The structure and function of cytochrome *c* dependent Nitric Oxide Reductase from *Paracoccus denitrificans*.

Finja König, Katarzyna Jankowska, Maximilian Kahle, Sofia Appelgren* and Pia Ädelroth

Department of Biochemistry and Biophysics, Stockholm University, Stockholm, Sweden, * Department of Biology, Philipps University Marburg, Marburg, Germany,

The enzyme cytochrome *c* dependent Nitric Oxide Reductase (*c*NOR) is part of the branched respiratory chain of the organism *Paracoccus denitrificans (Pd)* which enables its anaerobic respiration of nitrogen compounds. *c*NOR catalyzes the reduction of nitric oxide to nitrous oxide. It consists of two subunits, NorC and NorB. NorC contains a *c*-heme which accepts electrons from a soluble electron donor. The electrons are passed to the NorB subunit which holds two *b*-hemes, one of the *b*-hemes and a non-heme iron make up the enzyme's active site. Previous work has shown that the insertion of a non-heme iron in the active site requires the expression of two chaperone proteins, NorQ and NorD [1]. Without co-expression of the chaperones the resulting *c*NOR is fully hemylated but lacking the non-heme iron, resulting in an inactive enzyme.

Using Cryo-EM we have solved the structure of *Pd c*NOR, it shows a dimer of two *c*NOR proteins ($NorB_2C_2$). This is in contrast to the only previously solved *c*NOR structure, a crystal structure of the enzyme from *P. aeruginosa* showing a monomeric *c*NOR (NorBC) [2]. A dimeric structure has been reported for a related protein, the quinol dependent Nitric Oxide Reductase (*q*NOR) [3]. In *q*NOR the dimeric form is more active than the monomer. However, the dimer interface in *q*NOR is different compared to our *c*NOR structure.

We have introduced mutations to residues in the dimer interface aiming to disrupt dimer formation to investigate the importance of dimerization. Blue Native PAGE results indicate that some of these mutations do affect dimer formation. The formation of a *c*NOR dimer is further investigated using Mass Photometry. We are also measuring the mutant's nitric oxide reduction activity and non-heme iron content. We hope to elucidate whether the dimer is biologically relevant for the activity of *c*NOR and/or for iron-insertion by the chaperones.

(1) Kahle, M.; Ter Beek, J.; Hosler, J. P.; Ädelroth, P. The Insertion of the Non-Heme FeB Cofactor into Nitric Oxide Reductase from P. Denitrificans Depends on NorQ and NorD Accessory Proteins. *Biochim. Biophys. Acta BBA - Bioenerg.* **2018**, *1859* (10), 1051–1058. https://doi.org/10.1016/j.bbabio.2018.05.020.

(2) Hino, T.; Matsumoto, Y.; Nagano, S.; Sugimoto, H.; Fukumori, Y.; Murata, T.; Iwata, S.; Shiro, Y. Structural Basis of Biological N_2 O Generation by Bacterial Nitric Oxide Reductase. *Science* **2010**, *330* (6011), 1666–1670. https://doi.org/10.1126/science.1195591.

(3) Jamali, M. A. M.; Gopalasingam, C. C.; Johnson, R. M.; Tosha, T.; Muramoto, K.; Muench, S. P.; Antonyuk, S. V.; Shiro, Y.; Hasnain, S. S. The Active Form of Quinol-Dependent Nitric Oxide Reductase from *Neisseria Meningitidis* Is a Dimer. *IUCrJ* **2020**, *7* (3), 404–415. https://doi.org/10.1107/S2052252520003656.