affect certain parts of aquaporin-2, which are relevant for quality control mechanisms within the cell, are not necessarily too detrimental to the structure of the protein. On the other hand, they may lead to decreased stability and water flux of the protein.

Sjögren's syndrome may occur as a consequence of disruptions in the regulation of aqauporin-5, as the excretion of water in mucous membranes is affected.

We have studied ther gulation of these aquaporins, the consequences of patient-derived mutations, and how all of these aspects function on a molecular level.

The study of these membrane proteins on a biochemical and biophysical level is very much dependant on our ability to isolate them from other materials within the cell through the use of detergents. These detergents may lead to certain defects which may be noticed if one is trying to solve the protein structure through the use of protein X-ray crystallography. X-ray crystallography of proteins requires protein crystals, which are formed by slowly concentrating protein in solution droplets, forcing them into supersaturation. The protein can then either aggregate, or form crystals. Crystals are tightly packed grids of molecules interacting with each other. If one shines a laser beam onto such a crystal, a diffraction pattern will show. This diffraction pattern may be used to solve the molecular structure of the protein. A problem which one may run into when studying membrane proteins, is that they often suffer defects as a consequence of the detergents used when purifying them. These defects may in certain cases lead to further information regarding the molecular structure. We have, aside from studying aquaporins, also studied the cause of these defects, and if they may be manipulated to a researcher's advantage through the addition of a selection of other molecules.

Populärvetenskaplig sammanfattning

Allt liv kretsar kring vatten. För att kunna reglera flöden av vatten i floder har vi dammar. För att kunna reglera vattnet inom våra kroppar krävs det en annan typ av konstruktion: aquaporiner.

Aquaporiner tillåter våra kroppar att antingen utsöndra vatten, i form av exempelvis saliv eller tårar, eller återuppta vatten från urin. Hade vi inte kunnat utsöndra saliv eller tårar hade våra slemhinnor i munnen och kring ögonen blivit väldigt torra och spruckna, och infektioner hade varit ett faktum. Hade vi inte kunnat återuppta vatten från urinen vi bildar, hade vi behövt urinera flera liter varje timme.

Vad händer då när någonting blir fel med den genetiska koden, DNA, som är ritningen till alla proteiner, inte minst dessa aquaporiner? Sjukdomar såsom gråstarr, Sjögrens syndrom, atopisk dermatit, och nefrogen diabetes insipidus är alla sjukdomar med kopplingar till antingen defekter, eller mutationer, på själva aquaporinerna, eller andra proteiner och mekanismer som hjälper till att reglera dem.

För att kunna studera proteiner såsom aquaporiner, som är membranproteiner benägna i cellens membran, och ta reda på vad som sker på en ren strukturell nivå med dessa proteiner krävs det att man först uttrycker dem i celler, som man sedan kan isolera cellmembranen från. Dessa membran kan sedan lösas upp med detergenter, och genom olika filtreringsmetoder kan dessa proteiner sedan renas upp och studeras.

Vi har studerat just tre av dessa aquaporiner: aquaporin-0, aquaporin-2, och aquaporin-5.

Ögats lins kräver en viss grad av cirkulation för att fungera, och då krävs det att aquaporin-0 är närvarande vid cellmembranet och är öppen för genomflöde av

vattenmolekyler. Mekanismen för att öppna aquaporin-0 sker genom fosforylering och interaktion med det reglerande proteinet calmodulin.

Patienter med nefrogen diabetes insipidus kan ha det av många anledningar, och en del av dessa är på grund av mutationer i genen för aquaporin-2. Dessa mutationer leder till att enstaka aminosyror, som är proteinernas byggstenar, byts ut, och därmed tillför andra kemiska egenskaper än de ursprungliga. Våra resultat visar att mutationer från patienter, som rör delar av aquaporin-2 som är relevanta för glykosylering och kvalitetskontrollmekanismer, inte nödvändigtvis påverkar integriteten av proteinets struktur till en större grad. Däremot kan de ofta ha en negativ inverkar på proteinets stabilitet och förmåga att leda vatten, men de är likväl funktionella.

Vid fel i reglering av aquaporin-5 kan sjukdomen Sjögrens syndrom uppstå, då utsöndringen av vatten från slemhinnor upphör att fungera.

Vi har studerat regleringen av dessa aquaporiner, konsekvenserna av mutationer som kan återfinnas hos patienter, och hur allt detta ter på en molekylär nivå.

Studierna kring dessa membranproteiner på en biokemisk och biofysikalisk nivå kretsar mycket kring möjligheten att kunna isolera dem från övrigt material i cellen genom att användningen av detergenter. Dessa detergenter ger dock vissa bieffekter, vilka ofta är märkbara när man försöker utröna proteinets struktur genom röntgenkristallografi. Röntgenkristallografi av proteiner kräver just proteinkristaller, vilka kan formas genom att man under väldigt specifika och kontrollerade former låter protein i lösning öka i koncentration, vilket långsamt tvingar in proteinet i övermättning. När detta sker kan proteinet antingen aggregera, eller bilda kristaller. Kristaller består av tätt packade molekyler, som binder till varandra på ett sådant sätt att en viss geometri uppstår. Belyser man dessa kristaller med en laserstråle får man ett diffraktionsmönster, som kan användas för att utröna de enstaka molekylernas struktur på molekylnivå. Ett problem som kan uppstå vid studier kring membranproteiner är att kristallerna som bildas ofta har defekter till följd av användningen av detergenter vid uppreningen. Dessa defekter kan dock i vissa fall kan leda till ytterligare information kring den molekylära strukturen. Vi har, utöver att studera aquaporiner, även försökt att ta reda på hur dessa defekter uppstår, och om de kan påverkas till en forskares fördel genom olika tillsatser.

Acknowledgements

I would never have made it to this point in my life without the guidance, friendship, love, and banter from a vast number of people in my life.

Let's start with the one most important to my career thus far, and this thesis: **Susanna**. Working together with you throughout the supposed "peak" of my youth, sickness, a pandemic, a recession, war, and God knows what else until I actually finish up here at CMPS, has been nothing less than an honour and a pleasure. I don't think I have always been the easiest student for you to supervise, but I truly think you helped me mature into who I am today. Thank you, for your kindness, understanding, and guidance!

I would also like to thank all current members of the group, as well as former: **Stefan**, starting off this aquaporin-journey together with you was a wild ride, and I feel like I ended up like some twisted version of you: continuing similar projects, teaching the same course, working on the same computer, and equally messing up my hair throughout the process - the difference is that you kept yours... **Jennifer**, you were like a kind elder sister when I first entered the group, and I guess you kind of remain so in the context of helping me survive all the Indians. **Veronika**, you saved my life in both the lab and the course lab many many times, and I miss having your cheekiness around. **Helin**, I could never have asked for a better office buddy, and I think we are both lucky that çavkaniyên mirovî barely is a thing here. Thanks for a tonne of laughs, distractions, and chaotic - yet hilarious - teaching sessions and late nights! Choooni. **Tamim**, it has been great working with you, especially with the benefits of chatting with you and your generous BBQs! I sorely need to repay the favour, maybe with a little whisky on the side. I would like to thank my student, **Zahraa**, I think you ended up teaching me more about myself academically than I had expected. And thanks for the food! **Yasmin**, it was great

having you around and getting to know you, and thanks for introducing me to sucuk. **Fahmidul**, it was a fun coincidence that we ended up working next to each other after having taken our very first course in Lund together. Best of luck with the kiddo! **Rita**, I'm in awe with you (and Helin) for working so hard while being so pregnant. It was fun hanging out with you in Kåseberga! **Markus**, you might not drink beer, but you're a pretty decent guy anyway. You have a good sense of style, and few people know how it is to walk in my shoes. **Simon**, it's been great having you around, first in the course lab, and then in the actual lab. I think you have a bright future ahead of you.

Urban, your beard will always be beautiful, and I hope that you one day will win the war against the barkborrar. In a way, your group was like a step-group to me, and it was interesting and fun to share ideas and progress together with you all, **Per**, **Michael**, **Balder**, **Oliwia**, **Elin**, and **Titus**.

I would also like the other people who have made my time at the department memorable: **Olof**, you are a hairy fella, but that's ok. You have educated me about many things outside of science, and I hope I will have more time for late night sessions at wine bars (or bars in general) again soon. **Fimpen**, the Beard Baron. I will forever cherish our beard journeys, your comments regarding "den bruna maten", and overall wisdom. **Magnus**, I don't know if the department will ever work without you. I didn't break anything (I hope), and I popping by your office to complain/chitchat/steal stuff was always a pleasure, especially with your impeccable taste in music. **Maryam**, you have been so kind to me since my first day at the department, and you always made me feel like there was someone looking out for me at all times. Thank you for your kindness, help, and food! **Johan**, you always have interesting thoughts regarding current events, and discussions with you have always been thought provoking and enlightening. **Eimantas**, coffee breaks (and snaps) with you were always a blast. Labas, and best of luck with the racing! **Martin**, I think we share a few frustrations in life, but none of my solutions have been as good as your disco-ball waste station. **Ryan** for our beer-fueled political chats. **Bhakat & Dev**, pagal chutiyas. Thank you for teaching the beautiful Hindi language, which turned out to be more useful than I had first bargained for. BC/MC. **Mathieu**, as fashion police you always kept me grounded, and your compliments were not handed out easily, they were earned. Merci cheri, tu me manques. **Archimede**, best roomate I've ever had in Tällberg. At least we didn't break the sauna. **Antonio**, I knew Spaniard were crazy, but Jesus. I hope **Carmen**

survives, as she keeps me humble at the climbing crag. You guys are great. **Henrik, Hinken**, you have a good sense of music and the punk scene in Malmö, cheers! **Abishek**, I hope your life in the North isn't too lacking of sun, but I hope you're doing fine up there. **Mikael**, I doubt I'll ever be good at NMR, but I think at least we can agree on two things; fashion, and that *"climbing is the ultimate sport"*. **Herwig**, you've been a fresh breeze at the department, and I hope you continue that habit. **Thompa**, hearing from you that my running technique was good made my week, maybe even month. Best of luck with the Labbot! **Sara**, you amaze me. Firstly, with your passion and skill for research, and everything surrounding it. Secondly, for being *so damn strong* in Sankt Hans. One day, I hope I can tackle the hills of both research and Sankt Hans at a fraction of your level! **Cecilia**, one of the few professors I could always trust to join for a beer and a chat. **Lo**, the piano music seeping out of your office was always a nice touch to the corridor. **Salam**, hearing your stories of your upbringing and education was always interesting and inspiring. **Folke**, even in retirement your involvement in the department has been heartfelt! **Derek**, it's always been nice chatting with you about Scotland, classical music, less classical music, KG&tLW, and many other subjects. **Björn**, thanks for all the honey! **Alex**, like an alpha and omega to my time at CMPS, with beautiful cowboy boots. **Ipsita**, our time together at the department started off at about the same time, and it was a joy to start off and to grow in academia alongside you. Your memory will be with me forever. **Sofia**, I wish you had joined the department earlier. Keep that espresso machine flowing, and those llama-marshmellow-whatnots tapping. **Simon**, I don't know where to begin or end with you, but you've been awesome. Our Rocket League careers never took off properly, but I think we have great potential in competitive Smash - I *will* own you with Kirby. **Max**, you have great potential, especially with the combo of BJJ and BC General. **Andreas**, keep the tennis going, and hopefully we can revive our unbeatable foosball team. **Lei**, it's been weird seeing you with pants on. Ja, precis. **Egle**, you're up next. Best of luck. **Signe**, eating cornflakes with water will never be a thing for me. **Tinna**, I can't believe it's been 5 years since you taught me in Advance Biochemistry. You gave me a good first impression of the department! **Ingemar**, I never expected to find someone else with such a diverse liking for music, and impeccable Stockholm accent. Tjeeeeenare! **Christin**, a strong runner, strong researcher, and a strong beer drinker. And a strong mum! **Jelica**, hope Sweden was a nice break from Serbia, despite our shitty weather. **Lovisa**, we were so close to winning that pub quiz in Umeå. I hope you get some CryoEM going in between those insane hikes. I hope to sprinkle a bit of both over my future. **Wojchiech**, your passion for data

and coding have been interesting to witness. Hope ESS is operational soon, hehe. **Niels**, what you did was not art. Neuken in de keuken. **Vera**, you've been a fun addition to the department as well, and I hope Niels is nice to you in that cave. **Angus**, thank you for you your kind lunch donation on the 3rd of September 2018, during my brief time of extreme poverty. **Thomas Björkman**, thank you for showing sympathy (and shared annoyance) when the finance office didn't pay my salary for three months, causing my brief time of extreme poverty.**Erik**, it's always been fun to have you around, gossiping about politics and everything else. You have my vote. **Nicholas**, cheers for all the chats, hot sauce, and tales of the American wilderness. **Emil** you crazy mountain monkey. Most social student I ever had in the course lab, and definitely the only one who got me into jumping off of cliffs, climb back up, and do it all over again. I'm forever glad you became my colleague. **Yonathan**, you might be Danish, but that's ok. You have been a great friend through thick and thin, and I'm happy we got to do that hellish beamtime in Grenoble years ago. **Vero**, you are crazy. That's what makes you special. **Virginia**, keep kicking ass, in your studies and on the wall. **Mads** - Another Dane...? Ah well, you have nice thighs, and you've been an invaluable help with Python/Rosetta, and the best climbing buddy one can ask for. It took a while, but you finally brought me the delicacies of your homeland, which I savoured while writing this line.

Papi's Jolly Gang of Adventurers, or whatever our name is. It's been pretty much as long as my entire PhD, but our adventures have been almost crazier than the PhD itself. Cheers to my eternal comrades **Mattias** and **Samuel** (who by the way has been the best inofficial legal counselor/career advisor I've had to date), and the constantly recast crazies **Mathias**, and **Camille & Sven** (and **Saga**!). Of course, none of this would ever have been possible without our incredibly generous and inventive in-house dieties, **Viktoria & Ben**.

I would also like to thank my fellow AQP peeps; **Pontus** and his gang, **Julie**, **Jonas**, **Malene**, **Kamil**, **Kamil**, **Niloofar**, **Anders**, **Christina**, **Ping**, **Eva**, **Nina**, **Pin Jie**, **Zongxin**, **Elena**, **Qiaoxia**, **Kaituo**, **Liying**, **Karin** and her gang, **Peng**, **Ramynta**, **Sofia**, **Sibel**, and **Hanna**. Thanks for all the great times at the joint meetings, the Christmas dinners, and Tällberg!

Oleksandr, I don't think I can or want to count the amount of abysmal crystals I have brought or sent to you (although I think they totalled to 261), but you

have measured them all, whilst providing insightful discussions during late nights at PETRA III.

I would like to thank **Chris Baker**, for offering me wafers throughout these years and being a reminder of how certain things in life remain constant throughout the passage of time.

Bengt, you have acted as another type of supervisor during my time in Lund. Wherever I may be in the future, I will at least have mementos of you with me, and hopefully still with snazzy shoe taps.

Another thanks goes to my climbing buddies **Theo, Martin**, and **Elin**, who I'm happy to entrust my life to (as long as they bring snacks).

Life would not be the same without my new, global, Indian family: **Emmy, Neha & Tarun** taking care of me in Lund and being an endless source of entertainment, **Nida & Kailash** taking care of me in Gothenburg, with your additions of **Rajiv**,**Ashwin** and **Shraddha**, **Rohit, Soni & Adiva** in Järfälla, **Chimmu** in the rest of Stockholm and the wilderness, and **Shruti & Kruti** in Munich.

Life also wouldn't be the same with my age old friends, who have been with me longer than what some might consider healthy: **Dylan**, **Arvid**, **Sahel**, **Erik**, **Christoffer**, and **Robert**.

Guidance comes in many shapes and forms, and some of it has come from my godparents: **Tom** and **Ikuko**, and **Hans** and **Annika**.

Lastly, I would like to thank my extended family, and my parents: **Mamma and Dad** Thank you both giving me life, and for the guidance and love you have given me my throughout it. Mamma, jag ber hemskt mycket om ursäkt att jag höll på att somna när du disputerade, men jag hoppas att jag har lyckats göra dig stolt likväl! I love you both.

Hafsa - Ek bas tu hi pyaar ke kabil saara jahaan hai nikamma! I love you.

Introduction

It all begun some odd 3.8 billion years ago, when compounds that would come to form what we now know as a cell likely floated freely in a *primordial soup*, as was hypothesised by Aleksandr Oparin [1] and John "JBS" Haldane [2] in the 1920's. Through fortune, be it through divine intervention or through any mechanisms perceived as likely today or in the future, these compounds formed nucleic acids (DNA and RNA), lipids, and amino acids, the latter eventually forming polypeptides and proteins. Once the cell had formed around the necessary compartments (which would later include other prokaryotic cells, forming mitochondria and chloroplasts), it had an incentive to propagate and survive to the best of its abilities. The interior could through decoding of the blueprint that is the genetic code produce the proteins necessary to carry out the housekeeping of the interior, and interactions and signalling with the exterior environment and other cells in its proximity, while the exterior was sealed off via a semipermeable lipid bi-layer that had formed around its interior. Water could *slowly* move across the bi-layer, as could O_2 and CO_2, but other molecules would need the development of specialised channels, or proteins which could mediate and propagate signals within the cell. Enter the membrane protein.

Membrane proteins

Membrane proteins are situated in the lipid bi-layer composing the outer perimeter of the cell, thus providing isolation from the outside world, while also allowing communication, of sorts, through the embedded membrane proteins.

The membrane has evolved to contain of a mixture of hydrophobic lipids, steroids (such as cholesterol), and a vast variety of membrane proteins.

Unlike soluble proteins, membrane proteins are required to operate within this lipid-rich environment, for which they have evolved to be stable within. As they are destined for the cell membrane, they are required to contain signal sequences which allows the cell to recognise where they belong. They may also be regulated via different mechanisms depending on the protein and the triggering mechanism, be it through trafficking to or from the membrane or gating mechanisms triggered by another protein, ion, pH, or signalling substance.

Of course, as organisms grew more complex, and even became multi-cellular, the need for the diverse specialisations of proteins became clear. Certain proteins evolved to channel ions across the membrane (like voltage-gated potassium channels), while others evolved to potentiate signalling cascades (like GPCRs). As such, membrane proteins are highly regarded as potential drug targets. Despite making up only 20-30% of the human proteome [3], membrane proteins are considered to make up 60% of drug targets [4][5][1].

Nonetheless, the most interesting aspect of the specialisation of membrane proteins [2], resulted in the regulation of the flow of water better than just through non-facilitated passive diffusion.

[1] Despite these reviews being from almost two decades ago, the consensus seems to remain the unchanged.

[2] *At least for this thesis!*

Aquaporins

Even though a single cell organism such as *Escherichia coli*, found in the lower gastrointestinal tract, may be somewhat limited in its complexity in relation to multicellular organisms, the environments around it may change, and sometimes rapidly. Thus, they require more than merely passive diffusion of water across their membranes in order to survive, and through evolution, as was originally proposed by Darwin [6], organisms adapt to their surroundings, or perish.[3] *Life finds a way*.

Aquaporins (AQPs) evolved to allow rapid movement of water molecules across membranes through facilitated diffusion, and allowed regulation of cellular water content depending on the osmotic gradient on the outside. As cells specialised and differentiated into various tissue types, the need for further specialised AQPs arose. In humans, these AQPs can be divided into a subset of water channels: Classical AQPs, aquaglyceroporins and superaquaporins.

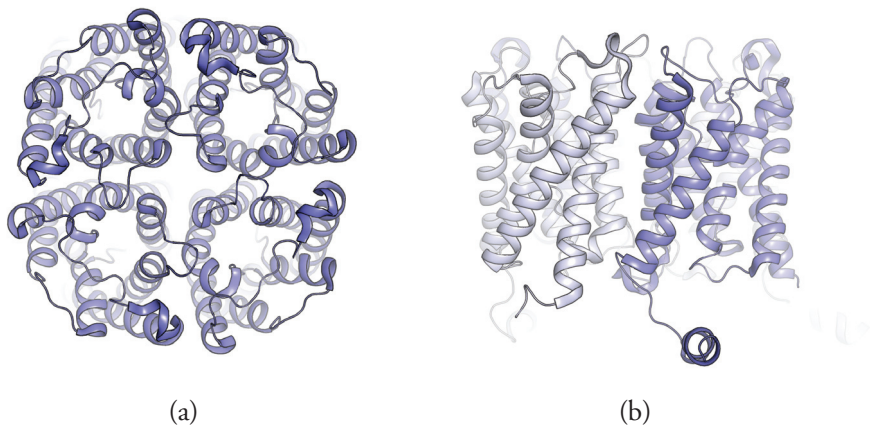
The classical AQPs have in general specialised in *only* transporting water, whereas aquaglyceroporins are capable transporters of both water *and* glycerol in *e.g.* adipose tissue, and superaquaporins function as water channels within specific cells [7].

[3]Or rather, the more successfully adapted assert dominance.

	Class	*Location*	*Role*	*PDB*	*Ref*
AQP0	I	Eye lens	Poor water channel, junction protein		
AQP1	I	CNS, kidneys, ear, eye, erythrocytes, muscle, lung	Water transport	1IH5	[8]
AQP2	I	Kidneys, ear, reproductive tract	Water re-absorption from urine in kidney	4NEF	[9]
AQP3	II	Skin, kidneys, lungs muscle, GIT			
AQP4	I	Brain, spine, heart, lungs	Swelling in oedemas, can form arrays	3GD8 2D57	
AQP5	I	Secretory glands, lungs, ear, eye, kidney, GIT	Mucous excretion	3D9S	[10]
AQP6	I	Ear, kidney	Poor water channel*		[?]
AQP7	II	Adipocytes, ovaries, testes	Junction protein*	6QZI 8AMX	[11] [12]
AQP8	I	Pancreas, liver, adipose tissue, GIT, reproductive tract	Water and ammonia transport		[13]
AQP9	II	Adipose tissue, ear, liver, CNS*, reproductive tract	Water and urea transport		
AQP10	II	Adipose tissue, reproductive tract	pH-gated glycerol flux	6F7H	[14]
AQP11	III	Kidney, liver, brain, ear, adipose tissue, reproductive tract	Unknown, essential for kidney development		[15]
AQP12	III	Pancreas	Unknown		[7] [15]

Table 1: Summary of known location, function, and structure (if known) of human aquaporins. Classes as follows: I - Classical aquaporins, II - Aquaglyceroporins, III - Superaquaporins. *Speculated.

As all aquaporins to some degree are selective toward water, and thus perform similar tasks, they also evolved to share certain chemical and structural traits. They all consist of four identical monomers, thus forming a *homo-tetramer*, which consist of six transmembrane α-helices with interconnecting loops, two of which form smaller α-helices which join half way through the membrane (Figure **??**). These helices form a pore in the centre of each monomeric structure, which contains two highly conserved NPA-motifs at the mid-point, protruding from each of the smaller helices (with exceptions for AQP11 and AQP12, which have one helix containing the NPA-motif, and the other either NPC or NPT, respectively), and aromatic-arginine motif close to the mouth of the pore. The aromatic-arginine motif acts as an initial selectivity filter, and in the case of aquaglyceroporins, the radius of said filter is larger in order to facilitate transport of glycerol [16]. The NPA-motif acts as a water selectivity filter, aligning water molecules one by one to pass through the pore in an orderly fashion.



(a) (b)

Figure 1: Extracellular view (a) and side view (b) of a typical AQP, here AQP2 (PDB:4NEF). From the extracellular view, one has a clear view of the water channelling pores of each monomer.

AQPs may be regulated in a multitude of ways [17]; some are regulated via pH-dependent gating or phosphorylation-dependent gating, others via a range of interacting proteins which are involved in the localisation of the AQP, including ezrin [18], LIP5 [19], calmodulin [20].

Aquaporins are not necessarily required to be active at the cell membrane at any given moment. Instead, evolution has resulted in aquaporins being able to either be regulated through pH-dependent gating [21] [22] [23], or by physically

removing them from the membrane via trafficking [24].

The mechanisms of AQP trafficking are rather well known for some of the proteins in the family, where post-translational modifications, such as phosphorylation (which may be regulated via hormonal signals) [25] or glycosylation, allow for both quality control and directed transport to the membrane where it needs to be put into use. In the case of aquaporin-2, once the physiological need for it to be present at the membrane subsides, it is dephosphorylated and either recycled via storage vesicles, degraded, or in some cases expelled via exosomes [26].

Since the discovery of AQPs in 1992 by Peter Agre [27] [4], they have been thoroughly studied and been the focus of many careers, including that of yours truly.

[4]Although some may argue Gheorghe Benga stumbled upon it first. [28, 29, 30, 31]

Aquaporin-0

Aquaporin-0 (AQP0) is a rather poor water channel [32], which is highly expressed in the lens of the eye. Here it has two functions; it regulates the microcirculation within the lens fibres, and upon maturation it is truncated [33], allowing it to form tight junctions with AQP0 in neighbouring cells. The circulation ensures that the cells within the lens are provided with oxygen and nutrients, as well as the removal of metabolites [34], whereas the junctions that mature AQP0 form not only keep the cells adhered to each other; they also ensure that the space between cells within the lens is kept at a constant distance which is smaller than that of the wavelength of visible light [35].

AQP0 has been reported to be regulated by pH [36] and by interactions with calmodulin [37], which in turn is influenced by the level of phosphorylation of the C-terminal domain of AQP0. Phosphorylation of AQP0 occurs at residues S229, S231, and S235 [38], and varies depending on the maturation of AQP0 and how far from the centre of the lens the cell is located [39].

We wished to explore the impact of the specific residues involved in the phosphorylation of AQP0 on its interaction with calmodulin, and what significance it would have for the transportation of water within the lens (Paper III).

Cataracts

Cataracts are often the consequence of mutations or other issues relating to the function, structure, or regulation of AQP0. Since the circulation within the eye is predominantly regulated via AQP0, reduced water channelling capabilities may result in buildup of metabolic waste within the lens. Similarly, if the junction properties of AQP0 were to be affected, the crucial spacing between cells in the lens would deteriorate, resulting in diminished translucency of the lens [35].

Aquaporin-2

As we go about our lives, our kidneys are continuously filtrating metabolites from our blood into the urine, at a rate of 1L per hour. If we were to urinate at the same rate, we would rapidly succumb to dehydration.

Aquaporin-2 (AQP2) was the second aquaporin to be discovered, by Fushimi *et al.* in the wondrous year of 1993 [40]. It is situated primarily in the collecting duct of the nephrons in the kidney, where it plays a key role in hormonally regulated reabsorption of water from the urine, thus preventing dehydration. While other AQPs are present further upstream of AQP2 and reabsorb a majority of the

water[41], AQP2 is the only one of them which is responds to hormonal regulation upon dehydration (Fig. 2).

AQP2 is regulated in response to arginine vasopressin (AVP). AVP activates the vasopressin V2 receptor (AVPR2), triggering a signalling cascade, which through cAMP promotes the expression of AQP2, as well as initiates the translocation of AQP2 situated in storage vesicles by C-terminal phosphorylation. AQP2 may be phosphorylated at S256 [42], S261 [42], S264 [36], and T269 [43], which in turn influences the interactions with regulatory proteins, and affects the time during which it will be localised at the membrane [44].

Nephrogenic diabetes insipidus

Following disruption in the regulation of AQP2 in the kidney, be it due to the disruption affecting the vasopressin pathway or AQP2 itself, nephrogenic diabetes insipidus (NDI) ensues. NDI may vary in its severity, but the disease is always characterised by the patients inability to properly concentrate their urine.

NDI may be either acquired or genetic. Acquired NDI often happens as a consequence of lithium treatment (not uncommon in treatment of bipolar disorder), or metabolic disturbances (*e.g.* hypokalemia or hypercalcemia) [45].

Depending on the location of the mutation, and whether it occurs alone or is combined with either another copy or another point-mutation, it will be processed by the quality control mechanisms of the cell in different ways, and thus have different fates. Certain mutations end up stuck in the endoplasmic reticulum (ER), or is sent for endoplasmic-reticulum-associated protein degradation (ERAD) [46], or might even be assumed functional, but to a lower degree [47, 46, 48].

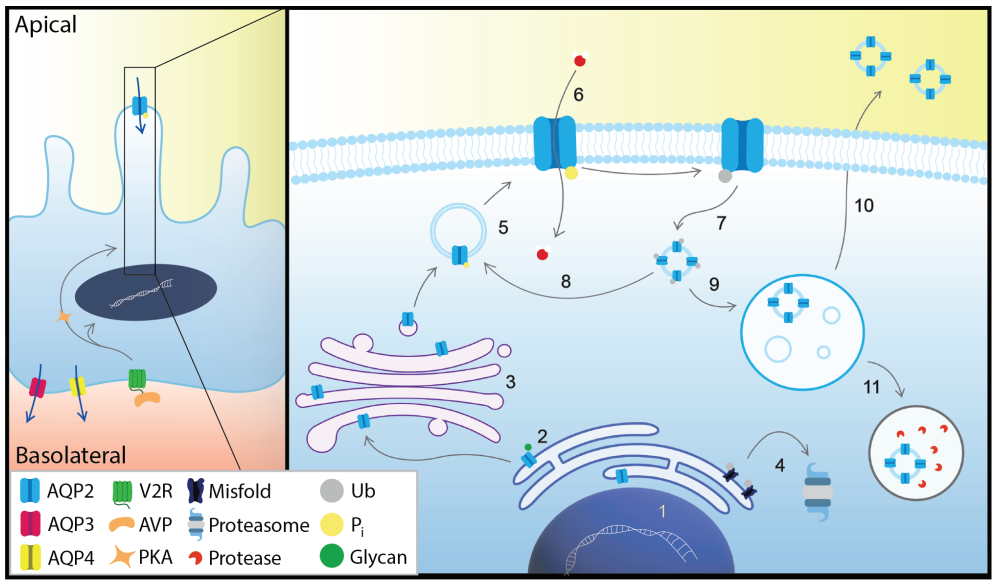


Figure 2: AQP2 expression is stimulated via AVP activation of AVPR2 (1). As AQP2 is expressed, it is co-translationally glycosylated in the ER (2), where it also undergoes initial quality control mechanisms. If it passes the control, it proceeds into the Golgi, where the glycosylation is further matured (3), whereas misfolded protein is directed to the proteasome for degradation (4). After passing through the Golgi, AQP2 is relocated into storage vesicles, awaiting phosphorylation, which in turn will target it for translocation into the apical membrane(5). Once in the apical membrane, it reabsorbs water form the urine, concentrating it (6). The absorbed water re-enters the blood via AQP3 and AQP4, located in the basolateral membrane. When the body deems hydration to be sufficient, AQP2 is downregulated, and is thus dephosphorylated and ubiquitinated, promoting endocytosis (7), and either recycled (8) or relocated into multivesicular bodies (MVBs) (9). Once in MVBs, it is either removed via exosomes (10), or degraded in the lysosome (11).

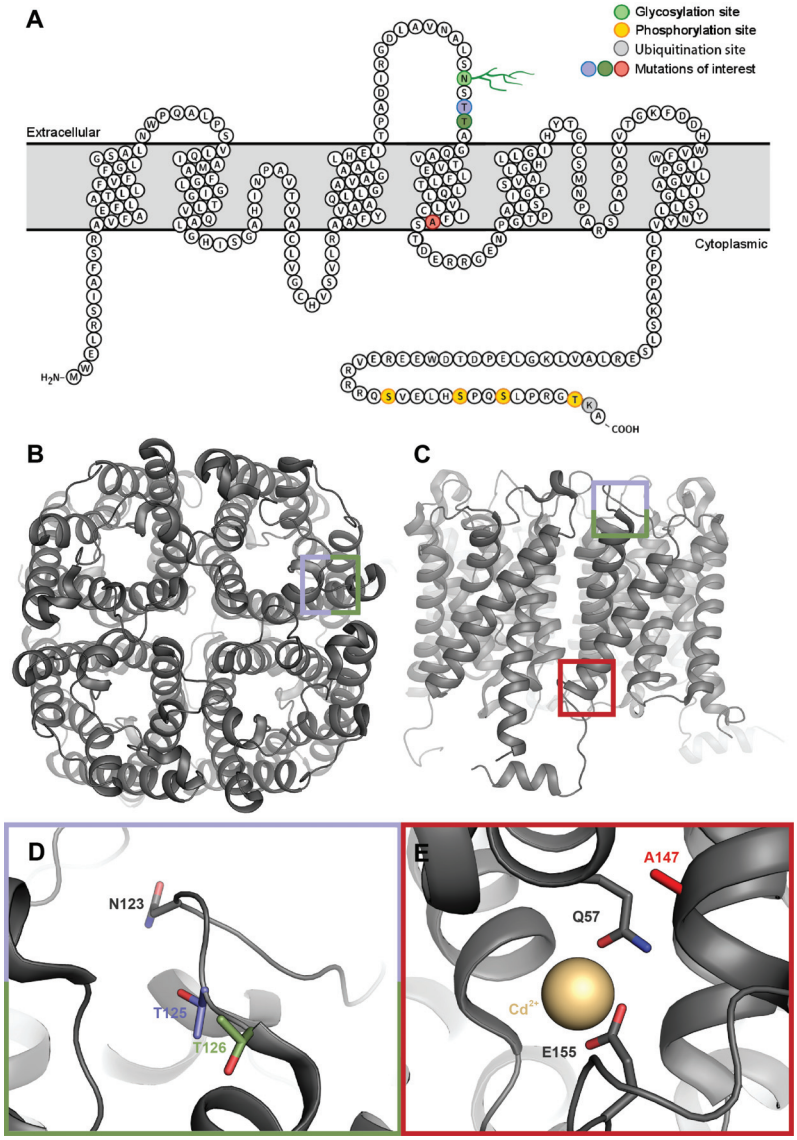


Figure 3: A) Overview of the domains of AQP2, with glycosylation and phosphorylation sites indicated, as well as mutated residues studied in Paper IV (T125, T126, and A147). B) Extracellular view of AQP2. C) Side view of AQP2. D) Close-up of loop C, with residues involved in the N-linked glycosylation process, as well as the mutation sites studied (T125 and T126), indicated. E) Close-up of the Cd^{2+} binding site, the location of A147.

Residue	Mutation	Domain	Reference
M1	I	N-term	[49]
A19	V	TM-I	[50]
L22	V	TM-I	[51]
V24	A	TM-I	[52]
L28	P	TM-I	[46]
G29	S	TM-I	[49]
A47	V	TM-II	[53]
Q57	P	TM-II	[54]
G64	R	Loop B	[55]
N68	S	Loop B	[56]
A70	D	Loop B	[57]
V71	M	Loop B	[46]
R85	W	TM-III	[58]
A86	V	TM-III	[59]
G96	Q	TM-III	[60]
G100	R/V	TM-III	[61, 54]
I107	N	Loop C	[62]
T108	M	Loop C	[63]
T125	M	Loop C	[64]
T126	M	Loop C	[56]
A130	V	TM-IV	[65]
L137	P	TM-IV	[66]
A147	T	TM-IV	[56]
D150	E	Loop D	[67]
V168	M	TM-V	[58]
G175	R	TM-V	[64]
G180	S	TM-V	[61]
C181	W	Loop E	[51]
N184	H	Loop E	[65]
P185	A	Loop E	[46]
R187	C/H	Loop E	[68, 57]
A190	T	Loop E	[64]
V194	I	Loop E	[46]
G196	D	Loop E	[69]
H201	Y	Loop E	[70]
W202	C	Loop E	[71]
G211	R	TM-VI	[70]
G215	C/S	TM-VI	[67, 72]
S216	P/F	TM-VI	[68, 73]
K228	E	C-term	[52]
R254	Q/L/W	C-term	[74, 75, 76]
E258	K	C-term	[56]
P262	L	C-term	[64]
R267	G	C-term	[77]

Table 2: Known NDI-causing point-mutations of AQP2, reported in patients, and which region of the protein they are situated in.

Among the point mutations studied in NDI, certain mutations seem to have a fate within the cell which may not represent their functionality. We elected to study point mutations T125M, T126M, and A147T, which have previously been highlighted as targets of interest to better understand the underlying mechanisms of congenital NDI (Paper IV).

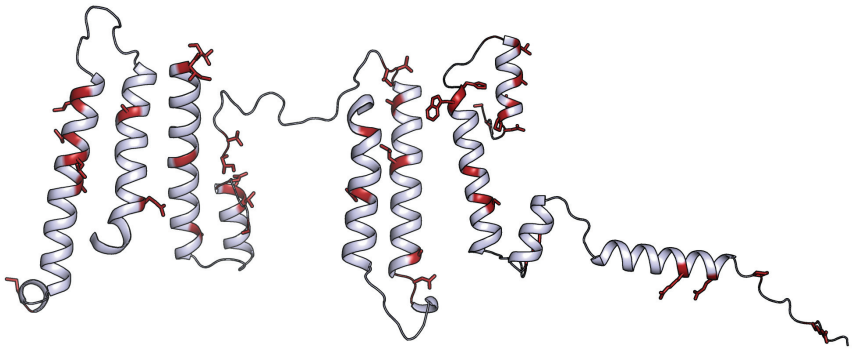


Figure 4: AQP2 splayed out, with point mutations found in NDI patients indicated in red.

Aquaporin-5

Aquaporin-5 (AQP5), much like aforementioned AQPs 0 and 2, is a water-selective channel. Unlike the other two, it is highly involved in the production of saliva, sweat, and tears (and pulmonary secretions)[78].

It is regulated via translocation to the membrane in response to hypotonic conditions and phosphorylation [79], although the exact mechanisms behind its regulation are not very well understood.

In order to better understand the trafficking and regulation of AQP5, we investigated its interactions with ezrin, and how this interaction might look on a molecular level (Papers I and II).

Sjögren's syndrome

Sjögren's syndrome primarily affects the exocrine glands of post-menopausal women, and may lead to acute dryness of eyes and mouth, as it often affects the salivary and lacrimal lands the most.

The importance of AQP5 in these tissues has been reported [80], especially for the production and excretion of saliva and tears. It has also been reported that patients with Sjögren's syndrome show abnormal localisation of AQP5 within their lacrimal and salivary glands [81, 82]. As AQP5 is regulated through trafficking [79], it is likely that the interaction with a regulatory protein may be at fault.

AQP5 is also involved in the pathology of *(diffuse) palmoplantar keratoderma* [83, 84] and *atopic dermatitis* [85].

Proteins involved in aquaporin regulation

Ezrin

In order to traffic membrane proteins, such as aquaporins, along the complex network of scaffold proteins, such as actin, within the cell, cargo-binding proteins may be employed. One such group of proteins is the Ezrin, Radixin, and Moesin family, also known as the ERM-family, which is essential for cell morphogenesis, polarisation, motility, and adhesion. The ERM-family proteins share a high level of sequence identity (>70%), and consist of three domains; a C-terminal actin-binding domain, a linker domain, and an N-terminal *FERM-domain* (named after high levels of conservation within the ERM-family, as well as the oddly named Protein 4.1. These ERM proteins are highly involved in vesicle trafficking and maturation, and reorganisation of the cytoskeleton [86].

Ezrin is composed of three main domains: a C-terminal domain which tethers to the cytoskeleton, a long linker domain, and the N-terminal FERM domain (Figure 5), which binds to amphipathic helices [87], such as those occurring in certain AQPs C-terminal domains, and it has been shown that knockdown of ezrin results in membrane accumulation of AQP2 as a result of inhibited endocytosis [18]. We were intrigued to see whether we could shed some light on these interactions, in particular with AQP2, and AQP5, resulting in Papers I and II.

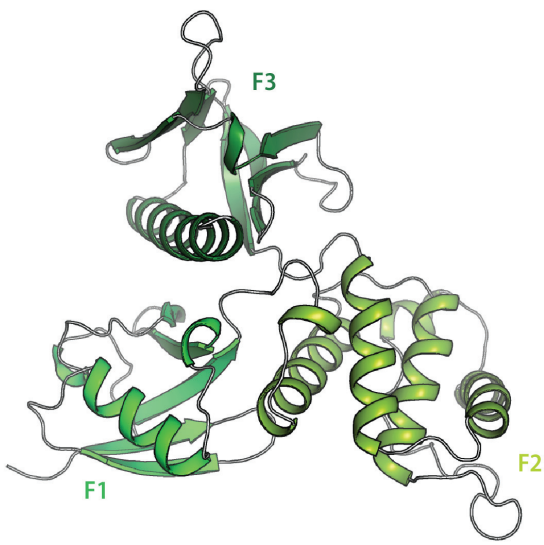


Figure 5: Overview of the ezrin FERM-domain and its subdomains.

Calmodulin

Calmodulin is a small ubiquitous helical protein, involved in a vast amount of regulatory processes throughout the body. It consists of two EF-hand motifs, connected via an extended helix, which are highly dependant on calcium binding for the functionality of the protein [88]. Upon binding calcium, calmodulin undergoes a conformational change, unfolding the two EF-hand motifs from the connecting helix, allowing it to bind with interacting partners.

Calmodulin has been shown to bind to AQP0, AQP4, and AQP6 [89, 90, 91].

Methodology in membrane protein biophysics

Protein expression and purification

In order to study proteins, it is necessary to find ways to isolate them from the source material. For certain applications and for certain proteins, it is enough to prepare them from tissue samples, *e.g.* AQP0 has been successfully purified from sheep eye lenses [92], and microtubuli can be prepared by putting calf brains in a blender [93][5].

In most cases however, it is necessary to introduce the gene sequence of the protein of interest into a new host, most commonly *E. coli*, *Saccharomyces cerevisiae*, *Spodoptera frugiperda*, or *Pichia pastoris*. This is done through selection of a suitable circular DNA vector (*plasmid*), which is then cut open with endonucleases, and the gene of interest may be inserted. Once the plasmid containing our insert has been constructed, it may be inserted into a host. The gene may then be expressed through some trigger, and protein produced and further purified.

In the case of aquaporins, we have chosen to express them in a particular version of *Pichia pastoris*, with an AOX1 promoter. This allows us to increase the biomass of *P. pastoris* by feeding it glycerol until it has reached a level we deem high enough (usually for 24 hours), after which protein expression may be induced by switching the feed to methanol. After 48-72 hours of induction, the cells are harvested.

From the harvested cells, one may now isolate the membranes through lysis via BeadBeater (a type of high-power blender with 0.1 mm glass beads breaking the cells) followed by a series of centrifugations and homogenisation into buffers which are suitable for the protein of interest, but also harsh enough to remove

[5]Rajiv has described that this is the reason he no longer finds brain palatable. Understandable.

unwanted impurities (such as proteases).

The isolated membranes are then solubilised using a suitable detergent, after which there are a few options with which to proceed. When constructing the gene sequence for the protein of interest, one must consider which methods of liquid chromatography techniques one wishes to utilise. Commonly, one may introduce a repeat of histidines at either end of the protein sequence, which have a high degree of affinity for binding to Ni^{2+} ions. Thus, once bound to a column containing immobilised Ni^{2+} ions (*immobilised metal ion affinity chromatography*), one may out-compete their interaction, and thus *elute* the protein, by applying a (sometimes step-wise) gradient of imidazole.

Further purification may be performed by isolating the correct conformation of the protein, or remove any remaining impurities, through *size exclusion chromatography*. As the name implies, the method isolates particles according to their size, making them pass a porous stationary phase, trapping smaller molecules for longer and letting larger ones pass through faster.

We now have protein to conduct studies on!

Structural methods

A handful of different methods have been developed in order to perform structural studies of proteins. *Nuclear magnetic resonance imaging* (NMR) allows dynamic studies in solution, but sacrifices the size of the protein that can be investigated. *Electron microscopy* (EM) was for a long time good at getting low resolution images that could hint at the overall structure of larger proteins or complexes, similarly to *small angle x-ray scattering* (SAXS), although SAXS is performed in solution (and uses X-ray diffraction), thus arguably in more ”native” conditions. However, *cryo-EM* has made significant advances toward atomic resolution during the last decade.

Cryo-EM allows a rather simplified approach for the sample preparation compared to what most structural biologists have been used to, with much lower sample volumes and concentrations needed than previously, but still suffers from the fact that it is rather difficult to predict how a protein will behave on the grids that are used for the measurements [94], the protein cannot be too small, and the computational resources required for the processing of the data are vast and not the easiest to come by for all researchers. I was curious to see if there was any work done on the distribution of particles across the grids, and what sample concentration would be optimal for a protein of a given size, and did a quick survey of

the Protein Data Bank (PDB) [95]. As the standard is moving from .pdb-files to .cif-files for structure submissions, it is possible to include more data on the experimental conditions in the structure file itself. By screening all deposited structures since 2014 of higher resolution than 4Å (5824) for submissions containing information on the sample concentration (2237) and converting the concentration from mg/ml to mM, Figure 6 was produced. There does indeed seem to be a certain correlation between what concentration works for a protein of a given size, but considering that only 38% of structures had the information necessary, a more rigorous study would need to be conducted (potentially also taking the blotting method used into account).

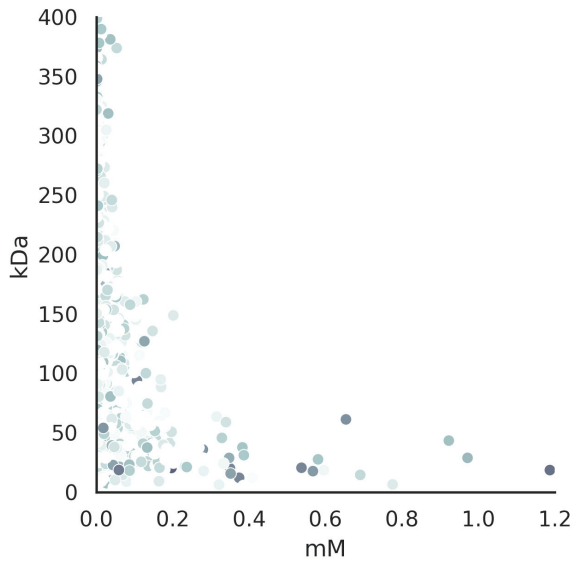


Figure 6: Distribution of PDB entries using CryoEM yielding structures of $\leq$ 4Å in relation to their size and concentration of sample applied to the grid. Data procured 2022-05-13.

But what if we want to study a protein of any size, and maybe even allow for studies on conformational changes[6]? Luckily, great minds came up with a solution for that during the first half of the previous century [96, 97, 98]: Protein X-ray crystallography.

[6]Although, NMR is usually a preferred method in this case.

X-ray crystallography

"If no crystals form, dump the samples in the sink and curse the darkness."
- Alexander McPherson, 1982 [7]

If you were to leave a drop of water containing regular table salt (NaCl) to dry at 25°C, you would eventually see small salt crystals appear. This is a consequence of the water content in the drop diminishing, thus increasing the concentration of salt in the drop, and at a certain point it will reach the *solubility limit* for NaCl at the given temperature (357 mg/ml), and the molecules would start to fall out of the solution, or *precipitate*. If the precipitation occurs in such a way that the molecules may start to form homogeneous structures, which are identical along all axes of the structure, a crystal forms.

For a small molecule like NaCl, weighing 58.443 g/mol (or *Dalton* (Da)), this is nothing out of the ordinary. For a molecule weighing thousands of times more, like a protein, this is indeed rather out of the ordinary. Proteins are complex molecules, comprised of long chains of amino acids, all with their unique chemical properties adding to the properties of the different sections of the protein, and all behaving differently in relation to solvents (such as water) and hydrophobic environments (such as lipids or other hydrophobic residues). As such, protein crystals are not the easiest to form, and often take many months (or years) to produce successfully. Even then, they may not diffract optimally, and the process may start anew with further optimisation. McPherson's words of encouragement shed a touch of levity over the field, which I believe many of us can relate a bit too well to...

Growing a protein crystal

Once one has purified the desired protein and concentrated it to an arbitrary concentration, one may decide how to approach the crystallisation. There are a few different methods one may employ, which all evolve around different approaches with which how to push the concentration of protein into the *nucleation zone* of the *phase diagram* (Figure7). The most common method is vapour diffusion [99], which is usually performed as a *hanging drop* or *sitting drop*. In this setup, a reservoir containing selected reagents is situated at the bottom of a well, over which one either *hangs* (attached to a cover slip) or *sits* (commonly on a small elevated

[7]Preparation and Analysis of Protein Crystals, McPherson, A. (1982). New York: John Wiley & Sons.

platform) a drop of protein solution mixed with reservoir solution and desired additives. As the drop now contains a lower concentration of the reagents found in the reservoir, water will (as vapour) gradually diffuse from the drop into the reservoir, thus bringing the protein concentration toward *supersaturation*. Here, depending on the nature of the protein in relation to the conditions used, the protein commonly form disordered precipitate. However, if the conditions are *just* right, the protein will enter the *nucleation zone*, and form crystals. Once a crystal has begun to form, more protein molecules will be incorporated due to the crystal being an energetically favourable state in relation to protein in solution, and thus the crystal will grow. If many small crystals form, they may sometimes shrink in favour of the growth of a larger one, in a process known as *Ostwald ripening* [100].

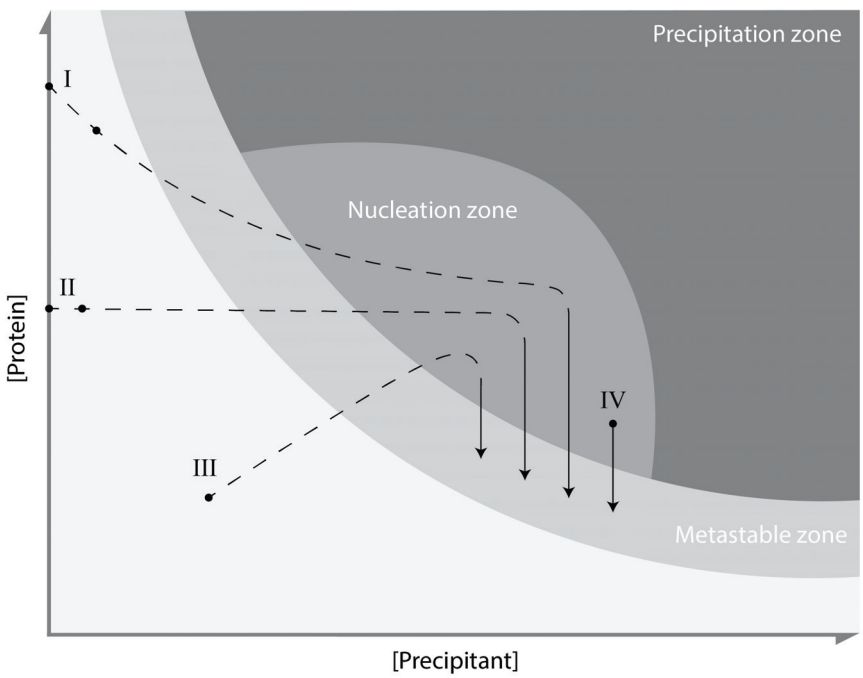


Figure 7: Diagram over how different crystallisation methods function in relation to protein and precipitant concentration. I: Batch, II: Dialysis, III: Vapour diffusion, IV: Free interface diffusion. Adapted from Chayen (2004) [101].

The perfect crystal

In a perfect world, all protein crystals would be approximations of salt crystals in their crystal packing; well organised, highly symmetric, and well diffracting. When working with soluble proteins, or the occasional (and rare) well-behaving membrane protein, this might sometimes (almost) be the case. In theory, this would mean that all protein molecules composing the crystal are homogeneously dispersed in a well ordered manner, with low levels of solvent between the molecules, strong crystal contacts, and hopefully leading to *Bragg peaks* at subatomic (<1Å) resolution. Such a lattice may look a lot like Figure 12A.

Space groups

When molecules are arranged into a crystal lattice, they may assume to one of 230 different ways of packing, called *space groups*. The space group essentially refers to the particular way the molecules have arranged themselves in relation to each other in Euclidian space through various symmetry operations (mirroring, chirality, rotation, or inversion). If view a *unit cell*, the smallest repeating unit of the crystal, it might contain a single molecule. If this crystal belongs to space group $P1$ (Figure 8), the next molecule within the crystal will be located at the same position and orientation in the neighbouring unit cells. However, if we look at a molecule in a more complex space group, things get a little harder to conceptualise, and so does the nomenclature.

Space groups are noted according to the following format,

$$XN_n$$

where X is the *Bravais lattice* (the facing of the molecule within the asymmetric unit; P for primitive (cell corners), I for body centred (one point is at the centre of the unit cell), A, B, or C for centring on on a particular face of the unit cell, R for rhombohedral, F for face centring (toward each face of the unit cell), N describes the rotation required to perform the symmetry operation ($\frac{1}{N}$; 3 would mean a 120° rotation), and n describes the translation required for the symmetry operation in relation to the lattice vector.

Thus, a $P2_1$ crystal would indicate a primitive centring motif of the molecule (one per unit cell) with a twofold screw axis (180°) and a 1/2 translation of the lattice vector.

As proteins are entirely composed of L-amino acids, they are limited to which symmetry operations they can abide by. Thus, the number of various space groups

a protein may crystallise into reduces significantly, from 230 to 65, shown in Table 3.

Depending on how a particular protein is structured, what chemical properties and charges are distributed around its surface and what the particular crystallising conditions are, each protein may crystallise into a range of different space groups, depending on the crystal contacts that are possible to form under the given circumstances. Certain space groups (such as $I4$) may allow for a dense packing of the molecules, with little void space, whereas other space groups (such as $P4$) may result in a crystal packing with more void space. This space may be referred to as *solvent channels*, and thus may result in a higher solvent content of the crystal. Both of these space groups are represented in Figure 8.

Crystal system	Point group	Space groups
Triclinic	C_1	$P1$
Monoclinic	C_2	$P2$, $P2_1$, $C2$
Orthorombic	D_2	$P222$, $P222_1$, $P2_12_12$, $P2_12_12_1$, $C222$, $C222_1$, $F222$, $I222$, $I2_12_12_1$
Tetragonal	C_4	$P4$, $P4_1$, $P4_2$, $P4_3$, $I4$, $I4_1$
	D_4	$P422$, $P42_12$, $P4_122$, $P4_222$, $P4_322$, $P4_12_12$, $P4_22_12$, $P4_32_12$, $I422$, $I4_122$
Trigonal	C_3	$P3$, $P3_1$, $P3_2$, $R3$
	D_3	$P312$, $P3_112$, $P3_212$, $P321$, $P3_121$, $P3_221$, $R32$
Hexagonal	C_6	$P6$, $P6_1$, $P6_2$, $P6_3$, $P6_4$, $P6_5$
	D_6	$P622$, $P6_122$, $P6_222$, $P6_322$, $P6_422$, $P6_522$
Cubic	T	$P23$, $P2_13$, $F23$, $I23$, $I2_13$
	O	$P432$, $P4_132$, $P4_232$, $P4_332$, $F432$, $F4_132$, $I432$, $I4_132$

Table 3: List of all possible space groups proteins may crystallise into. Crystal systems indicate the geometry of the asymmetric unit. Point groups indicate the cyclic (C), dihedral (D), tetrahedral T, and octahedral O. Subscript number indicates the number of reflections or rotations. Taken from https://www.ccp4.ac.uk/ccp4-ed/misc/tables/ (accessed 2023-02-20).

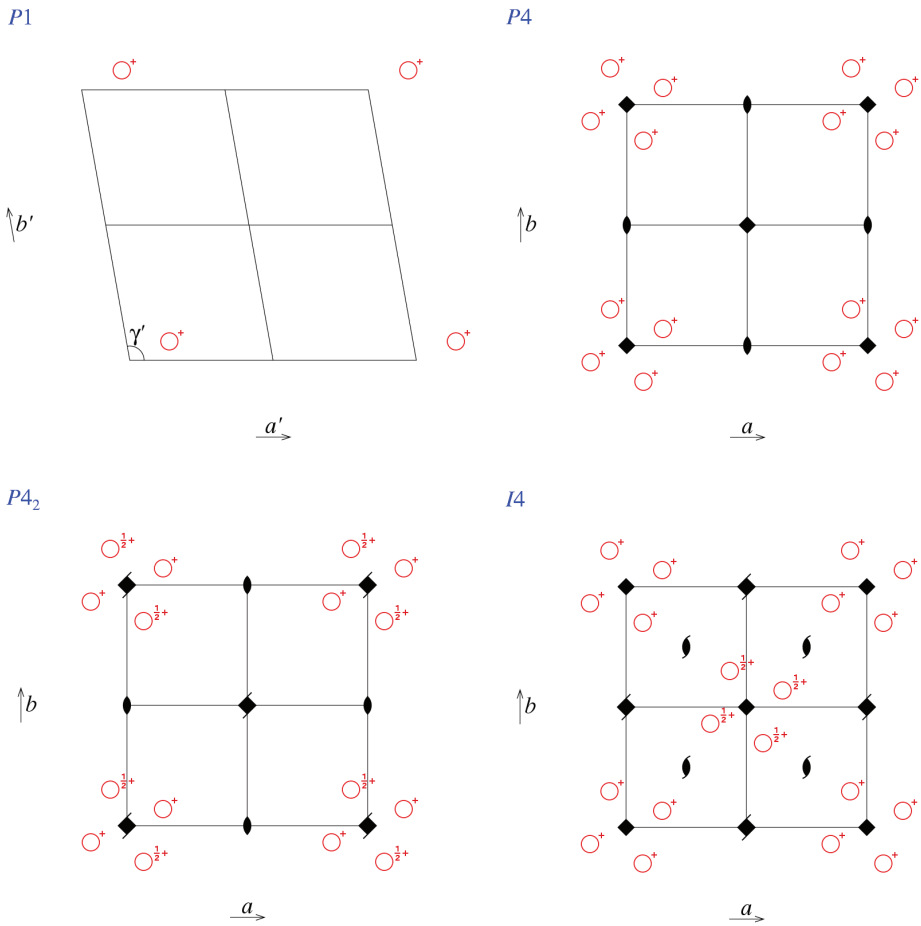


Figure 8: Space group diagrams of $P1$ (A), $P4$, $P4_2$, and $I4$. Images from *A Hypertext Book of Crystallographic Space Group Diagrams and Tables* [102].

The perfect diffraction

If one were to shine a focused beam of light through two thin parallel slits onto a wall, one would observe a rather interesting phenomenon; the light would create a pattern, as seen in Figure 10. If one were to increase the number of slits, the pattern would multiply, and if one were to add another dimension to the slits (thus making a grid of holes), the pattern would change further. The phenomenon occurs due to the wave properties of light, creating peaks and troughs. If two scattered waves have different phases, they may interact in such a way that the peaks of one overlap with the troughs of the other, thus resulting in no signal (destructive interference). If they overlap in such a way that both of their peaks occur at the same point in their phases, one will see a bright spot of light (constructive interference).

This was first shown by Thomas Young in 1804 [103], and is essentially the basis for modern X-ray crystallography, as a well ordered material, such as a crystal, functions as a grid. If light of a given wavelength (λ) passes through a plane of *Miller indices* (plane of Bravais lattice) in a crystal (h, k, l), a portion of the light will act as if interacting with a weak mirror, and is reflected at an angle (θ). When the distance between the planes of the crystal (d) are so that the difference in path length between two incoming waves of light (one being reflected at a higher plane than the other) is equal to a whole number (n) of wavelengths to the other, the conditions for *Bragg's law* [104] are met:

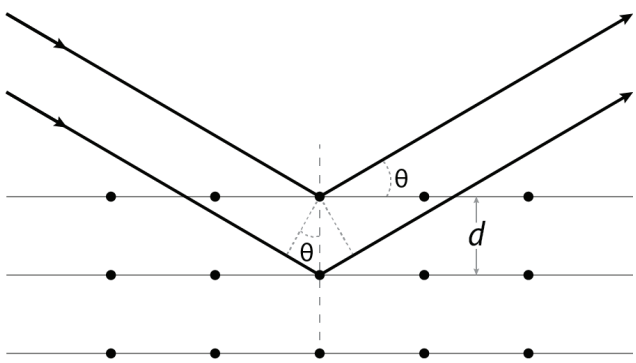


Figure 9: Conditions for Bragg's law. Adapted from *Protein Crystallography: A Concise Guide* [105].

$$n\lambda = 2d \sin\theta \tag{1}$$

For a given unit cell in a crystal containing a single atom at position x, y, z, it is possible to get the diffraction pattern via the following equation;

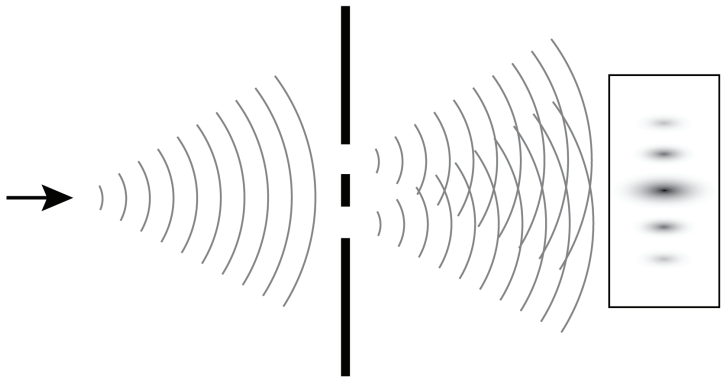


Figure 10: Depiction of the famous *double slit experiment*. An incoming wave of light passes through the two slits; the resulting waves will affect each other and produce *constructive interference* (spots), and *destructive interference* (spaces between spots).

$$\mathbf{F}(h,k,l) = f(h,k,l)e^{2\pi i(hx+ky+lz)} \tag{2}$$

where $f(hkl)$ is the atomic scattering factor. However, if we look at a complex crystal, such as those containing macromolecules, there will be a significant increase in the amount of atoms within each unit cell. In order to study these, we need to consider the sum of the diffracting atoms (N). Thus, we end up with something referred to as the *structure factor equation*;

$$\mathbf{F}(h,k,l) = \sum_{j=1}^{N} f_j(h,k,l)e^{2\pi i(hx_j+ky_j+lz_j)} \tag{3}$$

In order to calculate the densities of electrons within the unit cell (ρ), one may use the total number of electrons in the unit cell, given by the structure factor ($\mathbf{F}$);

$$\rho(xyz) = \frac{1}{V}\sum_{-\infty}^{\infty} \mathbf{F}(h,k,l)e^{-2\pi i(hx_j+ky_j+lz_j-\phi(hkl))} \tag{4}$$

where V is the volume of the unit cell, and ϕ is the phase. But how do we get the phase?

The phase problem

As the diffracted light hits the detector, we are able to visually inspect the diffraction pattern and intensities, and infer the amplitudes. All of these factors are necessary to solve equation 4, but what we cannot see, are the phases of the light. The phases contain the most essential information regarding what we are trying to observe; the three-dimensional information of the molecules we are studying. As one may see in Figure 11, it is possible to construct an image without the phases, but it will not be possible to extract much information from it. If we input incorrect phases (introducing *bias*), we end up with an image which we might think is correct, but in fact is not.

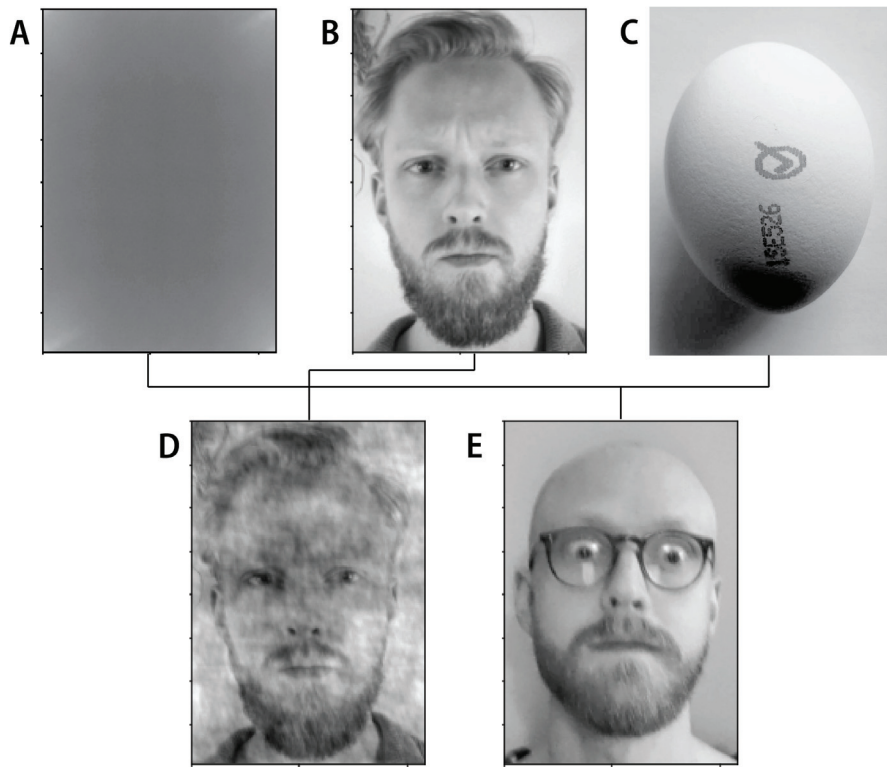


Figure 11: The importance of phase: If we have the amplitudes of an unknown object (A), and use the phases of a known, hypothetically similar object (B, *who has yet to start his PhD studies*), we might end up with what we think is a correct representation of the object (D). However, if we use the phases of an object which is *actually* more representative of the object, we will get a more successful reconstruction (E, *about to submit his PhD thesis*).

Over the last century, a lot of effort has been put into overcoming what has been come to be the *phase problem*. Methods such as SAD/MAD (single-/multi-wavelength anomalous dispersion) and SIR/MIR (single/multiple isomorphous replacement) use the presence of atoms which scatter at a higher angle than those naturally in a protein, and can thus be used to a high level of success in combination with a *Patterson map*, which utilises the *Patterson function*;

$$\mathbf{P}(u,v,w) = \sum_{hkl} \mathbf{F}(hkl)^2 e^{-2\pi i(hu+kv+lw)} \tag{5}$$

The Patterson function does not utilise the structure factors, but rather the intensities to calculate interatomic vectors within the unit cell. Thus, it is possible to map out the three-dimensional structure of the protein, however with a caveat: anomalous scattering *or* a known homolog may be used, however the former requires the addition of a heavy atom, and the latter requires input of a solved structure with minimum 20% homology. Using a homolog, one may perform *molecular replacement*, which allows the creation of a Patterson map of the homolog and overlapping it with the map of the unknown structure. This method is widely used, but has a risk of introducing model bias, as the generated phases may deviate from what is actually there. Therefore, it is important to approach this method bearing the risk of bias in mind.

Reality

Lattice imperfections and thermal motion

As we are dealing with macromolecular crystals, the likelihood for the crystal lattice to be entirely uniform and the symmetry operations to be absolutely representative of every single molecule diminishes, be it due to lack of stable crystal contacts, or higher solvent content of the crystal (as may happen in the case of certain space groups). Instead of the pristine lattice that we see in Figure 12A and B, instead we may be dealing with a lattice which looks a little bit more like Figure 12C and D.

As these deviations from the mean in the crystal structure (u^2) impacts the intensity of the scattered X-rays negatively ($|\mathbf{F}|$), it is necessary to implement considerations for these deviations in the structure factor equation, by taking atomic displacement into consideration, caused by thermal motion or imperfect unit cell alignment. We may implement the *temperature factor*, B ($B = 8\pi^2 u^2$), and thus end up with the *Debye-Waller factor*, and the structure factor equation can be adjusted accordingly;

$$\mathbf{F}(h,k,l) = \sum_{j=1}^{N} f_{j(h,k,l)} e^{-B\frac{\sin^2\theta}{\lambda^2}} e^{2\pi i(hx_j+ky_j+lz_j)} \quad (6)$$

Continuous diffraction

If there are imperfections in the crystal lattice causing irregularities, the resulting constructive diffraction is affected. There may not be as many successfully met conditions required for Bragg peaks to occur, and there may instead be a certain level of *diffuse*, or *continuous diffraction* occurring (see Figure12). This phenomenon has been known within the field of SAXS for a longer time, but within the realm of X-ray crystallography it is a relatively poorly studied topic. It was recently discovered that this continuous diffraction may contain structural information, and in conjunction with Bragg peaks may lead to even higher resolution data than merely by the implementation of Bragg peaks alone [106]. This continuous diffraction also contains information which has allowed *ab initio* phasing - thus allowing the structural determination of a protein without using methods introducing strong scatterers or molecular replacement; without introducing bias.

So far, this phenomenon has only been documented in a few model systems, but interestingly membrane proteins show potential for targets of this novel method, due to the crystals they form having a tendency to have larger solvent channels and higher solvent content.

There have been speculations regarding the core of this phenomenon, and what information may be extracted from it. Are there only rigid-body irregularities from which we can extract higher resolution information [107][106][108], or is there also potential for information relating to biochemically relevant dynamic motions of the protein [109][110]? Since the proteins are embedded in crystals, the latter would seem unlikely due to the unnatural environment a protein crystal unavoidably is.

Nonetheless, we wished to study the nature of continuous diffraction. Firstly, if it would be possible to observe it in novel crystal systems, and secondly if it could be affected via the addition of secondary additives (Paper V).

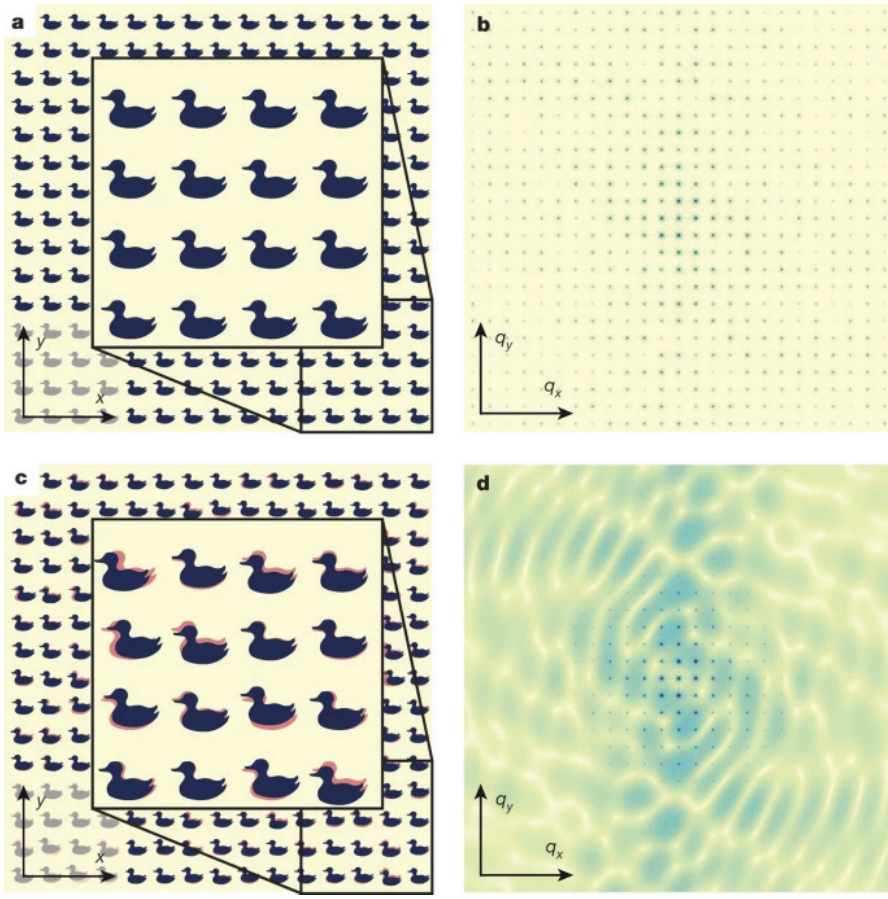


Figure 12: Example of diffraction patterns from a well ordered crystal lattice, versus the diffraction pattern from a crystal lattice with imperfections. From Ayyer et al. [106].

Characterising a protein - other biophysical techniques

Stopped-flow spectroscopy

In order to measure the functional aspects of a protein, one may employ different methods depending on what exact aspect of the protein one wishes to study. When it comes enzymes, enzymatic assays are a common approach.

When it comes to aquaporins however, we are interested in studying the capability of them to transport water across a membrane. This may be done by reconstituting them into lipid vesicles (*liposomes*), after which we can subject them to rapid mixing with an osmotic gradient, using *stopped-flow spectroscopy*. During the reconstitution, one may introduce a fluorophore into the liposomes, which will scatter differently depending on the swelling or shrinkage of the liposomes. One may also measure the scattered light from the liposomes without any fluorophore added. The increase in scattering during this rapid mixing may then be fitted with the following equation;

$$y = y_0 + A_1 e^{-k_1(x-x_0)} + A_1 e^{-k_2(x-x_0)} \tag{7}$$

where A is the amplitude of the scattering, and k is the swelling rate of the liposomes once exposed to the osmotic pressure. A_1 and k_1 are assumed to be representative of the passive water transportation through the aquaporin, whereas A_2 and k_2 are assumed to be representative of the passive diffusion across the lipid membrane.

Using k_1 from 7, one may calculate the *permeability factor* (Pf);

$$P_f = \frac{k_1}{\frac{S_0}{V_0} V_W C_{out}} \tag{8}$$

where S_0 is the initial surface area of the liposomes, V_0 is the initial surface volume of the liposomes, V_w is the partial molar volume of water ($18 cm^3/mol$), and C_{out} is the external osmolality from the mixing solution.

It is essential to correct for successful reconstitution of protein into liposomes, as there is no guarantee that all protein will be incorporated. This may be done in various ways, but I have found that running a Western blot and analysing the bands with ImageJ [111] allows for an easy approach. Integrate the resulting peaks, and either correlate them to each other or a known standard. The Pf values may thus be adjusted for the *actual* protein incorporated.

One of the main reasons to consider studies using proteoliposomes, is the simplicity of them. Many studies have been conducted using oocytes to investigate

the effect of *e.g.* osmotic gradients on a particular protein, but these studies will always include the entire oocyte, which actually is a rather complex entity. In contrast, a proteoliposome constructed with selected lipids and purified protein will be a relatively *true* representation of that particular protein under very precise conditions.

Circular dichroism

The secondary structure composition of a protein may be to some degree elucidated by measuring the absorption of polarised light due to their strict chiral nature (being composed of solely L-amino acids), using circular dichroism (CD). [8]

The ellipticity (θ) of a protein is usually measured between 190nm and 250nm, and the *mean residual ellipticity* (*MRE*) may be calculated as;

$$MRE = \frac{\theta \times MRW}{10 \times P \times C} \tag{9}$$

where MRW is the mean residual weight, P is the pathlength of the cuvette used for the measurement, and C is the concentration of the sample.

Typical α-helical proteins display characteristic minimas at 208nm and 222nm [113], whereas β-sheets display a minimum at 195nm.

While it may be interesting to investigate the exact secondary composition of a protein using this method, it heavily relies on very precise concentration determination of the protein sample, as shown in equation 9. However, as a method for determining the integrity and stability of the secondary structures of a protein, it may prove rather useful. As the temperature of a protein sample increases, the effect on secondary structure may be studied as the absorbance changes. At a certain point, 50% of the protein in a sample will be denatured. This point is referred to as the *melting point*, and the temperature at which this happens the *melting temperature* (T_m). The T_m of a protein may be calculated as follows;

$$\theta = \frac{(T\alpha_n + \beta_n) + (T\alpha_d + \beta_d)e^{\frac{\Delta G}{RT}}}{1 + e^{\frac{\Delta G}{RT}}} \tag{10}$$

where α_n and α_d are the native and denatured states at 0 K respectively, β_n and β_d are the slopes with respect to temperature of the native and denatured states

[8] Little is known regarding why this norm was established, but there have been recent advances in the synthesis of "mirror proteins" [112].

respectively, R is the ideal gas constant, and ΔG is the free energy of unfolding. Given these conditions, one may calculate the T_m using the Gibbs-Helmholtz equation, assuming that the heat capacity of the protein, $\Delta C_p = 0$;

$$\Delta G = \Delta H \frac{1 - T}{T_m} \tag{11}$$

where ΔH is the enthalpy, and T is the measured temperature.

It may also be of interest to compare the *onset* of the response to temperature, as merely looking at the T_m may in certain cases be misleading; if the rate of unfolding may be slower for protein A than for protein B, they may still have equal T_m. Thus, one may calculate when T deviates from the baseline, resulting in T_{onset} [114].

Differential scanning fluorometry

While circular dichroism uses the chirality of both proteins and light, differential scanning fluorometry (DSF) uses excitable fluorescent compounds with various chemical properties, most often hydrophobic. The concept is rather simple; as a protein is subjected to increasing temperatures, it will eventually unfold. As it unfolds, the residues normally buried within the protein are exposed, and the fluorescent compound may bind to them. As the compound binds, the intensity of its fluorescence decreases proportionally to the amount of exposed residues. The resulting curve may be fitted with equation 10, and the T_m calculated with equation 11.

What if we want to measure the stability membrane proteins? Suddenly the approach using hydrophobic compounds no longer works, as membrane proteins are inherently hydrophobic along their membrane-embedded regions. Lately, there have been significant advances in rather convenient ways of circumventing the need of a fluorophore, while also offering speed and convenience; *nano*DSF.

NanoDSF uses the intrinsic fluorescence of the amino acid tryptophan, which upon unfolding will change in intensity for both soluble proteins and membrane proteins, while also allowing data to be collected in just over and hour *and* using only $< 10\mu l$ sample. Beautiful.

Microscale Thermophoresis

There are various ways of studying the energies of protein-protein interactions, but microscale thermophoresis (MST), is probably the most convenient method I have

encountered. The sample preparation consists of labelling one of the hypothesises interacting proteins with a fluorescent dye, after which one creates a titration series with the other protein. Each sample in the series is then loaded into thin glass capillaries, each housing $10\mu l$. Provided the operator has dispensed the components of the samples with sufficient precision, the initial scan of the capillaries will show the fluorescence of each sample. If there are any larger deviations, the sample preparation must be redone. Once the capillary scan is successful, measurements may commence.

The measurements are performed by heating a central spot of the capillary, using an infra-red laser. As the sample heats up, the measured fluorescence of the labelled protein changes according to the speed at which it moves either away from the locally heated area. This speed depends on the size, conformation, charge, and hydration shell of the protein, which would all be affected if there indeed is an interaction between the two proteins (and both of the interacting proteins are sufficiently large enough to affect these properties of the other). Larger molecules, or complexes, move slower than smaller molecules. The difference in this *thermoporetic mobility* within the concentration series of the sample (F_{norm}) may thus be plotted in relation to the ligand concentration, using the local florescence of the heated spot *before* (F_{cold}) and *after* (F_{hot}) heating;

$$F_{norm} = \frac{F_{hot}}{F_{cold}} \tag{12}$$

The resulting plot may be fitted using the following equation;

$$y = S_1 + (S_1 - S_2)\left(\frac{L_{free}}{L_{free} + K_D}\right) \tag{13}$$

where S_1 and S_2 are the measured signals from the unbound and bound states of the labelled protein, respectively, K_D is the dissociation constant, and L_{free} is the concentration of free ligand, defined by;

$$L_{free} = 0.5(L_{tot} - P_{tot} - K_D) + \sqrt{\frac{(K_D + P_{tot} - L_{tot})^2}{4} + L_{tot}K_D} \tag{14}$$

where L_{tot} is the total amount of ligand, and P_{tot} is the total amount of the labelled protein.

In silico approaches

Even though the amount of protein structures deposited to the PDB since its inception has increased almost exponentially every year, the amount of work required to produce merely a single protein structure still remains rather vast. As discussed previously, obtaining a protein crystal can take months, or in some cases years [], and even when the crystals do appear, they may not provide sufficient diffraction. Even in these days with increasing use of CryoEM, sample optimisation is needed, and once the data has been acquired there may be issues with processing the terabytes of data (which may end up showing preferred orientation).

Alongside the work of classical wet lab scientists, *in silico* researchers have been using the empirical data achieved in the wet lab to guide their attempts at creating algorithms which may predict the fold of a protein from its amino acid sequence, with the goal of eventually being able to avoid the arduous work that goes into solving structures historically and today.

As nature has developed such a vast set of proteins in all living organisms (*and viruses* from merely *21* amino acids, there is bound to be a certain level of similarities between certain regions of proteins which possess similar properties.

Certain secondary structures have certain combination of amino acids that allow them to form in the environment in which they exist. For example, alpha-helical structures for soluble proteins likely have an amphipathic composition, with hydrophilic residues exposed toward the cytosol, and hydrophobic residues buried inside the structure, away from the cytosol.

If we look at aquaporins, there are multiple sites that have a degree of conservation between them, but they all have the central NPA-motif (with a slight variation in AQP11 and AQP12, as mentioned previously). Thus, if a protein has this motif within a central pore, it would not be unreasonable to assume that this is used for water selectivity.

Furthermore, if we look at regions that may be used for post-translational modifications or protein-protein interactions, we may be able to figure out how the protein interacts with its environment, and how it might be regulated. If we wish to study the nature of these interactions, we may choose to do so experimentally through *i.e. proximity ligation assays*, *microscale thermophoresis*, *isothermal titration calorimetry*, or via any of the structural methods discussed previously. However, if we lack prior knowledge of these interactions, and if we want to study importance of particular amino acid residues, it would be very useful if we could perform predictions as to how these interactions form structurally. These predictions may

vary depending on what tool we decide to use, and how they are constructed.

One tool which is commonly used is the online docking server HADDOCK (*High Ambiguity Driven protein-protein Docking*) [115], developed by the group of Alexandre Bonvin developed a web server for online protein-protein interaction predictions based on the docking of rigid structures to each other while aiming for low overall energy levels, achieved through buried hydrophobic residues and appropriate polar interactions where applicable. The caveat is that prior knowledge of the interaction is required, and a slight variation in the postulated interaction site may lead to significantly different results.

Another approach has been used by David Baker and cohorts, in their software Rosetta [116]. Rosetta focuses on protein structure prediction, and rigid body docking for protein-protein interactions. The rather intriguing aspect of Rosetta is the rather large and interactive community, leading to developments for more flexible alpha carbon structure prediction for protein-protein interactions, such as EvoDOCK [117].

AlphaFold

Protein structure prediction algorithms have been bench marked against each other at biennial events named *Critical Assessment of Techniques for Protein Structure Prediction* (CASP) since its inception in 1994.

At the 13th event, CASP13, in 2018, a new competitor entered the scene. DeepMind, with their artificial intelligence oriented software *AlphaFold*, performed vastly better than the competition, and with AlphaFold 2 they scored even higher in 2020 at CASP14, almost well enough to be considered as good as experimentally determined structures [118].

While AlphaFold is seemingly doing rather impressive progress for singular protein prediction, it is uncertain whether the specificity for the modelling of protein-protein interactions is sufficient for replacing experimental work. For even more complex structures, such as disordered regions or *intrinsically disordered proteins*, the predictions are simply not good enough. Nonetheless, it can provide data which may be used in *conjunction* with experimental data, maybe even confirmed. Nonetheless, since AlphaFold relies on a a deep learning algorithm, it is only as biased as the data it has been trained upon, and serves as a rather good initial estimation for interactions for which there is little prior information for, but which shares properties with previously solved structures.

At its current stage, AlphaFold has evolved to be a rather useful tool for experimentalists for the design of experimental studies, and likely will become an ever

increasingly important tool for everyone involved in the field of structural biology, be it *in vitro* or *in silico*.

Results & Discussion

Aquaporin regulation

Paper I: Aquaporin-5 interactions with ezrin

AQP5 was confirmed to interact with ezrin via proximity ligation assays in salivary gland cells, as well as through immunoprecipitation and LC/MS-MS. As the C-terminal domain of AQP5 is highly similar to that of AQP0 and AQP2, which have both been suggested to interact with ezrin via this domain [119, 18], it was assumed to be the main interacting site.

In order to investigate the nature of the interaction, *in silico*, it was necessary to predict the structure of the C-terminal domain of AQP5, as it has yet to be experimentally determined in its entirety [10]. We utilised Robetta [120] in order to predict the full-length AQP5, resulting in two models; one predicted using the crystal structure for AQP5 (which does not show the full C-terminal domain due to its flexibility) [10], and one predicted using TrRosetta. The resulting models were used for docking simulations using PyRosetta [121], by generating 1000 decoys. The highest scoring predictions were further analysed using HADDOCK [122] and PRODIGY [123]. The results were then compared with the structures of comparable structures where a helical peptide interacted with a FERM domain: *Mus musculus* NHERF-1 (PDB:2D10) and NHERF-2 (2D11) C-terminal peptides in complex with radixin-FERM [124], *Drosophila melanogaster* merlin C-terminal in complex with merlin-FERM (PDB:7EDR) [125], and human NHERF-1 in complex with moesin-FERM [126].

The predicted interactions between AQP5 and ezrin-FERM were shown to be rather similar to those of the aforementioned helical peptides, although there we were somewhat bewildered by the fact that AQP5 seemed to bind to ezrin-FERM in the *opposite direction* of the other peptides.

Paper II: *More* aquaporin interactions with ezrin

As we remained a bit uncertain regarding the *exact* binding mode of AQP5 and ezrin, a novel development allowed us to try a new approach; the release of AlphaFold [9] [127].

Using AlphaFold, we set out to study two other proteins which had been confirmed to interact with ezrin, with highly similar C-terminal domains (Figure 13); AQP0 [119] and AQP2 [18].

Figure 13: Alignment of C-terminal domains of AQP0, AQP2, and AQP5. Blue; hydrophobic residues, red; charged residues; pink; polar residues, yellow; glycine and proline.

One of the main problems with generating the prediction for Paper I was the flexibility of the AQP5 C-terminus. In comparison to the methods used there, AlphaFold suggested a rather different structure for the C-terminal domain of AQP5, which was very similar to that of the predicted C-terminal domain of AQP2. However, the predicted structure of the C-terminal domain of AQP0 gave little new information, due to it likely being intrinsically disordered (Figure 14).

It is however worth noting that the all three predictions assume that the proximal helix of the C-terminal domains is directed toward the cytosolic loops of the main body of the AQP. This has been shown to be the case in AQP5 [10], but is not the case for AQP2, probably due to the two prolines unique to AQP2 [21].

The docking generated two different binding modes, depending on if either full length or merely the C-terminal domain of the AQP was used.

The full length predictions suggested, in contrast to our previous data, that the distal part of the C-terminal domain adds to the β-sheet of ezrin-FERM, similarly to ICAM-2 [128]. All models still showed the same structure of the proximal C-terminal domain (Figure 15A, C).

In contrast, the docking predictions using *only* the C-terminal domains indicated a potential allosteric interaction, where the proximal C-terminal domain binds more like our previous prediction for AQP5 (although not in the "flipped" mode) (Figure 15D), as well as with the distal C-terminal domain, like the full length docking (Figure 15B, C).

[9] Published just as we submitted Paper I.

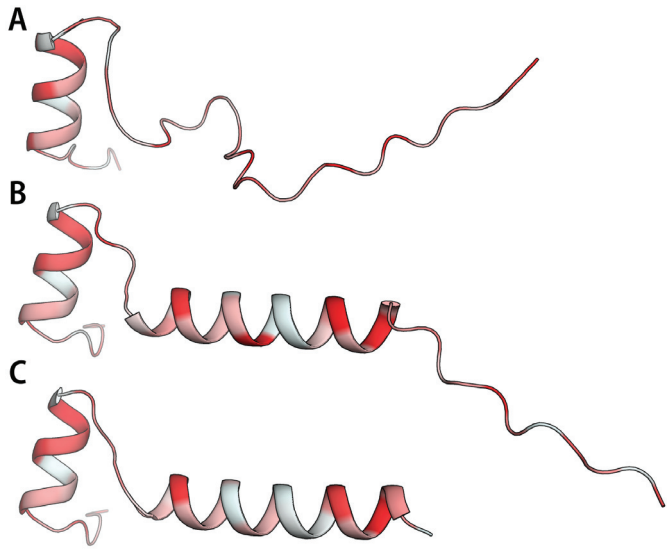


Figure 14: Structure of C-terminal domains of A) AQP0, B) AQP2, and C) AQP5, predicted with AlphaFold. Colour according to hydrophobicity, with white being highly hydrophobic, and red highly hydrophilic.

Thus, these interactions are potentially more similar to those of NHERF-1 and NHERF-2 than previously suggested. Even more interesting is the binding mode of moesin-FERM to the moesin C-terminal domain, which binds in *both* of these suggested modes [129], just like our predicted models. This behaviour has only been documented for this particular interaction however, so further experimental work is required to understand it fully.

However, due to our experimental data progressing in parallel with these simulations focusing on AQP2 and AQP5 at the time of writing, we elected to focus on these two for Paper II.

The interactions between the C-termini of AQP2 and AQP5 with ezrin-FERM were studied using GST-fused constructs, due to our interest in only studying the interaction of the C-terminal domains. GST-fusion proteins also have the advantages of yielding high amounts of protein with little optimisation needed. The interaction between the produced GST-fusion constructs were confirmed to interact with ezrin-FERM through co-elution followed by Western Blot.

The affinity of the interactions were studied using MST (Figure 16), showing that AQP2 and AQP5 have similar affinity to ezrin-FERM ($K_D = 7.76 \pm 2.88$

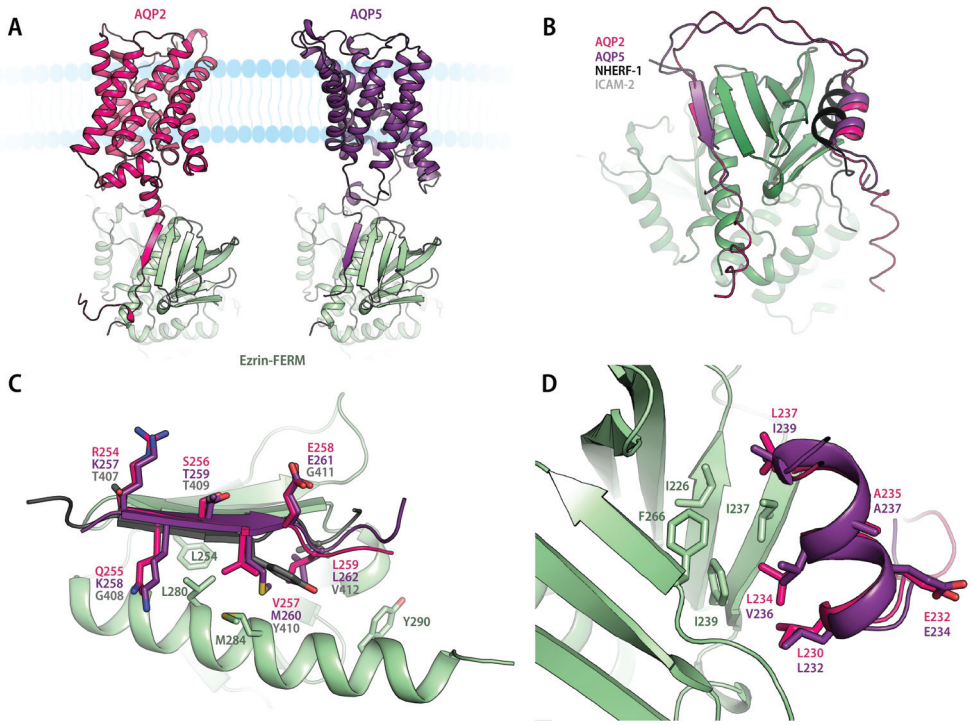


Figure 15: A) Full length AQP2 and AQP5 docked with ezrin-FERM. B) C-terminal peptides of AQP2, AQP5, ICAM-2 and NHERF-1 interacting with ezrin-FERM. C) Close up of the predicted β-sheet interaction of the distal C-termini. D) Close up of the predicted interaction of the a-helical proximal C-termini.

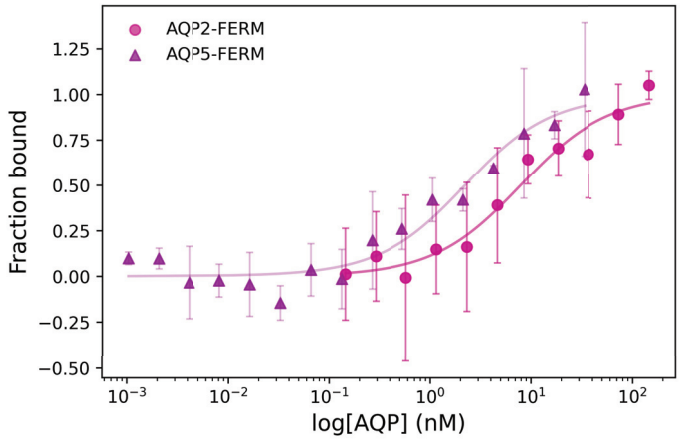


Figure 16: Fitted F_{norm} of AQP2 and AQP5 binding to ezrin-FERM.

µM and $K_D = 2.13 \pm 0.70$ µM, respectively).

One of the potential regulating factors of he AQP2-ezrin interaction may be phosphorylation of the C-terminal. Initial *in silico* studies, created by generating S256D, S261D, S264D, and T269D mutants in AlphaFold, where docked with ezrin-FERM, and analysed with PRODIGY. The data showed increased binding affinity with phosphorylation, gradually increasing from S256D and S261D to S264D (Figure 17). The highest affinity was was however predicted for the combination of S256D and T269D, which occurs *in vivo* only once S256 has been phosphorylated. The additional phosphorylation of T269 decreases the rate of internalisation of AQP2, thus increasing its retention time at the membrane [130]. These phosphorylation data are merely initial studies, and at this stage provide little more than a direction for the continued studies of the interactions between AQP2 and ezrin in particular, and AQP0 and AQP5 in general, when it comes to the involvement of phosphorylation.

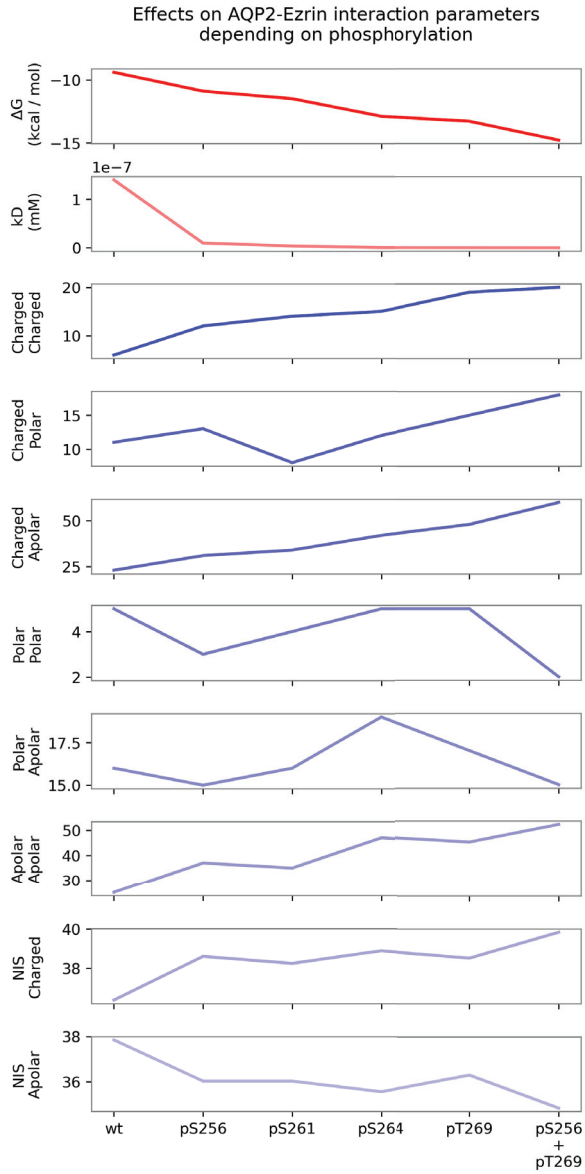


Figure 17: Energy levels and residue type interactions between AQP2 C-terminal domain and Ezrin FERM-domain depending on site of phosphorylation, as predicted by PRODIGY [123].

Overall, our data shows a novel binding modes for AQP0, AQP2, and AQP5 interactions with ezrin, and gives a good initial indication for the nature of these interactions. Nonetheless, further studies, mainly *in vitro*, are required to understand the accuracy and relevance of our initial studies.

Paper III: Aquaporin-0 regulation via CaM

In order to investigate the effect of phosphorylation of the C-terminal domain of AQP0 at residues S229, S231, and S235, phosphomimicking mutations were created, from serine to aspartate, thus resulting in separate constructs with the following mutations: S229D, S231D, and S235D.

In order to be able to compare the constructs with wild-type AQP0, it was necessary to confirm two things: if it is phosphorylated by the expression system (*P. pastoris*, and if it retains the full C-terminal domain. Fortunately, a Western blot using a Phos-tag was able to confirm the lack of phosphorylation of the wild-type AQP0, and the C-terminal domain was confirmed to be intact via linear mode MALDI spectrometry. Thus, comparison between the wild-type AQP0 and the phosphomimicking mutants was possible.

The interaction between AQP0 and calmodulin was initially studied via MST. Previous studies of this interaction has reported that AQP0 binds two calmodulin molecules, although this was in based upon simulations and electron microscopy to a resolution of 25Å [92]. The MST studies (Figure 18) showed that AQP0 bound to calmodulin similarly to what has previously been suggested, and that S229D and S235D did not. Interestingly, S231D *did* show interaction with calmodulin (with a K_D similar to that of wild-type AQP0), however in a peculiar fashion; the MST trace went the "opposite" way, thus indicating *slower* thermophoretic movement (a phenomenon that requires further study), and the data suggested a 1:1 AQP0:calmodulin interaction.

Using oocytes, it has been shown that the water permeability of unphosphorylated AQP0 is reduced upon interacting with calmodulin, and that the phosphorylation inhibits this gating mechanism [38]. In order to study this phenomenon without the potential interference with other proteins, which may happen in such a complex system, we reconstituted the purified AQP0 and the phosphomimicking constructs into liposomes. The resulting proteoliposomes were studied through stopped-flow spectrometry, where they were subjected to an osmotic gradient and the subsequent shrinkage of the liposomes could be measured. Due to AQP0 being a rather poor water channel, a rather high lipid to protein ratio was required (10). As can be seen in Figure 19, wild-type AQP0 responds well to calmodulin binding, whereas the addition of EGTA, a chelating agent, has no effect on AQP0, but prevents calmodulin from binding. S229D, S231D, and S235D all displayed unaffected water permeability under all conditions.

We have thus observed that AQP0 binds calmodulin and its water permeability is regulated via this interaction, and that phosphorylation at S229 and S235

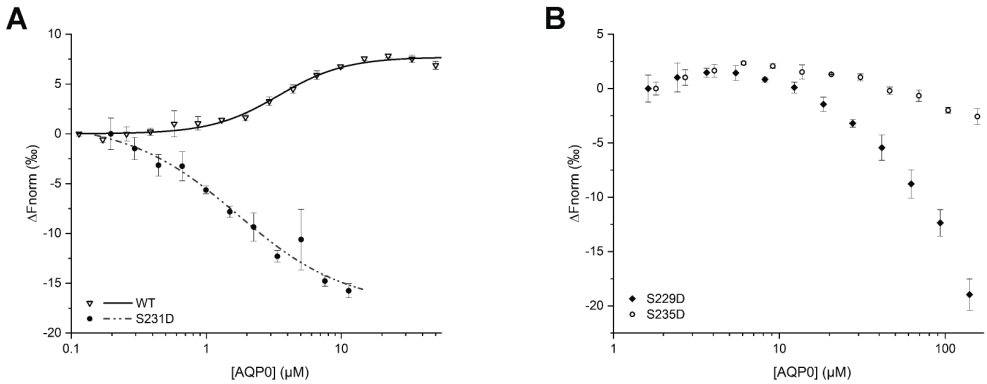


Figure 18: F_{norm} plots of of AQP0-calmodulin interactions. A) Wild-type and S231D displaying similar, yet *opposite* binding modes. B) S229D and S235D displaying inhibited binding.

prevents this gating mechanism, which aligns well with previously reported data [89, 38]. However, S231D seems to bind calmodulin, *without* it affecting the water permeability.

It has previously been shown that C-terminal peptides of AQP0 bind calmodulin, although with lower affinity than the full length AQP0 [131]. There are indications that this could be due to interactions with other parts of the cytosol-facing side of AQP0 [132, 133], which might also support the alternative binding mode of calmodulin interactions with pS231. It could also involve the distal C-terminal, thus involving an allosteric interaction, which also has been suggested for LIP5 in interactions with AQP2 [134] - and ezrin!

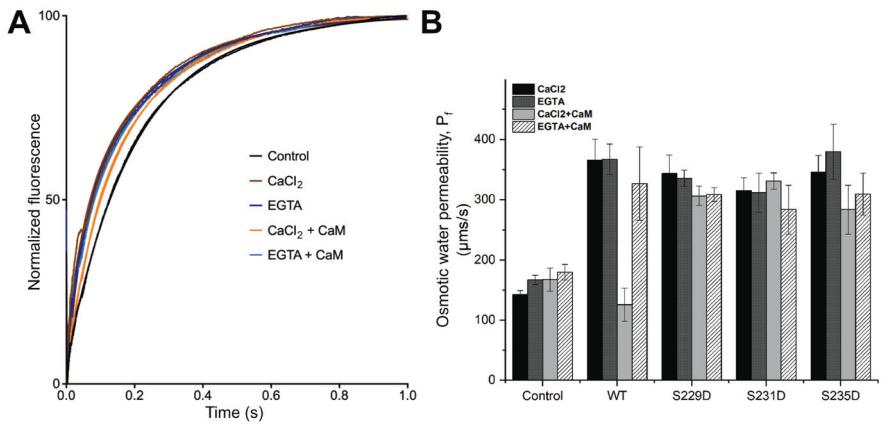


Figure 19: A) Stopped-flow spectrometry of AQP0 and phosphomimicking mutants. Note the rather long time (1 s) for the liposomes to reach a plateau, despite the high lipid to protein ratio. B) Water permeability of the constructs showing wild-type AQP0 being sensitive to calmodulin regulation, whereas all phosphomimicking mutations inhibited this regulation.

Aquaporin quality control

Paper IV: Impact of point mutations on AQP2 in NDI

Two groups of point mutations in AQP2 were generated, based on their importance to glycosylation and potential Ca^{2+} binding aspects [135]; T125M and T126M (situated in the N-linked glycosylation site of loop C), and Q57P and A147T (situated in the presumably important Ca^{2+} binding site).

All constructs were possible to express in *P. pastoris*, although Q57P proved too unstable for purification without significant further optimisation.

In order to investigate the secondary structure and stability of the constructs, they were studied using both CD and nanoDSF.

CD measurements, performed between $20°C$ and $95°C$ (Figure 20A and B) showed that the mutations resulted in structures which were highly similar to the wild-type AQP2, with highly α-helical patterns. As for stability, wild-type, T125M and T126M were found to display similar stability, with T_ms of $71.03 \pm 0.16°C$, $73.45 \pm 0.65°C$, and $72.14 \pm 0.25°C$, respectively. T125M did however show an increase in signal from $20°C$ to $40°C$, but we remain uncertain of the significance of this. A147T was found to be significantly less stable, with a T_m of 59.71 ± 0.34.

nanoDSF gave a similar indication (Figure 20C and D), with wild-type AQP2 seemingly being the most stable ($T_m = 70.39 \pm 0.27°C$), followed by T126M ($T_m = 67.87 \pm 0.39°C$), T125M ($T_m = 65.27 \pm 0.21°C$), and A147T ($T_m = 59.71 \pm 0.34°C$).

The impact of the mutations on the water permeability of AQP2 was investigated through stopped-flow spectrometry, by reconstituting them into liposomes. Since AQP2 is a more efficient water channel than AQP0, a lipid to protein ratio of 100 was used. The proteoliposomes were then subjected to rapid mixing with an osmotic gradient, and the rate of shrinkage could be observed (Figure 21A). The observed values were fitted according to equation 7, and the P_f was calculated according to equation 8 (Figure 21). Wild-type AQP2 and T126M displayed the same water permeability, whereas T125M displayed a slight decrease, and A147T even more so.

We sought to study the molecular structure of the mutants through X-ray crystallography, in hopes of being able to compare them with that of the wild-type AQP2. For the initial trials, we opted to replicate the conditions used for crystallisation of the wild-type AQP2 [21]. This did luckily result in crystals of very similar nature to that of wild-type AQP2; long rod-shaped crystals, which

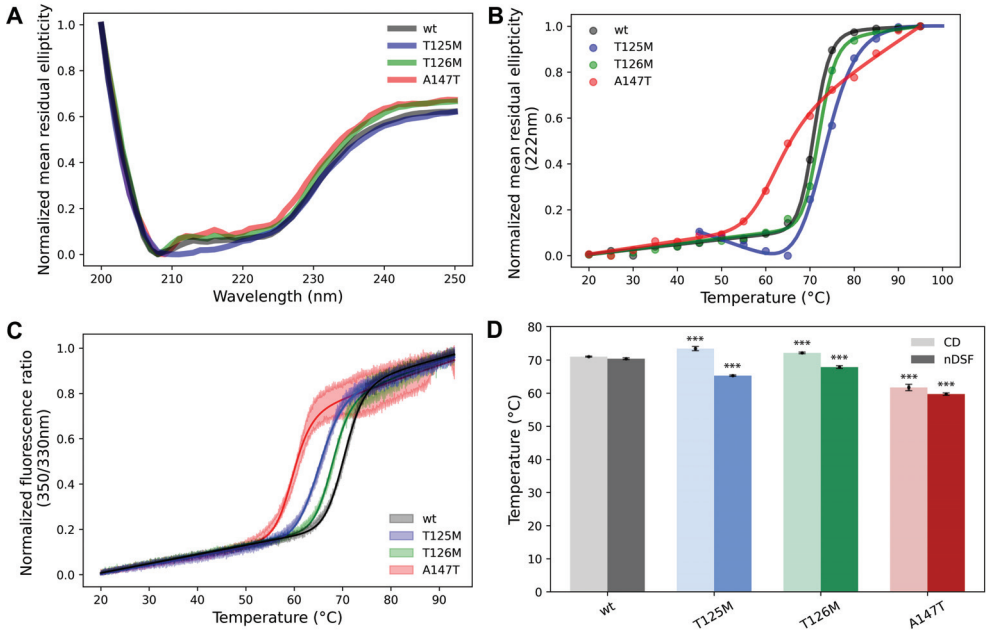


Figure 20: A) Normalised mean residual ellipticity for the AQP2 constructs, from CD measurements. B) Normalised mean residual ellipticity at 222nm, between $20°C$ and $95°C$. C) Normalised fluorescence from nanoDSF measurements. D) T_m values derived from both CD and nanoDSF measurements.

appeared within 3 days[10]. The diffraction of the crystals varied a lot, but regardless it was possible to collect data for T126M at 3.16Å and T125M at 3.90Å. A147T diffracted to only to 5Å, but it proved difficult to determine the cell parameters, and thus the molecular replacement was unsuccessful.

The resulting structures for T125M (PDB:8GHJ) and T126M (PDB:8OEE) displayed highly similar structures to that of the wild-type AQP2 (PDB:4NEF), with even loop C retaining its structure (Figure 22). A slight displacement of loop C could be seen in the structure of T125M, possibly due the steric hindrance cause by the introduced methionine.

We were also interested in seeing whether it would be possible to perform *in silico* studies on the interactions during the glycosylation process within OST-B. Attempts using AlphaFold failed, but attempts using HADDOCK resulted in po-

[10]Which, just like the wild-type crystals, would for an unknown reason cease to diffract if fished beyond 5 days of setting up.

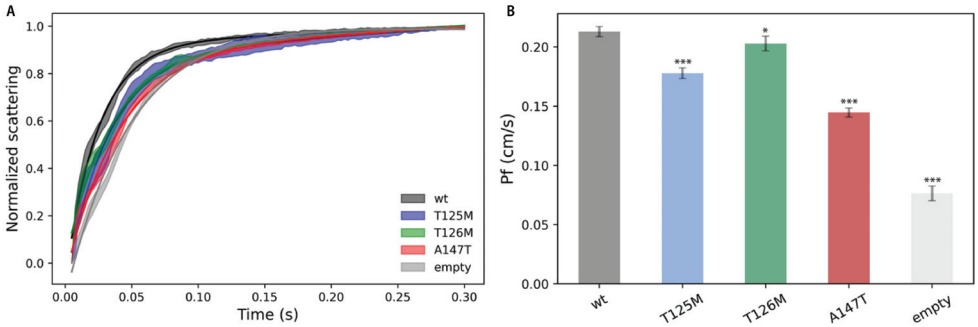


Figure 21: A) Stopped-flow spectrometry of AQP2 and NDI-derived point mutations. B) Calculated water permeability P_f of the constructs.

tential indications toward the steric hindrance which might occur during the glycosylation process. A forgetful mind did however lead to reattempting the docking, as I had forgotten which residues I had entered as potentially involved in the interaction. This lead to a significantly different result, showing little consequence of the mutations. We deemed the results as inconclusive, but it was an interesting lesson in how AlphaFold may be of high value, but it may not be entirely ready for the type of input we gave it, and how HADDOCK may be heavily biased by the input you give it. In both cases, we are dealing with bias: AlphaFold from its training data, HADDOCK from user input.

In summary, the mutations do to some degree cause destabilisation, but the overall structure is retained, as is the water permeability, albeit less so. The main cause of the disease model lies in the quality control mechanisms of the cell, as has been suggested previously [46, 47, 136, 56]. As AQP2 is glycosylated co-translationally in the ER, the protein undergoes a quality control mechanism through step-wise trimming of the complex glycan, as may be seen in Figure 23. The process is somewhat of a *dance* between the proteins UGT, CNX and CRT [137]. Sufficiently stable, correctly folded proteins are translocated for further maturation in the Golgi, while proteins which are not sufficiently stable are redirected for degradation in the proteasome. T126M is however sufficiently stable, and the mutation is *not* directly in the N-linked glycosylation recognition sequence, so why is it degraded? It has been shown that UGT has a higher affinity for peptides with a hydrophobic residue following the N-linked glycosylation sequence [138], which would be caused by a methionine mutation, which would explain why T126M leads to NDI.

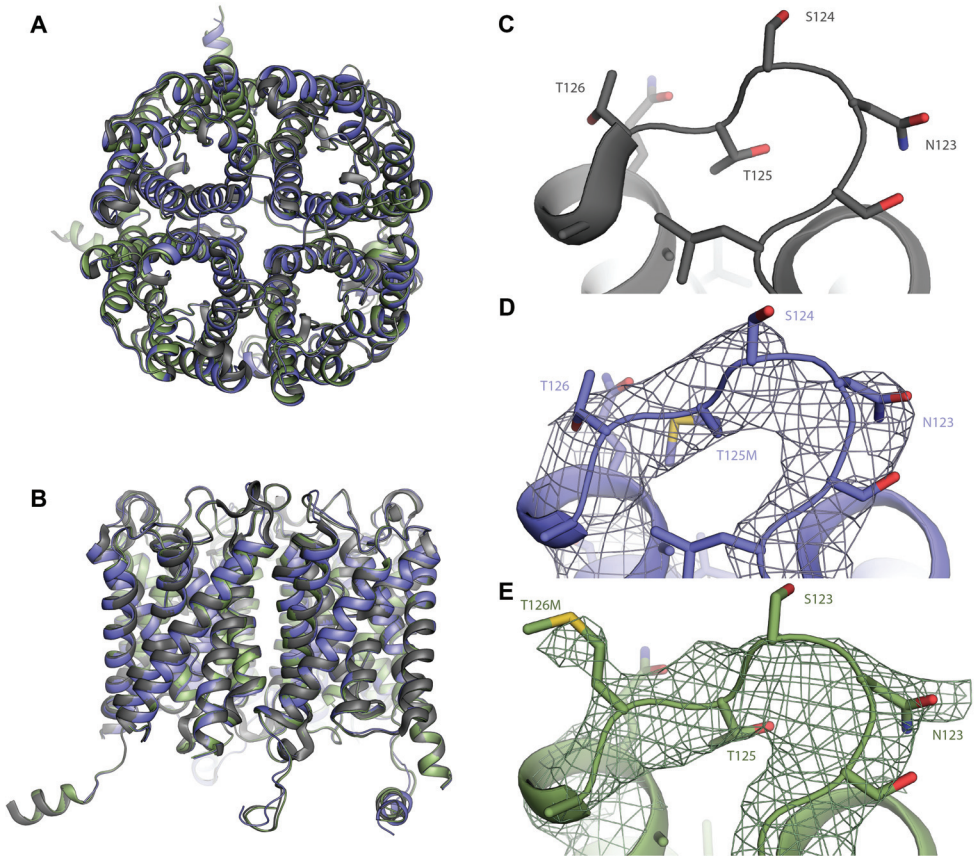


Figure 22: A) Cytosolic view of wild-type AQP2 (PDB:4NEF), AQP2 T125M (PDB:8GHJ), and AQP2 T126M (PDB:8OEE), superimposed. B) Side view of the protein structures, as in (A). C) Loop C of wild-type AQP2. D) Loop C of T125M. E) Loop C of T126M. 2Fo-Fc electron density map contoured at 1.0σ shown for both T125M and T126M.

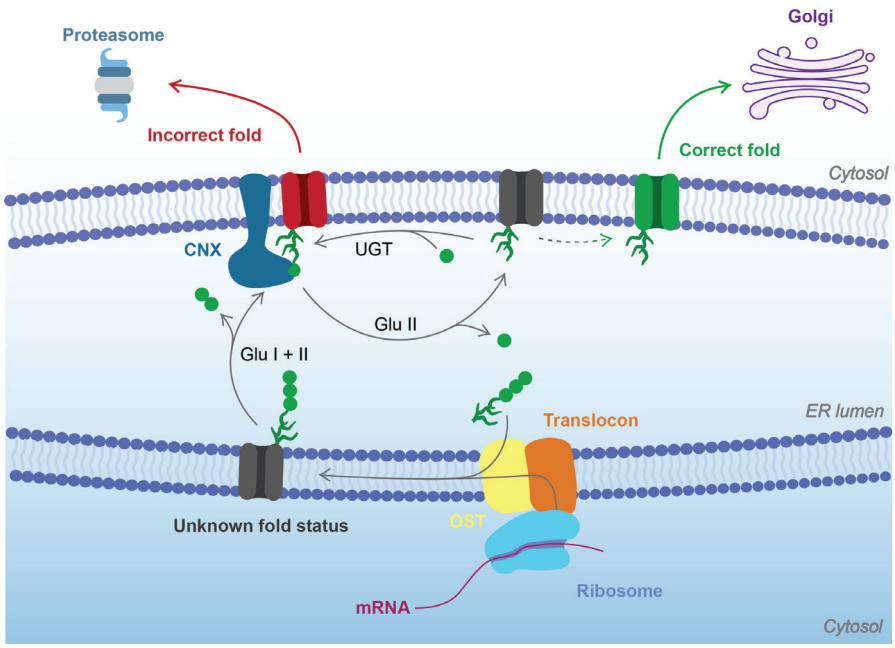


Figure 23: Proposed quality control pathway of AQP2. Cotranslationally glycosylated AQP2 is trimmed and undergoes quality control through interactions with CNX, UGT, and Glu I & II. Correctly folded AQP2 undergoes further maturation in the Golgi, while incorrectly folded AQP2 is marked for degradation in the proteasome.

Improving the study of membrane proteins

Paper V: Continuous diffraction - *a novel approach*

The implementation of continuous diffraction has been shown to have the potential to allow structural information beyond the range of Bragg diffraction to be collected and used in the structural determination of proteins. As prior studies had only shown continuous diffraction in photosystem II [106], we aimed to investigate whether this phenomenon could be observed in other systems as well. Our initial experiments were performed at the X-ray free electron laser (XFEL) at LCLS, and showed that AQP2 crystals *did* show strong continuous diffraction, extending beyond the Bragg peaks.

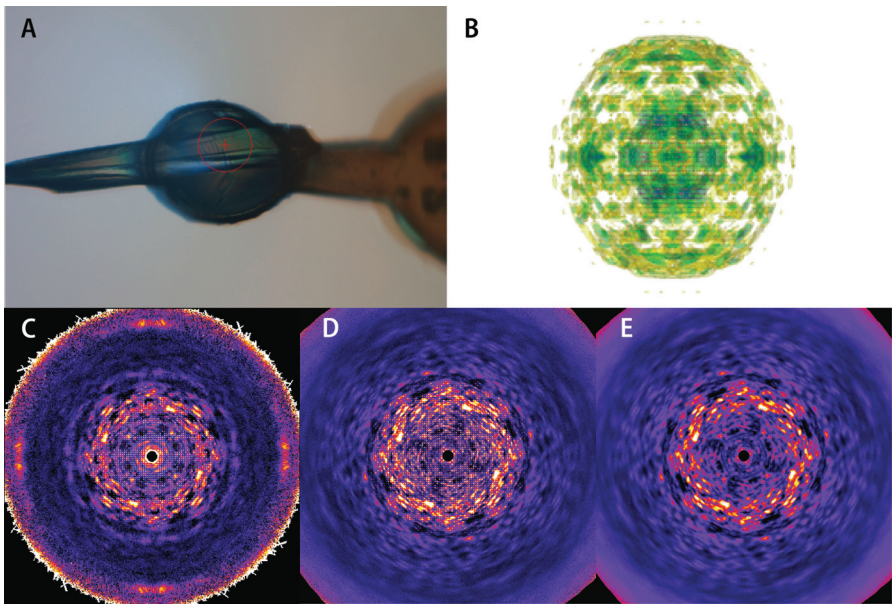


Figure 24: A) Crystal of AQP2 mounted in a cryo-loop. B) 3D merge of reciprocal space of AQP2 diffraction. C) 2D slice of the merged reciprocal space of AQP2, measured at an XFEL (LCLS). D) 2D slice of the merged reciprocal space of AQP2, measured at a synchrotron (DESY). E) 2D slice of the merged reciprocal space of AQP2, with Bragg peaks subtracted, measured at a synchrotron (DESY). Detector edge for the images from DESY corresponding to 1.7 Å.

As XFELs are not the easiest to access, it was also interesting to see whether the it would be sufficient to study continuous diffraction at a synchrotron, which it turned out to be. The diffraction patterns of multiple crystals were merged into a 3D reciprocal space volume using Merge3D [11]. 2D slices of these volumes are shown in Figure 24.

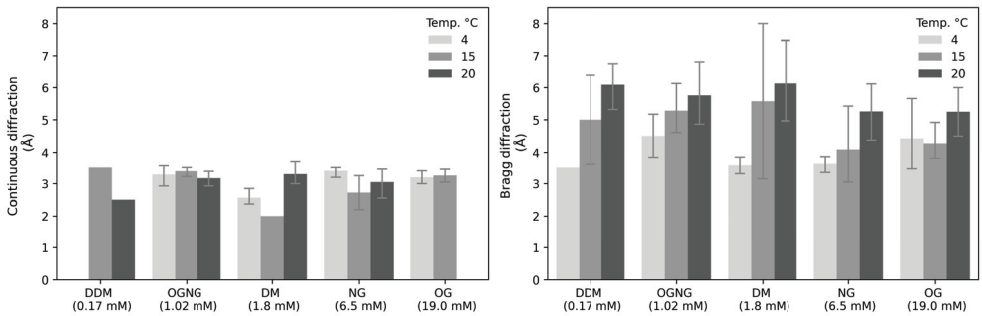


Figure 25: Effect of secondary detergents added to the crystallisation conditions of AQP2; A) continuous diffraction, B) Bragg diffraction.

Since AQP2 was a highly suitable candidate for studying continuous diffraction, we were also interested in seeing whether if it was possible to affect the quality of it through addition of secondary additives (Table 4) to the crystallisation conditions, as well as *inducing* it into a system that showed only weak signs of continuous diffraction; SoPIP2;1, an AQP from spinach with high structural similarity to AQP2 [139].

After setting up a a vast amount of drops, and bringing a total of 261 AQP2 crystals and 89 SoPIP2;1 crystals to DESY, we were able to conclude that the secondary additive did not impact the Bragg diffraction of the AQP2 crystals. Instead, the crystallisation temperature seemed to play a larger role (Figure 25).

In contrast, the secondary additive impacted the SoPIP2;1 crystals (Figure 26 C). However, it did *not* impact the continuous diffraction of either crystal system. Nonetheless, after merging the diffractions of SoPIP2;1, there were indications of *weak* continuous diffraction.

[11] https://stash.desy.de/projects/M3D/repos/merge3d

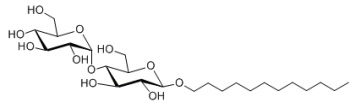
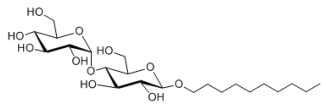
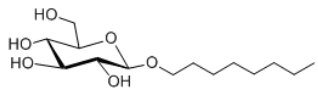
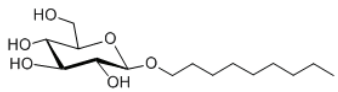
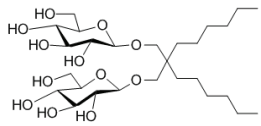
Detergent	CMC (mM)	MW (Da)	Structure
DDM	0.17	510.6	
DM	1.8	482.6	
OG	19.0	292.4	
NG	6.5	306.4	
OGNG	1.02	568.7	

Table 4: Added detergents, their chemical structures, and their properties; dodecyl maltoside (DDM), decyl maltoside (DM), octyl glucoside (OG), nonyl glucoside (NG), and octyl glucose neopentyl glycol (OGNG).

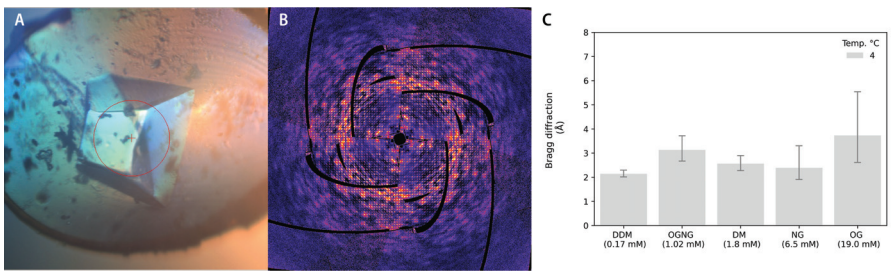


Figure 26: A) Crystal of SoPIP2;1 mounted in a cryo-loop. B) 2D slice of the merged reciprocal space of SoPIP2;1 diffraction, measured at a synchrotron (DESY), with the edge of the detector corresponding to 1.7 Å. C) Bragg diffraction of SoPIP2;1 crystals in relation to the secondary detergent added to the crystallisation conditions.

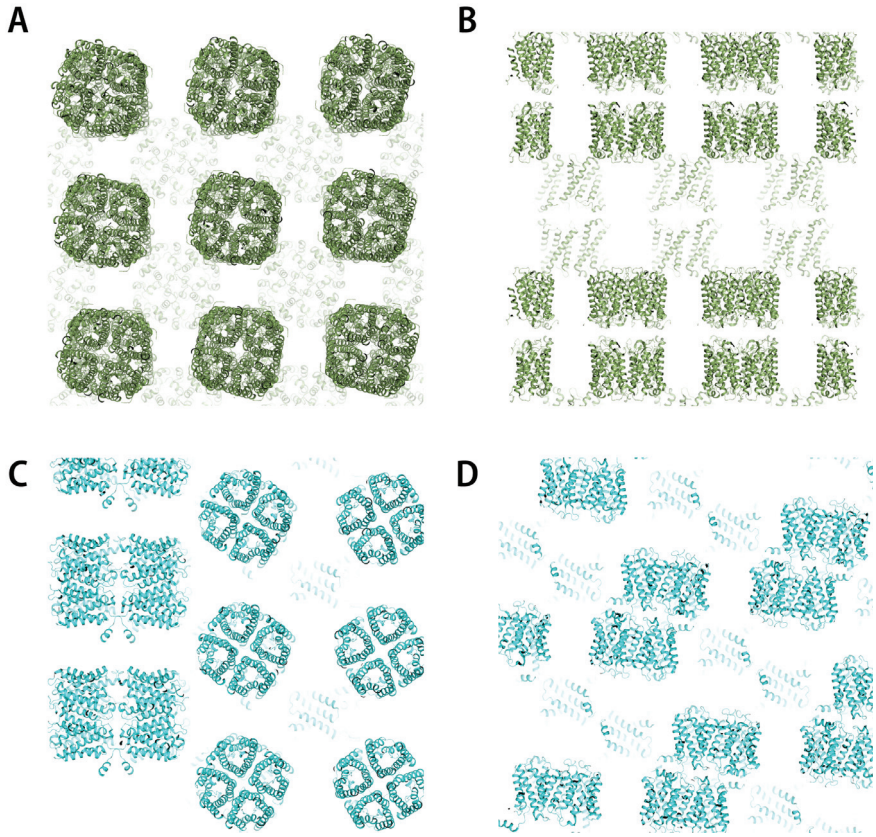


Figure 27: Crystal packing for SoPIP2;1 (packed in space group $I4$, A and B), compared with AQP2 (packed in space group $P4_2$, C and D), both views of respective packings with 90° rotation in relation to each other.

The nature of continuous diffraction remains elusive, but a clue might lie in manner of packing within the crystal system. SoPIP2;1 crystallises into $I4$, with somewhat dense packing and, compared to AQP2 packing into $P4_2$, has relatively small solvent channels and stronger crystal contacts (Figure 27). It should however be noted that the addition of maltoside detergents could affect the space group of SoPIP2;1, leading it to pack into $P42_12$, although without affecting translational disorder and the continuous diffraction.

Outlook

Since the dawn of my time as a doctoral student in the late summer of 2018, the field has evolved at a rate that was difficult to foresee. Suddenly, there are algorithms for protein structure prediction which are accurate to a level which few could imagine possible within such a short time frame. There are structural methods which are highly competitive with X-ray crystallography, a method which has dominated the field of structural biology for decades. The use of AlphaFold and cryoEM is almost household in structural biology labs these days, and they are becoming increasingly accessible for researchers outside of our field as well.

While *standard* X-ray crystallography may no longer be as alluring as it once was, competing methods are no guarantee for an easy structure, and crystallography remains the go-to method for smaller proteins and time resolved experiments.

However, the future likely lies in neither method, but rather the *combination* of them, and the lessons learned from them in terms of sample preparation, data processing, and final interpretation. In conjunction with endlessly improving computational power and increasingly sophisticated algorithms, the complexities of cells and tissues might be easier to elucidate sooner than we might believe today.

With the evolution of biochemical and biophysical methods, naturally the problems we may investigate and solve are often the driving factors behind these advancements.

The regulatory mechanisms behind AQPs are becoming increasingly better understood, although a lot of work still remains in order to be able to paint a wider picture on the extensive network of proteins involved in ”merely” regulating water regulation of tissues. With this understanding, it will eventually be possible to circumvent improperly functioning mechanisms within the body, such as quality control mechanisms acting on high alert to targets which may not pose a threat to the cell, and regulating *e.g.* water flow in particular events in our bodies which do not serve us as well as they so far have evolved to be, like in brain oedemas.

Overall, the field of structure biology remains an exciting field to be in, and

in the advent of highly accurate structure prediction algorithms, I curiously look forward to how we may apply our skills to problems which remain, for the time being, beyond the grasp of automation.

Bibliography

[1] Alexander Ivanovich Oparin. Proikhozndenie Zhizni. *Izd., Moskowski Rabochi.*, 1924.

[2] John Burdon Sanderson Haldane. Origin of Life. *The Rationalist Annual*, (148):3–10, 1929.

[3] Markus Sällman Almén, Karl JV Nordström, Robert Fredriksson, and Helgi B. Schiöth. Mapping the human membrane proteome: a majority of the human membrane proteins can be classified according to function and evolutionary origin. *BMC Biology*, 7(1):50, August 2009.

[4] Gunnar von Heijne. Membrane-protein topology. *Nature reviews. Molecular cell biology*, 7(12):909–18, 2006. ISBN: doi:10.1038/nrm2063.

[5] John P. Overington, Bissan Al-Lazikani, and Andrew L. Hopkins. How many drug targets are there? *Nature Reviews Drug Discovery*, 5(12):993–996, December 2006. Number: 12 Publisher: Nature Publishing Group.

[6] Charles Darwin. *On the Origin of Species by Means of Natural Selection*. Murray, London, November 1859.

[7] Kenichi Ishibashi, Yasuko Tanaka, and Yoshiyuki Morishita. The role of mammalian superaquaporins inside the cell. *Biochimica et Biophysica Acta (BBA) - General Subjects*, 1840(5):1507–1512, May 2014.

[8] G. Ren, V. S. Reddy, A. Cheng, P. Melnyk, and A. K. Mitra. Visualization of a water-selective pore by electron crystallography in vitreous ice. *Proceedings of the National Academy of Sciences of the United States of America*, 98(4):1398–1403, February 2001.

[9] Anna Frick, Urszula Kosinska Eriksson, Fabrizio de Mattia, Fredrik Oberg, Kristina Hedfalk, Richard Neutze, Willem J de Grip, Peter M T Deen, and Susanna Törnroth-Horsefield. X-ray structure of human aquaporin 2 and its implications for nephrogenic diabetes insipidus and trafficking. *Proceedings of the National Academy of Sciences of the United States of America*, 111(17):6305–10, 2014. ISBN: 1091-6490 (Electronic)\r0027-8424 (Linking).

[10] Rob Horsefield, Kristina Nordén, Maria Fellert, Anna Backmark, Susanna Törnroth-Horsefield, Anke C. Terwisscha van Scheltinga, Jan Kvassman, Per Kjellbom, Urban Johanson, and Richard Neutze. High-resolution x-ray structure of human aquaporin 5. *Proceedings of the National Academy of Sciences*, 105(36):13327–13332, September 2008. Publisher: Proceedings of the National Academy of Sciences.

[11] Sofia W. de Maré, Raminta Venskutonytė, Sandra Eltschkner, Bert L. de Groot, and Karin Lindkvist-Petersson. Structural Basis for Glycerol Efflux and Selectivity of Human Aquaporin 7. *Structure*, 28(2):215–222.e3, February 2020.

[12] Peng Huang, Raminta Venskutonytė, Rashmi B. Prasad, Hamidreza Ardalani, Sofia W. de Maré, Xiao Fan, Ping Li, Peter Spégel, Nieng Yan, Pontus Gourdon, Isabella Artner, and Karin Lindkvist-Petersson. Cryo-EM structure supports a role of AQP7 as a junction protein. *Nature Communications*, 14(1):600, February 2023. Number: 1 Publisher: Nature Publishing Group.

[13] Rebecca E. Day, Philip Kitchen, David S. Owen, Charlotte Bland, Lindsay Marshall, Alex C. Conner, Roslyn M. Bill, and Matthew T. Conner. Human aquaporins: regulators of transcellular water flow. *Biochimica Et Biophysica Acta*, 1840(5):1492–1506, May 2014.

[14] Kamil Gotfryd, Andreia Filipa Mósca, Julie Winkel Missel, Sigurd Friis Truelsen, Kaituo Wang, Mariana Spulber, Simon Krabbe, Claus Hélix-Nielsen, Umberto Laforenza, Graça Soveral, Per Amstrup Pedersen, and Pontus Gourdon. Human adipose glycerol flux is regulated by a pH gate in AQP10. *Nature Communications*, 9(1):4749, November 2018. Number: 1 Publisher: Nature Publishing Group.

[15] Kenichi Ishibashi, Yasuko Tanaka, and Yoshiyuki Morishita. The role of mammalian superaquaporins inside the cell: An update. *Biochimica et Biophysica Acta (BBA) - Biomembranes*, 1863(7):183617, July 2021.

[16] D. Fu. Structure of a Glycerol-Conducting Channel and the Basis for Its Selectivity. *Science*, 290(5491):481–486, 2000. ISBN: 0036-8075 (Print)\r0036-8075 (Linking).

[17] Jennifer Roche and Susanna Törnroth-Horsefield. Aquaporin Protein-Protein Interactions. *International Journal of Molecular Sciences*, 18(11):2255, October 2017. ISBN: 0902124459255.

[18] Wei Li, William W. Jin, Kenji Tsuji, Ying Chen, Naohiro Nomura, Limin Su, Naofumi Yui, Julian Arthur, Susanna Cotecchia, Teodor G. Păunescu, Dennis Brown, and Hua A. J. Lu. Ezrin directly interacts with AQP2 and promotes its endocytosis. *Journal of Cell Science*, 130(17):2914–2925, September 2017.

[19] Bas W. M. van Balkom, Michelle Boone, Giel Hendriks, Erik-Jan Kamsteeg, Joris H. Robben, H. Christiaan Stronks, Anne van der Voorde, Francois van Herp, Peter van der Sluijs, and Peter M. T. Deen. LIP5 interacts with aquaporin 2 and facilitates its lysosomal degradation. *Journal of the American Society of Nephrology: JASN*, 20(5):990–1001, May 2009.

[20] Steve L. Reichow and Tamir Gonen. Non-canonical binding of calmodulin to aquaporin-0: implications for channel regulation. *Structure (London, England : 1993)*, 16(9):1389–1398, September 2008.

[21] Anna Frick, Michael Järvå, and Susanna Törnroth-Horsefield. Structural basis for pH gating of plant aquaporins. *FEBS Letters*, 587(7):989–993, 2013. Publisher: Federation of European Biochemical Societies ISBN: 00145793.

[22] Sigurd Friis Truelsen, Julie Winkel Missel, Kamil Gotfryd, Per Amstrup Pedersen, Pontus Gourdon, Kresten Lindorff-Larsen, and Claus Hélix-Nielsen. The role of water coordination in the pH-dependent gating of hAQP10. *Biochimica et Biophysica Acta (BBA) - Biomembranes*, 1864(1):183809, February 2022.

[23] Claudia Rodrigues, Andreia Filipa Mósca, Ana Paula Martins, Tatiana Nobre, Catarina Prista, Fernando Antunes, Ana Cipak Gasparovic, and Graça

Soveral. Rat Aquaporin-5 Is pH-Gated Induced by Phosphorylation and Is Implicated in Oxidative Stress. *International Journal of Molecular Sciences*, 17(12):2090, December 2016.

[24] Robert A. Fenton, Sathish K. Murali, and Hanne B. Moeller. Advances in Aquaporin-2 trafficking mechanisms and their implications for treatment of water balance disorders. *American Journal of Physiology. Cell Physiology*, May 2020.

[25] Veronika Nesverova and Susanna Törnroth-Horsefield. Phosphorylation-Dependent Regulation of Mammalian Aquaporins. *Cells*, 8(2):82, 2019.

[26] Jonathan M Street, Willem Birkhoff, Robert I Menzies, David J Webb, Matthew A Bailey, and James W Dear. Exosomal transmission of functional aquaporin 2 in kidney cortical collecting duct cells. *The Journal of Physiology*, 589(Pt 24):6119–6127, December 2011.

[27] G. M. Preston, T. P. Carroll, W. B. Guggino, and P. Agre. Appearance of water channels in Xenopus oocytes expressing red cell CHIP28 protein. *Science (New York, N.Y.)*, 256(5055):385–387, April 1992.

[28] G. Benga, O. Popescu, V. I. Pop, and R. P. Holmes. p-(Chloromercuri)benzenesulfonate binding by membrane proteins and the inhibition of water transport in human erythrocytes. *Biochemistry*, 25(7):1535–1538, April 1986.

[29] G. Benga, O. Popescu, V. Borza, V. I. Pop, A. Muresan, I. Mocsy, A. Brain, and J. M. Wrigglesworth. Water permeability in human erythrocytes: identification of membrane proteins involved in water transport. *European Journal of Cell Biology*, 41(2):252–262, August 1986.

[30] P W Kuchel. The story of the discovery of aquaporins: convergent evolution of ideas–but who got there first. *Cellular and molecular biology (Noisy-le-Grand, France)*, 52(7):2–5, October 2006.

[31] Gheorghe Benga. The first discovered water channel protein, later called aquaporin 1: Molecular characteristics, functions and medical implications. *Molecular Aspects of Medicine*, 33(5):518–534, October 2012.

[32] Patrick O. Saboe, Chiara Rapisarda, Shreyas Kaptan, Yu-Shan Hsiao, Samantha R. Summers, Rita De Zorzi, Danijela Dukovski, Jiaheng Yu,

Bert L. de Groot, Manish Kumar, and Thomas Walz. Role of Pore-Lining Residues in Defining the Rate of Water Conduction by Aquaporin-0. *Biophysical Journal*, 112(5):953–965, March 2017. Publisher: Biophysical Society.

[33] Anastasia Korlimbinis, Yoke Berry, Danielle Thibault, Kevin L. Schey, and Roger J. W. Truscott. Protein aging: truncation of aquaporin 0 in human lens regions is a continuous age-dependent process. *Experimental Eye Research*, 88(5):966–973, May 2009.

[34] S. Sindhu Kumari, Neha Gupta, Alan Shiels, Paul G. FitzGerald, Anil G. Menon, Richard T. Mathias, and Kulandaiappan Varadaraj. Role of Aquaporin 0 in lens biomechanics. *Biochemical and biophysical research communications*, 462(4):339–345, July 2015.

[35] Sindhu S. Kumari, Jason Gandhi, Mohammed H. Mustehsan, Semih Eren, and Kulandaiappan Varadaraj. Functional characterization of an AQP0 missense mutation, R33C, that causes dominant congenital lens cataract, reveals impaired cell-to-cell adhesion. *Experimental eye research*, 116:10.1016/j.exer.2013.09.019, November 2013.

[36] Robert A. Fenton, Hanne B. Moeller, Jason D. Hoffert, Ming-Jiun Yu, Søren Nielsen, and Mark A. Knepper. Acute regulation of aquaporin-2 phosphorylation at Ser-264 by vasopressin. *Proceedings of the National Academy of Sciences of the United States of America*, 105(8):3134–3139, February 2008.

[37] S. J. Girsch and C. Peracchia. Lens cell-to-cell channel protein: I. Self-assembly into liposomes and permeability regulation by calmodulin. *The Journal of Membrane Biology*, 83(3):217–225, 1985.

[38] Katalin Kalman, Karin L. Németh-Cahalan, Alexandrine Froger, and James E. Hall. Phosphorylation determines the calmodulin-mediated Ca2+ response and water permeability of AQP0. *The Journal of Biological Chemistry*, 283(30):21278–21283, July 2008.

[39] Lauren E. Ball, Donita L. Garland, Rosalie K. Crouch, and Kevin L. Schey. Post-translational modifications of aquaporin 0 (AQP0) in the normal human lens: spatial and temporal occurrence. *Biochemistry*, 43(30):9856–9865, August 2004.

[40] K. Fushimi, S. Uchida, Y. Hara, Y. Hirata, F. Marumo, and S. Sasaki. Cloning and expression of apical membrane water channel of rat kidney collecting tubule. *Nature*, 361(6412):549–552, February 1993.

[41] Jinzhao He and Baoxue Yang. Aquaporins in Renal Diseases. *International Journal of Molecular Sciences*, 20(2):366, January 2019.

[42] Grazia Tamma, Joris H. Robben, Christiane Trimpert, Michelle Boone, and Peter M. T. Deen. Regulation of AQP2 localization by S256 and S261 phosphorylation and ubiquitination. *American Journal of Physiology. Cell Physiology*, 300(3):C636–646, March 2011.

[43] Hanne B. Moeller, Mark A. Knepper, and Robert A. Fenton. Serine 269 phosphorylated aquaporin-2 is targeted to the apical membrane of collecting duct principal cells. *Kidney international*, 75(3):295–303, February 2009.

[44] Jason D. Hoffert, Robert A. Fenton, Hanne B. Moeller, Brigitte Simons, Dmitry Tchapyjnikov, Bradley W. McDill, Ming Jiun Yu, Trairak Pisitkun, Feng Chen, and Mark A. Knepper. Vasopressin-stimulated increase in phosphorylation at Ser269 potentiates plasma membrane retention of aquaporin-2. *Journal of Biological Chemistry*, 283(36):24617–24627, 2008. ISBN: 0021-9258 (Print)\r0021-9258 (Linking).

[45] Apurv Khanna. Acquired nephrogenic diabetes insipidus. *Seminars in Nephrology*, 26(3):244–248, May 2006.

[46] Nannette Marr, Daniel G. Bichet, Susan Hoefs, Paul J. M. Savelkoul, Irene B. M. Konings, Fabrizio de Mattia, Michael P. J. Graat, Marie-Franc[COMBINING CEDILLA]oise Arthus, Michele Lonergan, T. Mary Fujiwara, Nine V. A. M. Knoers, Daniel Landau, William J. Balfe, Alexander Oksche, Walter Rosenthal, Dominik Mu[Combining Diaeresis]ller, Carel H. van Os, and Peter M. T. Deen. Cell-Biologic and Functional Analyses of Five New Aquaporin-2 Missense Mutations that Cause Recessive Nephrogenic Diabetes Insipidus. *Journal of the American Society of Nephrology*, 13(9):2267, September 2002.

[47] N. Marr, E. J. Kamsteeg, M. Van Raak, C. H. Van Os, and P. M.T. Deen. Functionality of aquaporin-2 missense mutants in recessive nephrogenic diabetes insipidus. *Pflugers Archiv European Journal of Physiology*, 442(1):73–77, April 2001. ISBN: 0031-6768 (Print)\r0031-6768 (Linking).

[48] Cristina Boccalandro, Fabrizio De Mattia, Dong-Chuan Guo, Li Xue, Philip Orlander, Terri M. King, Prateek Gupta, Peter M. T. Deen, Victor R. Lavis, and Dianna M. Milewicz. Characterization of an aquaporin-2 water channel gene mutation causing partial nephrogenic diabetes insipidus in a Mexican family: evidence of increased frequency of the mutation in the town of origin. *Journal of the American Society of Nephrology: JASN*, 15(5):1223–1231, May 2004.

[49] Taninee Sahakitrungruang, Suttipong Wacharasindhu, Thivaratana Sinthuwiwat, Vichit Supornsilchai, Kanya Suphapeetiporn, and Vorasuk Shotelersuk. Identification of two novel aquaporin-2 mutations in a Thai girl with congenital nephrogenic diabetes insipidus. *Endocrine*, 33(2):210–214, April 2008.

[50] Sei Sasaki. Aquaporin 2: From its discovery to molecular structure and medical implications. *Molecular Aspects of Medicine*, 33(5-6):535–546, 2012. Publisher: Elsevier Ltd ISBN: 0098-2997.

[51] Marie C. Canfield, B. K. Tamarappoo, Arnold M. Moses, A. S. Verkman, and Eli J. Holtzman. Identification and Characterization of Aquaporin-2 Water Channel Mutations Causing Nephrogenic Diabetes Insipidus with Partial Vasopressin Response. *Human Molecular Genetics*, 6(11):1865–1871, October 1997.

[52] Alexandre Leduc-Nadeau, Yoann Lussier, Marie-Françoise Arthus, Michèle Lonergan, Alejandro Martinez-Aguayo, Eva Riveira-Munoz, Olivier Devuyst, Pierre Bissonnette, and Daniel G. Bichet. New autosomal recessive mutations in aquaporin-2 causing nephrogenic diabetes insipidus through deficient targeting display normal expression in Xenopus oocytes. *The Journal of Physiology*, 588(12):2205–2218, 2010. _eprint: https://onlinelibrary.wiley.com/doi/pdf/10.1113/jphysiol.2010.187674.

[53] Dominik Müller, Nannette Marr, Tobias Ankermann, Paul Eggert, and Peter MT Deen. Desmopressin for nocturnal enuresis in nephrogenic diabetes insipidus. *The Lancet*, 359(9305):495–497, February 2002.

[54] Shih-Hua Lin, Daniel G. Bichet, Sei Sasaki, Michio Kuwahara, Marie-Francoise Arthus, Michele Lonergan, and Yuh-Feng Lin. Two Novel Aquaporin-2 Mutations Responsible for Congenital Nephrogenic Diabetes

Insipidus in Chinese Families. *The Journal of Clinical Endocrinology & Metabolism*, 87(6):2694–2700, June 2002.

[55] A. F. van Lieburg, M. A. Verdijk, V. V. Knoers, A. J. van Essen, W. Proesmans, R. Mallmann, L. A. Monnens, B. A. van Oost, C. H. van Os, and P. M. Deen. Patients with autosomal nephrogenic diabetes insipidus homozygous for mutations in the aquaporin 2 water-channel gene. *American Journal of Human Genetics*, 55(4):648–652, October 1994.

[56] S. M. Mulders, N. V. Knoers, A. F. Van Lieburg, L. A. Monnens, E. Leumann, E. Wühl, E. Schober, J. P. Rijss, C. H. Van Os, and P. M. Deen. New mutations in the AQP2 gene in nephrogenic diabetes insipidus resulting in functional but misrouted water channels. *Journal of the American Society of Nephrology*, 8(2):242, February 1997.

[57] Hae Il Cheong, Su Jin Cho, Shou Huan Zheng, Hee Yeon Cho, Il Soo Ha, and Yong Choi. Two Novel Mutations in the Aquaporin 2 Gene in a Girl with Congenital Nephrogenic Diabetes Insipidus. *Journal of Korean Medical Science*, 20(6):1076–1078, December 2005.

[58] Rosa Vargas-Poussou, Lionel Forestier, Marie Dominique Dautzenberg, Patrick Niaudet, Michèle Déchaux, and Corinne Antignac. Mutations in the Vasopressin V2 Receptor and Aquaporin-2 Genes in Twelve Families with Congenital Nephrogenic Diabetes Insipidus. In Hans H. Zingg, Charles W. Bourque, and Daniel G. Bichet, editors, *Vasopressin and Oxytocin: Molecular, Cellular, and Clinical Advances*, Advances in Experimental Medicine and Biology, pages 387–390. Springer US, Boston, MA, 1998.

[59] Alejandro García Castaño, Gustavo Pérez de Nanclares, Leire Madariaga, Mireia Aguirre, Sara Chocron, Alvaro Madrid, Francisco Javier Lafita Tejedor, Mercedes Gil Campos, Jaime Sánchez del Pozo, Rafael Ruiz Cano, Mar Espino, Jose Maria Gomez Vida, Fernando Santos, Victor Manuel García Nieto, Reyner Loza, Luis Miguel Rodríguez, Emilia Hidalgo Barquero, Nikoleta Printza, Juan Antonio Camacho, Luis Castaño, Gema Ariceta, and RenalTube Group. Novel mutations associated with nephrogenic diabetes insipidus. A clinical-genetic study. *European Journal of Pediatrics*, 174(10):1373–1385, October 2015.

[60] Rottanat Rugpolmuang, Asma Deeb, Yousef Hassan, Tawatchai Deekajorndech, Vorasuk Shotelersuk, and Taninee Sahakitrungruang. Novel

AQP2 mutation causing congenital nephrogenic diabetes insipidus: challenges in management during infancy. *Journal of Pediatric Endocrinology and Metabolism*, 27(1-2):193–197, January 2014. Publisher: De Gruyter.

[61] Pamela Carroll, Hamad Al-Mojalli, Abbas Al-Abbad, Ibrahim Al-Hassoun, Mohamed Al-Hamed, Rana Al-Amr, Abdul Islam Butt, and Brian Francis Meyer. Novel mutations underlying nephrogenic diabetes insipidus in Arab families. *Genetics in Medicine*, 8(7):443–447, July 2006.

[62] Mohamed Zaki, Torsten Schöneberg, Tareq Al Ajrawi, Abdul Nasser Al Said, Katrin Sangkuhl, and Holger Römpler. Nephrogenic diabetes insipidus, thiazide treatment and renal cell carcinoma. *Nephrology Dialysis Transplantation*, 21(4):1082–1086, April 2006.

[63] Youn Jong Park, Haing Woon Baik, Hae Il Cheong, and Ju Hyung Kang. Congenital nephrogenic diabetes insipidus with a novel mutation in the aquaporin 2 gene. *Biomedical Reports*, 2(4):596–598, July 2014. Publisher: Spandidos Publications.

[64] Michio Kuwahara. 2. Aquaporin-2, a Vasopressin-Sensitive Water Channel, and Nephrogenic Diabetes Insipidus. *Internal Medicine*, 37(2):215–217, 1998.

[65] Masanobu Fujimoto, Shin-ichi Okada, Yuki Kawashima, Rei Nishimura, Naoki Miyahara, Yasuo Kawaba, Keiichi Hanaki, Eiji Nanba, Yoshiaki Kondo, Takashi Igarashi, and Susumu Kanzaki. Clinical Overview of Nephrogenic Diabetes Insipidus Based on a Nationwide Survey in Japan. *Yonago Acta Medica*, 57(2):85–91, June 2014.

[66] Emel Saglar Ozer, Hanne B. Moeller, Tugce Karaduman, Robert A. Fenton, and Hatice Mergen. Molecular characterization of an aquaporin-2 mutation causing a severe form of nephrogenic diabetes insipidus. *Cellular and Molecular Life Sciences*, 77(5):953–962, March 2020.

[67] Achille Iolascon, Veruska Aglio, Grazia Tamma, Maria D'Apolito, Francesco Addabbo, Giuseppe Procino, Maria Carmela Simonetti, Giovanni Montini, Loreto Gesualdo, Erik W. Debler, Maria Svelto, and Giovanna Valenti. Characterization of Two Novel Missense Mutations in the AQP2 Gene Causing Nephrogenic Diabetes Insipidus. *Nephron Physiology*, 105(3):p33–p41, 2007. Publisher: Karger Publishers.

[68] Peter M. T. Deen, Marian A. J. Verdijk, Nine V. A. M. Knoers, Bé Wieringa, Leo A. H. Monnens, Carel H. van Os, and Bernard A. van Oost. Requirement of Human Renal Water Channel Aquaporin-2 for Vasopressin-dependent Concentration of Urine. *Science*, 264(5155):92–95, April 1994. Publisher: American Association for the Advancement of Science.

[69] Cécile Guyon, Yoann Lussier, Pierre Bissonnette, Alexandre Leduc-Nadeau, Michèle Lonergan, Marie-Françoise Arthus, Rafael Bedoya Perez, Anatoly Tiulpakov, Jean-Yves Lapointe, and Daniel G. Bichet. Characterization of D150E and G196D aquaporin-2 mutations responsible for nephrogenic diabetes insipidus: importance of a mild phenotype. *American Journal of Physiology-Renal Physiology*, 297(2):F489–F498, August 2009. Publisher: American Physiological Society.

[70] RD Liberatore-Jr, JG Carneiro, FB Leidenz, R Melilo-Carolino, HC Sarubi, and L Marco. Novel compound aquaporin 2 mutations in nephrogenic diabetes insipidus. *Clinics*, 67(1):79–82, January 2012. ISBN: 1980-5322 (Electronic) 1807-5932 (Linking).

[71] Alexander Oksche, Andreas Möller, John Dickson, Werner Rosendahl, Wolfgang Rascher, Daniel G. Bichet, and W. Rosenthal. Two novel mutations in the aquaporin-2 and the vasopressin V2 receptor genes in patients with congenital nephrogenic diabetes insipidus. *Human Genetics*, 98(5):587–589, September 1996.

[72] Qian Li, Bichao Lu, Jia Yang, Chao Li, Yanchun Li, Hui Chen, Naishi Li, Lian Duan, Feng Gu, Jianmin Zhang, and Weibo Xia. Molecular Characterization of an Aquaporin–2 Mutation Causing Nephrogenic Diabetes Insipidus. *Frontiers in Endocrinology*, 12, 2021.

[73] Seong-Su Moon, Han-Jong Kim, Yeon-Kyung Choi, Hyun-Ae Seo, Jae-Han Jeon, Jung-Eun Lee, Ju-Young Lee, Tae-Hwan Kwon, Jung-Guk Kim, Bo-Wan Kim, and In-Kyu Lee. Novel Mutation of <I>Aquaporin-2 </I>Gene in a Patient with Congenital Nephrogenic Diabetes Insipidus. *Endocrine Journal*, 56(7):905–910, 2009.

[74] Paul JM Savelkoul, Fabrizio De Mattia, Yuedan Li, Erik-Jan Kamsteeg, Irene BM Konings, Peter van der Sluijs, and Peter MT Deen. p.R254Q mutation in the aquaporin-2 water channel causing dominant nephrogenic

diabetes insipidus is due to a lack of arginine vasopressin-induced phosphorylation. *Human Mutation*, 30(10):E891–E903, October 2009.

[75] Fabrizio de Mattia, Paul J.M. Savelkoul, Erik-Jan Kamsteeg, Irene B.M. Konings, Peter van der Sluijs, Rudolf Mallmann, Alexander Oksche, and Peter M.T. Deen. Lack of Arginine Vasopressin–Induced Phosphorylation of Aquaporin-2 Mutant AQP2-R254L Explains Dominant Nephrogenic Diabetes Insipidus. *Journal of the American Society of Nephrology*, 16(10):2872–2880, October 2005.

[76] Pia Dollerup, Troels Møller Thomsen, Lene N. Nejsum, Mia Færch, Martin Österbrand, Niels Gregersen, Søren Rittig, Jane H. Christensen, and Thomas J. Corydon. Partial nephrogenic diabetes insipidus caused by a novel AQP2 variation impairing trafficking of the aquaporin-2 water channel. *BMC Nephrology*, 16(1):217, December 2015.

[77] Gitte R. Hinrichs, Sandrine Baltzer, Tamara Pallien, Per Svenningsen, Emil B. Dalgaard, Jens Michael Hertz, Claus Bistrup, Boye L. Jensen, and Enno Klussmann. A Novel AQP2 Sequence Variant Causing Aquaporin-2 Retention in the Cytoplasm and Autosomal Dominant Nephrogenic Diabetes Insipidus. *Kidney International Reports*, 7(10):2289–2294, October 2022.

[78] S. Raina, G. M. Preston, W. B. Guggino, and P. Agre. Molecular cloning and characterization of an aquaporin cDNA from salivary, lacrimal, and respiratory tissues. *The Journal of Biological Chemistry*, 270(4):1908–1912, January 1995.

[79] Philip Kitchen, Fredrik Öberg, Jennie Sjöhamn, Kristina Hedfalk, Roslyn M. Bill, Alex C. Conner, Matthew T. Conner, and Susanna Törnroth-Horsefield. Plasma Membrane Abundance of Human Aquaporin 5 Is Dynamically Regulated by Multiple Pathways. *PLOS ONE*, 10(11):e0143027, November 2015. Publisher: Public Library of Science.

[80] Carissa M. Krane, James E. Melvin, Ha-Van Nguyen, Linda Richardson, Jennifer E. Towne, Thomas Doetschman, and Anil G. Menon. Salivary Acinar Cells from Aquaporin 5-deficient Mice Have Decreased Membrane Water Permeability and Altered Cell Volume Regulation *. *Journal of Biological Chemistry*, 276(26):23413–23420, June 2001. Publisher: Elsevier.

[81] S. Steinfeld, E. Cogan, L. S. King, P. Agre, R. Kiss, and C. Delporte. Abnormal distribution of aquaporin-5 water channel protein in salivary glands from Sjögren's syndrome patients. *Laboratory Investigation; a Journal of Technical Methods and Pathology*, 81(2):143–148, February 2001.

[82] K. Tsubota, S. Hirai, L. S. King, P. Agre, and N. Ishida. Defective cellular trafficking of lacrimal gland aquaporin-5 in Sjögren's syndrome. *Lancet (London, England)*, 357(9257):688–689, March 2001.

[83] Yumeng Wang, Jianbo Wang, Yue Li, Chaolan Pan, Wange Zhou, Qiaoyu Cao, Zhirong Yao, Jianwen Han, and Ming Li. AQP5 pathogenic variants induce palmoplantar keratoderma Bothnia type in two Chinese families. *The Journal of Dermatology*, 49(4):463–468, April 2022.

[84] Y. Pan, Y. Men, and Z. Lin. Palmoplantar keratoderma Bothnia type with acrokeratoelastoidosis‐like features due to AQP5 mutations. *Clinical and Experimental Dermatology*, 44(1):88–91, January 2019.

[85] Paola Maura Tricarico, Donatella Mentino, Aurora De Marco, Cecilia Del Vecchio, Sabino Garra, Gerardo Cazzato, Caterina Foti, Sergio Crovella, and Giuseppe Calamita. Aquaporins Are One of the Critical Factors in the Disruption of the Skin Barrier in Inflammatory Skin Diseases. *International Journal of Molecular Sciences*, 23(7):4020, January 2022. Number: 7 Publisher: Multidisciplinary Digital Publishing Institute.

[86] Sabrina Marion, Eik Hoffmann, Daniela Holzer, Christophe Le Clainche, Marianne Martin, Martin Sachse, Iva Ganeva, Paul Mangeat, and Gareth Griffiths. Ezrin promotes actin assembly at the phagosome membrane and regulates phago-lysosomal fusion. *Traffic (Copenhagen, Denmark)*, 12(4):421–437, April 2011.

[87] Katharine A. Michie, Adam Bermeister, Neil O. Robertson, Sophia C. Goodchild, and Paul M. G. Curmi. Two Sides of the Coin: Ezrin/Radixin/Moesin and Merlin Control Membrane Structure and Contact Inhibition. *International Journal of Molecular Sciences*, 20(8):1996, April 2019.

[88] Corey Andrews, Yiting Xu, Michael Kirberger, and Jenny J. Yang. Structural Aspects and Prediction of Calmodulin-Binding Proteins. *International Journal of Molecular Sciences*, 22(1):308, December 2020.

[89] Karin L. Németh-Cahalan and James E. Hall. pH and Calcium Regulate the Water Permeability of Aquaporin 0*. *Journal of Biological Chemistry*, 275(10):6777–6782, March 2000.

[90] Hiroaki Ishida, Hans J. Vogel, Alex C. Conner, Philip Kitchen, Roslyn M. Bill, and Justin A. MacDonald. Simultaneous binding of the N- and C-terminal cytoplasmic domains of aquaporin 4 to calmodulin. *Biochimica et Biophysica Acta (BBA) - Biomembranes*, 1864(2):183837, February 2022.

[91] Kulandaiappan Varadaraj, Sindhu Kumari, Alan Shiels, and Richard T. Mathias. Regulation of Aquaporin Water Permeability in the Lens. *Investigative Ophthalmology & Visual Science*, 46(4):1393–1402, April 2005.

[92] Tamir Gonen, Yifan Cheng, Joerg Kistler, and Thomas Walz. Aquaporin-0 Membrane Junctions Form Upon Proteolytic Cleavage. *Journal of Molecular Biology*, 342(4):1337–1345, September 2004.

[93] Gisela Brändén, Greger Hammarin, Rajiv Harimoorthy, Alexander Johansson, David Arnlund, Erik Malmerberg, Anton Barty, Stefan Tångefjord, Peter Berntsen, Daniel P. DePonte, Carolin Seuring, Thomas A. White, Francesco Stellato, Richard Bean, Kenneth R. Beyerlein, Leonard M. G. Chavas, Holger Fleckenstein, Cornelius Gati, Umesh Ghoshdastider, Lars Gumprecht, Dominik Oberthür, David Popp, Marvin Seibert, Thomas Tilp, Marc Messerschmidt, Garth J. Williams, N. Duane Loh, Henry N. Chapman, Peter Zwart, Mengning Liang, Sébastien Boutet, Robert C. Robinson, and Richard Neutze. Coherent diffractive imaging of microtubules using an X-ray laser. *Nature Communications*, 10(1):2589, June 2019. Number: 1 Publisher: Nature Publishing Group.

[94] Alex J Noble, Venkata P Dandey, Hui Wei, Julia Brasch, Jillian Chase, Priyamvada Acharya, Yong Zi Tan, Zhening Zhang, Laura Y Kim, Giovanna Scapin, Micah Rapp, Edward T Eng, William J Rice, Anchi Cheng, Carl J Negro, Lawrence Shapiro, Peter D Kwong, David Jeruzalmi, Amedee des Georges, Clinton S Potter, and Bridget Carragher. Routine single particle CryoEM sample and grid characterization by tomography. *eLife*, 7:e34257, May 2018. Publisher: eLife Sciences Publications, Ltd.

[95] Helen M. Berman, John Westbrook, Zukang Feng, Gary Gilliland, T. N. Bhat, Helge Weissig, Ilya N. Shindyalov, and Philip E. Bourne. The Protein Data Bank. *Nucleic Acids Research*, 28(1):235–242, January 2000.

[96] J. D. Bernal and D. Crowfoot. X-Ray Photographs of Crystalline Pepsin. *Nature*, 133(3369):794–795, May 1934. Number: 3369 Publisher: Nature Publishing Group.

[97] D. W. Green, Vernon Martin Ingram, and Max Ferdinand Perutz. The structure of haemoglobin - IV. Sign determination by the isomorphous replacement method. *Proceedings of the Royal Society of London. Series A. Mathematical and Physical Sciences*, 225(1162):287–307, March 1954. Publisher: Royal Society.

[98] J. C. Kendrew, G. Bodo, H. M. Dintzis, R. G. Parrish, H. Wyckoff, and D. C. Phillips. A Three-Dimensional Model of the Myoglobin Molecule Obtained by X-Ray Analysis. *Nature*, 181(4610):662–666, March 1958. Number: 4610 Publisher: Nature Publishing Group.

[99] Jared A. Delmar, Jani Reddy Bolla, Chih-Chia Su, and Edward W. Yu. Crystallization of Membrane Proteins by Vapor Diffusion. *Methods in enzymology*, 557:363–392, 2015.

[100] W. Ostwald. Studien über die Bildung und Umwandlung fester Körper: 1. Abhandlung: Übersättigung und Überkaltung. *Zeitschrift für Physikalische Chemie*, 22U(1):289–330, February 1897. Publisher: De Gruyter (O).

[101] Naomi E Chayen. Turning protein crystallisation from an art into a science. *Current Opinion in Structural Biology*, 14(5):577–583, October 2004.

[102] J. K. Cockcroft. *A Hypertext Book of Crystallographic Space Group Diagrams and Tables*. Birkbeck College, University of London, 1999.

[103] Thomas Young. I. The Bakerian Lecture. Experiments and calculations relative to physical optics. *Philosophical Transactions of the Royal Society of London*, 94:1–16, December 1804. Publisher: Royal Society.

[104] William Henry Bragg and William Lawrence Bragg. The reflection of X-rays by crystals. *Proceedings of the Royal Society of London. Series A, Containing Papers of a Mathematical and Physical Character*, 88(605):428–438, July 1913. Publisher: Royal Society.

[105] E.E. Lattman and P.J. Loll. *Protein Crystallography: A Concise Guide*. Protein Crystallography. Johns Hopkins University Press, 2008.

[106] Kartik Ayyer, Oleksandr M. Yefanov, Dominik Oberthür, Shatabdi Roy-Chowdhury, Lorenzo Galli, Valerio Mariani, Shibom Basu, Jesse Coe, Chelsie E. Conrad, Raimund Fromme, Alexander Schaffer, Katerina Dörner, Daniel James, Christopher Kupitz, Markus Metz, Garrett Nelson, Paulraj Lourdu Xavier, Kenneth R. Beyerlein, Marius Schmidt, Iosifina Sarrou, John C. H. Spence, Uwe Weierstall, Thomas A. White, Jay-How Yang, Yun Zhao, Mengning Liang, Andrew Aquila, Mark S. Hunter, Joseph S. Robinson, Jason E. Koglin, Sébastien Boutet, Petra Fromme, Anton Barty, and Henry N. Chapman. Macromolecular diffractive imaging using imperfect crystals. *Nature*, 530(7589):202–206, February 2016. ISBN: 0000000000000.

[107] Parichita Mazumder and Kartik Ayyer. Understanding conformational dynamics from macromolecular crystal diffuse scattering, March 2021. Pages: 2021.02.11.429988 Section: New Results.

[108] Henry N. Chapman, Oleksandr M. Yefanov, Kartik Ayyer, Thomas A. White, Anton Barty, Andrew Morgan, Valerio Mariani, Dominik Oberthuer, and Kanupriya Pande. Continuous diffraction of molecules and disordered molecular crystals. *Journal of Applied Crystallography*, 50(Pt 4):1084–1103, August 2017.

[109] T. de Klijn, A. M. M. Schreurs, and L. M. J. Kroon-Batenburg. Rigid-body motion is the main source of diffuse scattering in protein crystallography. *IUCrJ*, 6(2):277–289, March 2019.

[110] Steve P. Meisburger, David A. Case, and Nozomi Ando. Diffuse X-ray scattering from correlated motions in a protein crystal. *Nature Communications*, 11(1):1271, March 2020. Number: 1 Publisher: Nature Publishing Group.

[111] Caroline A. Schneider, Wayne S. Rasband, and Kevin W. Eliceiri. NIH Image to ImageJ: 25 years of image analysis. *Nature Methods*, 9(7):671–675, July 2012. Number: 7 Publisher: Nature Publishing Group.

[112] Le Zhao and Wuyuan Lu. Mirror Image Proteins. *Current opinion in chemical biology*, 22:56–61, October 2014.

[113] N J Greenfield. Using circular dichroism spectra to estimate protein secondary structure. *Nature Protocols*, 1(6):2876–2890, 2006. ISBN: 1750-2799 (Electronic)\r1750-2799 (Linking).

[114] Vadim Kotov, Georg Mlynek, Oliver Vesper, Marina Pletzer, Jiri Wald, Celso M. Teixeira-Duarte, Herve Celia, Maria Garcia-Alai, Stephan Nussberger, Susan K. Buchanan, João H. Morais-Cabral, Christian Loew, Kristina Djinovic-Carugo, and Thomas C. Marlovits. In-depth interrogation of protein thermal unfolding data with MoltenProt. *Protein Science: A Publication of the Protein Society*, 30(1):201–217, January 2021.

[115] Cyril Dominguez, Rolf Boelens, and Alexandre M. J. J. Bonvin. HADDOCK: A Protein–Protein Docking Approach Based on Biochemical or Biophysical Information. *Journal of the American Chemical Society*, 125(7):1731–1737, February 2003. Publisher: American Chemical Society.

[116] Carol Rohl, Charlie Strauss, Kira Misura, and David Baker. Protein structure prediction using ROSETTA. *Methods in enzymology*, 383:66–93, February 2004.

[117] Daniel Varela, Vera Karlin, and Ingemar André. A memetic algorithm enables efficient local and global all-atom protein-protein docking with backbone and sidechain flexibility, March 2022. Pages: 2021.04.12.437963 Section: New Results.

[118] Mohammed AlQuraishi. AlphaFold at CASP13. *Bioinformatics*, 35(22):4862–4865, November 2019.

[119] Zhen Wang and Kevin L. Schey. Aquaporin-0 Interacts with the FERM Domain of Ezrin/Radixin/Moesin Proteins in the Ocular Lens. *Investigative Ophthalmology & Visual Science*, 52(8):5079–5087, July 2011.

[120] Jianyi Yang, Ivan Anishchenko, Hahnbeom Park, Zhenling Peng, Sergey Ovchinnikov, and David Baker. Improved protein structure prediction using predicted interresidue orientations. *Proceedings of the National Academy of Sciences*, 117(3):1496–1503, January 2020. Publisher: Proceedings of the National Academy of Sciences.

[121] Sidhartha Chaudhury, Sergey Lyskov, and Jeffrey J. Gray. PyRosetta: a script-based interface for implementing molecular modeling algorithms using Rosetta. *Bioinformatics (Oxford, England)*, 26(5):689–691, March 2010.

[122] G. C. P. van Zundert, J. P. G. L. M. Rodrigues, M. Trellet, C. Schmitz, P. L. Kastritis, E. Karaca, A. S. J. Melquiond, M. van Dijk, S. J. de Vries, and A. M. J. J. Bonvin. The HADDOCK2.2 Web Server: User-Friendly Integrative Modeling of Biomolecular Complexes. *Journal of Molecular Biology*, 428(4):720–725, February 2016.

[123] Rodrigo V. Honorato, Panagiotis I. Koukos, Brian Jiménez-García, Andrei Tsaregorodtsev, Marco Verlato, Andrea Giachetti, Antonio Rosato, and Alexandre M. J. J. Bonvin. Structural Biology in the Clouds: The WeNMR-EOSC Ecosystem. *Frontiers in Molecular Biosciences*, 8, 2021.

[124] Shin-ichi Terawaki, Ryoko Maesaki, and Toshio Hakoshima. Structural Basis for NHERF Recognition by ERM Proteins. *Structure*, 14(4):777–789, April 2006.

[125] Fayou Zhang, Beibei Liu, Yaqi Gao, Jiafu Long, and Hao Zhou. The crystal structure of the FERM and C-terminal domain complex of Drosophila Merlin. *Biochemical and Biophysical Research Communications*, 553:92–98, May 2021.

[126] Casey M. Finnerty, David Chambers, Janet Ingraffea, H. Richard Faber, P. Andrew Karplus, and Anthony Bretscher. The EBP50-moesin interaction involves a binding site regulated by direct masking on the FERM domain. *Journal of Cell Science*, 117(8):1547–1552, March 2004.

[127] John Jumper, Richard Evans, Alexander Pritzel, Tim Green, Michael Figurnov, Olaf Ronneberger, Kathryn Tunyasuvunakool, Russ Bates, Augustin Žídek, Anna Potapenko, Alex Bridgland, Clemens Meyer, Simon A. A. Kohl, Andrew J. Ballard, Andrew Cowie, Bernardino Romera-Paredes, Stanislav Nikolov, Rishub Jain, Jonas Adler, Trevor Back, Stig Petersen, David Reiman, Ellen Clancy, Michal Zielinski, Martin Steinegger, Michalina Pacholska, Tamas Berghammer, Sebastian Bodenstein, David Silver, Oriol Vinyals, Andrew W. Senior, Koray Kavukcuoglu, Pushmeet Kohli, and Demis Hassabis. Highly accurate protein structure prediction with AlphaFold. *Nature*, 596(7873):583–589, August 2021. Number: 7873 Publisher: Nature Publishing Group.

[128] Keisuke Hamada, Toshiyuki Shimizu, Shigenobu Yonemura, Shoichiro Tsukita, Sachiko Tsukita, and Toshio Hakoshima. Structural basis of

adhesion-molecule recognition by ERM proteins revealed by the crystal structure of the radixin—ICAM-2 complex. *The EMBO Journal*, 22(3):502–514, February 2003. Publisher: John Wiley & Sons, Ltd.

[129] Matthew A. Pearson, David Reczek, Anthony Bretscher, and P. Andrew Karplus. Structure of the ERM Protein Moesin Reveals the FERM Domain Fold Masked by an Extended Actin Binding Tail Domain. *Cell*, 101(3):259–270, April 2000.

[130] Susanna Törnroth-Horsefield. Chapter Five - Phosphorylation of human AQP2 and its role in trafficking. In Gerald Litwack, editor, *Vitamins and Hormones*, volume 112 of *Aquaporin Regulation*, pages 95–117. Academic Press, January 2020.

[131] Stefan Kreida, Jennifer Virginia Roche, Caroline Olsson, Sara Linse, and Susanna Törnroth-Horsefield. Protein-protein interactions in AQP regulation-biophysical characterization of AQP0-CaM and AQP2-LIP5 complex formation. *Faraday Discussions*, 209:35–54, 2018.

[132] Steve L. Reichow, Daniel M. Clemens, J. Alfredo Freites, Karin L. Németh-Cahalan, Matthias Heyden, Douglas J. Tobias, James E. Hall, and Tamir Gonen. Allosteric mechanism of water-channel gating by Ca2+-calmodulin. *Nature Structural & Molecular Biology*, 20(9):1085–1092, September 2013.

[133] James B. Fields, Karin L. Németh-Cahalan, J. Alfredo Freites, Irene Vorontsova, James E. Hall, and Douglas J. Tobias. Calmodulin Gates Aquaporin 0 Permeability through a Positively Charged Cytoplasmic Loop. *The Journal of Biological Chemistry*, 292(1):185, January 2017. Publisher: American Society for Biochemistry and Molecular Biology.

[134] Jennifer Virginia Roche, Sabeen Survery, Stefan Kreida, Veronika Nesverova, Henry Ampah-Korsah, Maria Gourdon, Peter M.T. Deen, and Susanna Törnroth-Horsefield. Phosphorylation of human aquaporin 2 (AQP2) allosterically controls its interaction with the lysosomal trafficking protein LIP5. *Journal of Biological Chemistry*, 292(35):14636–14648, September 2017. ISBN: 1083-351X (Electronic) 0021-9258 (Linking).

[135] Anna Frick. *Structural Studies of Aquaporins in Human Kidney and Plant*. 2014.

[136] Teresa M. Buck, Joel Eledge, and William R. Skach. Evidence for stabilization of aquaporin-2 folding mutants by N-linked glycosylation in endoplasmic reticulum. *American Journal of Physiology - Cell Physiology*, 287(5 56-5):1292–1299, 2004.

[137] Christiane Ritter, Katharina Quirin, Michael Kowarik, and Ari Helenius. Minor folding defects trigger local modification of glycoproteins by the ER folding sensor GT. *The EMBO Journal*, 24(9):1730–1738, May 2005.

[138] Sean C Taylor, Pierre Thibault, Daniel C Tessier, John J M Bergeron, and David Y Thomas. Glycopeptide specificity of the secretory protein folding sensor UDP–glucose glycoprotein:glucosyltransferase. *EMBO reports*, 4(4):405–411, April 2003. Publisher: John Wiley & Sons, Ltd.

[139] Susanna Törnroth-Horsefield, Yi Wang, Kristina Hedfalk, Urban Johanson, Maria Karlsson, Emad Tajkhorshid, Richard Neutze, and Per Kjellbom. Structural mechanism of plant aquaporin gating. *Nature*, 439(7077):688–694, 2006. ISBN: 0028-0836 1476-4679.

Research papers

Author contributions

Paper I:
Ezrin is a novel protein partner of aquaporin-5 in human salivary glands and shows altered expression and cellular localization in Sjögren's syndrome

I designed and performed the *in silico* analysis, made the corresponding tables and figures and participated in manuscript writing.

Paper II:
Structural basis for the interaction between the ezrin FERM-domain and human aquaporins

I designed and performed the *in silico* modelling, analysed the MST data and prepared the related figures. I took part in manuscript writing.

Paper III:
The role of phosphorylation in calmodulin-mediated gating of human AQP0

I helped design the stopped-flow experiments, performed the subsequent data analysis and helped prepare the corresponding figures and text.

Paper IV:
Structural and functional analysis of aquaporin-2 mutants in nephrogenic diabetes insipidus

I took a major in project design and did the protein expression, the majority of the purifications, nDSF, CD and crystallisation. I did the crystallographic work for one of the mutants. I wrote the code and performed the analysis of nDSF and CD data. I wrote the initial draft and made all figures but one.

Paper V:
Effect of growth conditions and detergents on continuous diffraction from membrane protein crystals

I took a major part in project design, did all the AQP2 expression, purification and crystallisation and produced some of the SoPIP2;1 crystals. I partook in collecting data at DESY and performed the statistical analysis of the data. I wrote the initial manuscript draft and prepared several of the figures.

Printed by Media-Tryck, Lund 2023 NORDIC SWAN ECOLABEL 3041 0903

Biochemistry and Structural Biology
Faculty of Science
Lund University

ISBN 978-91-7422-960-8