Structural and biochemical characterization of Roco proteins
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Chapter 7

Summary and Discussion

Nederlandse Samenvatting

Acknowledgements

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Summary and Discussion

Leucine-rich-repeat kinase 2 (LRRK2) is an extremely large and complex multi-domain protein which turned out to be incredibly difficult to study in many different aspects. Mutations in LRRK2 account for the majority of familial Parkinson’s Disease (PD) cases (Pringsheim et al., 2014). Many LRRK2 mediated pathways and interaction partners have been identified, but the cellular functions of LRRK2 and its malfunction in PD are still not understood in great detail (Boon et al., 2014; Wallings et al., 2015; Roosen and Cookson, 2016; Rosenbusch and Kortholt, 2016; Tang, 2016b). PD-linked mutations in LRRK2 are found in almost every domain, but are primarily located in the catalytic core of the protein (RocCOR-kinase domains) (Cookson and Bandmann, 2010). Several of the PD-mutations have been linked to a decrease in GTPase and/or an increase in kinase activity (West et al., 2005; Greggio et al., 2006; Guo et al., 2007; Jaleel et al., 2007; Lewis et al., 2007; Li et al., 2007; Luzón-Toro et al., 2007; Anand et al., 2009; Liao et al., 2014; Rudi et al., 2015b; Ho et al., 2016). However, the molecular mechanisms of G-protein and kinase activation remain to be determined. Here, our approach was to dissect the problem into smaller pieces, e.g. the kinase domain or the RocCOR tandem/LRR-RocCOR, and approach one after the other. My thesis was aimed to investigate the RocCOR domain tandem as the central and name giving part of Roco proteins.

Several lines of evidence suggest that the nucleotide binding state (GDP/GTP) of the Roc domain is important for kinase activation (West et al., 2005; Ito et al., 2007; Biosa et al., 2013). Nevertheless it is not clear how the Roc domain regulates the kinase domain on a molecular level. Classical G-proteins are inactive in the GDP-bound state and active when GTP is bound. The switch regions are flexible in the GDP-bound state but will be fixed in an active conformation when bound to the γ-phosphate of GTP, and thereby effectors can bind to this region (Vetter and Wittinghofer, 2001). For LRRK2, the situation was not clear: Liao et al. suggested that also the GTP bound form of the Roc domain is the active conformation that can stimulate LRRK2 kinase activity (Liao et al., 2014). However, several other studies showed that LRRK2 kinase activity does not change upon addition of GDP, GTP, or non-hydrolysable GTP analogues (Liu et al., 2011a; Taymans et al., 2011), while others suggested that an intermediate state during hydrolysis presents the active state of LRRK2 (Biosa et al., 2013; Rudi et al., 2015b). Also other aspects of the regulation of Roco protein besides RocCOR is still under debate (Nixon-abell et al., 2016).
Dimerization seems to be a major regulator of the Roco proteins’ G-protein cycle (Gotthardt et al., 2008; Gasper et al., 2009) and LRRK2 in particular (Berger et al., 2010; Daniëls et al., 2011b; Rudi et al., 2015b). Since biochemical evidence on this topic is limited and the LRRK2 protein is very difficult to work with in vitro, we employed prokaryotic Roco proteins as a model in order to study the biochemical and structural features of the RocCOR domain tandem.

In Chapter 2 and 3 we investigated the influence of dimerization on Roc activity. We confirmed that the C-terminal subdomain of COR (COR-B) is the dimerization device and that dimerization is important for the GTPase activity but not GDP or GTP binding (chapter 2, (Gotthardt et al., 2008)), highlighting the importance of dimerization for the G-protein cycle. In chapter 3, we could show that the Roco protein from Chlorobium tepidum (Ct) cycles between a monomer and dimer within half a minute in a nucleotide dependent manner, which is in the catalytically relevant time scale for GTP hydrolysis (10 minutes) (Deyaert et al., 2017a), implying that dimerization might have an important role in the hydrolysis mechanism. A mutant homologous to one mutated in LRRK2 PD patients (L487A, L1371V in LRRK2) shows impairment in this monomerization/dimerization cycle and a reduction in the single turnover GTP hydrolysis rate.

Both chapters show that that dimerization is important for the G-protein cycle. However the kinetic properties of the Roco G-protein cycle were not studied in great detail. Therefore, we set out to characterize several Roco proteins including LRRK2 biochemically in a systematic fashion (chapter 4). Consistent with previous theories and data (Gotthardt et al., 2008; Liao et al., 2014) we could confirm that all Roco proteins have nucleotide affinities in the micro-molar range, meaning that they don’t need a GEF for nucleotide exchange in contrast to classical small G-proteins. Moreover, we showed that the KM of all Roco proteins is consistently in the higher micro-molar range, enabling them to act as GTP sensors. Whether this is an important sensing function or just a kinetic feature of the protein remains to be shown in the context of the cell. Additionally the large difference between KM and KD points towards a more complex hydrolysis mechanism. We could show that this difference is a feature of the hydrolysis reaction itself and that P1 release is not the rate limiting step of the GTPase reaction. There seems to be a GTP dependent mechanism involved, independent of the canonical binding/hydrolysis site that we do not understand yet. All in all this shows that Roco proteins follow a unique G-protein cycle, different from classical G-proteins. Moreover, for LRRK2 it has been
demonstrated that the kinase is stimulated only in the presence of GTP but not GppNHp (a non-hydrolysable GTP analogue) or GDP, again indicating that no classical active or inactive conformations are present but rather that the cycling itself is the active form that enhances kinase activity.

Consistent with the hypothesis that not the GDP or GTP state is the active form, the structures of the \(Mb\) RocCOR tandem in the GppNHp and GDP bound states show no major differences in the switch region in contrast to conformational changes reported for classical small G-proteins such as Ras (Chapter 5). This again points out the difference to the classical small GTPases. Moreover we learned from these structures that the three subdomains (Roc, COR-A and COR-B) can obtain multiple conformations relative to each other. Also it seems clear that switch II and the RocCOR interface has an important role in the activation mechanism and function of the RocCOR domain tandem. Despite the fact that \(Mb\) RocCOR does not monomerize upon GTP binding, HDX experiments revealed that it undergoes a similar conformational change as the \(Ct\) Roco.

Taken all this data together we suggest the following activation mechanisms for Roco protein, here shown in the context of LRRK2 (Figure 1): Considering the nucleotide affinities, the majority of the LRRK2/Roco protein should be GTP bound. Exchange from GTP to GDP (and vice versa) is fast, but since the GTP concentration is usually 10 times higher than GDP, all protein should be bound to GTP (Traut, 1994). Moreover it has been demonstrated that the protein exists as a monomer in the cytosol and as a membrane bound dimer which is the more (kinase) active fraction (chapter 6). The cytosolic monomer is probably maintained by other proteins, namely 14-3-3. Recruitment to membranes is regulated by binding of Rabs to the N-terminus (Liu et al., 2017). At the membrane LRRK2 is a dimer and has its highest kinase activity. To be able to perform a hydrolysis reaction, the Roc domains need to come together by which the COR domains probably need to undergo a conformational change. Switch II and the hydrophobic interface between the Roc and the COR domain are very important for this process to mediate changes in the dynamic properties of the protein. In \(Ct\) it is possible that in order to allow this process, the COR domain needs to dissociate (chapter 3). The hydrolysis mechanism probably works in several steps, since we cannot explain the difference in \(K_M\) and \(K_D\) with a simple one step hydrolysis mechanism (chapter 4). Here, more research is clearly needed to answer during which step dimerization plays a role. As demonstrated in chapter 4, LRRK2 needs to hydrolyse GTP in order to fully activate its kinase. It is possible that membrane bound
LRRK2 has a higher GTP hydrolysis rate than the one we measured in vitro in solution as a dimer. Localization, dimerization and also interaction with other proteins and phosphorylation are important regulatory processes that influence each other and both kinase and GTPase activities.

With this thesis we could give a first combined insight into kinetic, structural and biochemical properties of the G-protein cycle of LRRK2 and Roco proteins. However, my work also raised important new questions. Since Roco proteins have only a moderate GTPase activity, it will be important to identify co-regulators; can for example membrane binding stimulate both GTPase and kinase activity? Also the dynamic changes of the protein especially in the RocCOR tandem need to be understood in order to explain the complex crosstalk of Roc and kinase domain via COR and the effect of mutations in this region. With the advances in the production of high quality LRRK2 protein, I believe it is now possible to tackle at least some of these questions employing LRRK2 full-length protein. Notwithstanding the important progress with LRRK2, still the panel of now available prokaryotic Roco proteins is still a valuable addition and, as demonstrated here, can give precious general insights and help to advance the field.
Figure 1: Proposed activation mechanism of LRRK2.
References


Nederlandse Samenvatting

De ziekte van Parkinson is een progressieve motorische aandoening, veroorzaakt door het verlies van dopaminerge neuronen in de middenhersenen. Algemeen bekende symptomen van Parkinson zijn tremor, stijfheid en posturale instabiliteit. Op cellulaire niveau wordt Parkinson gekenmerkt door de formatie van eiwitaggregaten. Deze zogenaamde “Lewy bodies” bestaan uit α-synucleïne, Leucine Rich Repeat Kinase 2 (LRRK2) en andere eiwitten. Ongeveer 2% van de personen ouder dan 80 jaar wordt wereldwijd getroffen door Parkinson. De meeste gevallen zijn sporadisch (zonder bekende oorzaak), maar genetische studies hebben aangetoond dat ten minste 10% van de gevallen verklaard kan worden door Mendelianaanse erfelijkheid. Parkinson-geassocieerde mutaties zijn gevonden in verschillende genen, waaronder SNCA / α-synucleïne, PINK1, LRRK2 en DJ-1. Het meest frequent gemuteerde gen is LRRK2, dat autosomaal dominante vormen van Parkinson veroorzaakt. Interessant is dat de symptomen van LRRK2 en sporadische Parkinson zeer vergelijkbaar zijn en daarom zou inzicht in de LRRK2-functie kunnen helpen bij het begrijpen van de ziekte van Parkinson in het algemeen.

LRRK2 is een zeer groot en complex eiwit dat uit meerder domeinen is opgebouwd en daarom erg lastig te onderzoeken is. Ondanks dat veel LRRK2 interactiepartners zijn geïdentificeerd, begrijpen we de cellulaire functies van LRRK2 nog steeds niet volledig. LRRK2 heeft twee enzymatische domeinen, een GTPase en een kinase domein. De meest voorkomende Parkinson mutaties hebben een verlaagde GTPase en verhoogde kinase activiteit. Het is echter onbekend hoe het G-domein en kinase precies geactiveerd worden en hoe de Parkinson mutaties de activiteit beïnvloeden. In dit proefschrift heb ik me gericht op het onderzoeken van het moleculaire activeringsmechanisme en functie van het G-domein. Omdat LRRK2 eiwit slechts in kleine hoeveelheden te isoleren is, heb ik ook gebruik van de homologe Roco eiwitten van lagere organismen als een model om de biochemische en structurele aspecten van het RocCOR domein tandem te bestuderen.

LRRK2 behoort tot de Roco eiwitfamilie dat gekenmerkt wordt door de aanwezigheid van een RocCOR domein tandem. Roc is het G-domein dat de GTPase-activiteit heeft. Om de juiste functie te vervullen zijn G-nucleotiden (GDP en GTP) essentieel. Ook dimerisatie, het samen komen van twee identieke eiwitmoleculen, is belangrijk voor de functie van LRRK2. Hoofdstuk 2 en 3 van dit proefschrift benadrukken het belang van dimerisatie voor de G-eiwit cyclus. Doormiddel van structurele en biochemische studies konden we bevestigen dat het C-terminale deel van COR essentieel is.
voor dimerisatie. Dimerisatie is belangrijk voor de GTPase-activiteit, maar niet voor GDP of GTP-binding.

In hoofdstuk 4 hebben we verschillende Roco eiwitten, waaronder LRRK2, op een systematische manier biochemisch gekarakteriseerd. In overeenstemming met eerdere theorieën en gegevens konden we bevestigen dat alle Roco eiwitten een unieke G-eiwit cyclus doorlopen. Ook laten we voor LRRK2 zien dat de kinase activiteit alleen gestimuleerd is in de aanwezigheid van GTP maar niet GppNHp (een niet-hydrolyseerbaar GTP analoog) of GDP. In tegenstelling tot klassieke G-eiwitten, schakelen Roco eiwitten dus niet tussen een actieve (GTP) en inactieve (GDP) conformatie, maar is de cyclus zelf de actieve vorm die de kinase activiteit verhoogd. Met andere woorden; LRRK2 moet GTP verbruiken om tot maximale kinase activiteit te komen.

In hoofdstuk 5 laten we drie verschillende kristalstructuren van de Mb RocCOR-tandem dimeer met atomaire resolutie zien. Consistent met de eerdere bevindingen wijst dit nogmaals op het verschil met de klassieke kleine GTPases. Bovendien hebben we van deze structuren geleerd dat de drie subdomeinen (Roc, COR-A en COR-B) meerdere conformaties ten opzichte van elkaar kunnen verwerven. Ook lijkt het duidelijk dat switch II en de RocCOR interface een belangrijke rol in het activatie mechanisme en de functie van het RocCOR domein tandem spelen.

In dit proefschrift hebben wij een gecombineerd inzicht kunnen geven in de kinetische, structurele en biochemische eigenschappen van de G-eiwit cyclus van LRRK2 en Roco eiwitten. Dit heeft niet alleen geleid tot een nieuw model voor het activeringsmechanisme (hoofdstuk 6 + 7), maar ook belangrijke nieuwe vragen aan het licht gesteld. Hoe kunnen conformatie veranderingen in de RocCOR tandem leiden tot een verhoogde kinase activiteit? Welke co-regulatoren beïnvloeden de GTPase activiteit? Welke rol speelt membraan-binding van LRRK2? Ondanks de belangrijke vooruitgang geboekt met de productie en isolatie van het humane LRRK2 eiwit, is het panel van de nu beschikbare bacteriële Roco-eiwitten een waardevolle toevoeging en kan het, zoals in dit proefschrift aangetoond, een belangrijke rol spelen in het beantwoorden van deze vragen.
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I don’t have an answer to that so I just start….

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