Summary

Nitrate reductase (NR) catalyses the first and rate-limiting step in nitrogen metabolism in plants, algae and fungi. It converts nitrate to nitrite by using NAD(P)H as electron donor. Nitrite is further reduced to ammonia (by the nitrite reductase), which is used as a nitrogen source for the biosynthesis of amino acids, nucleotides and other nitrogen-containing metabolites. The dimeric NR is a modular tri-domain-enzyme. The N-terminal Mo-domain, which harbours the molybdenum cofactor (Moco), is connected by linker1 with the heme b_5-containing heme-domain. This domain is joined via linker2 with the FAD-domain, which binds the FAD-cofactor and harbours an NAD(P)H-binding side. Electrons are transferred from NAD(P)H via FAD to the heme, followed by the transfer to the Moco, where the reduction of nitrate takes place. Because of nitrite toxicity, an overproduction of nitrite has to be prevented. Consequently, the NR is tightly regulated. The enzyme is inhibited due to a phosphorylation in linker1, which is followed by binding of a 14-3-3 protein. The molecular mechanism of the inhibition by phosphorylation and 14-3-3-binding of the NR was investigated in the work presented here.

NR, its domain fragments and an appropriate kinase (CDPK17) from *Arabidopsis thaliana* were heterologous expressed and purified. Eight out of the twelve 14-3-3 protein isoforms of *Arabidopsis* were cloned, heterologously expressed and purified to homogeneity. A phosphorylation assay was established and the inhibition efficiencies of the different 14-3-3 isoforms were determined.

By using different assays to study steady-state and pre-steady-state kinetics, the different electron transfer steps in the inhibited and non-inhibited NR were investigated. This resulted in the identification of the affected step, the electron transfer from heme to Moco, which was 9-fold reduced by 14-3-3 protein binding. Kinetic studies in viscous media led to the hypothesis of a domain movement in NR during electron transfer. Interaction analysis with surface plasmon resonance spectroscopy of phosphorylated N-terminal- and linker1-fragments of NR with 14-3-3 proteins, have shown that both regions are significantly able to bind 14-3-3 proteins in a phosphorylation-dependent manner. This demonstrates an addition role of the N-terminus in NR regulation.

From these results a new model of NR function and regulation including reduction-dependent domain movement has been established. The movement reduces the distance between the heme- and the Moco and causes a change in the reduction potential, both simplifying the electron transfer. The inhibition of NR is a consequence of the 14-3-3 protein mediated blocking of the intramolecular domain movement by binding to the linker1 and the N-terminus.