

Summary of Ph.D. thesis

**The role of the proteins involved in the *c*-type
cytochrome biogenesis and regulation of their genes in
*Sinorhizobium meliloti***

by

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2004

Introduction

The soil bacterium *Sinorhizobium meliloti* is able to form symbiotic interaction with the host plant alfalfa, and to fix atmospheric dinitrogen in the nodules formed on the roots of the host plant. Nitrogen fixation is an energetically expensive procedure. The required energy is provided by a symbiosis-specific electron transport chain, which comprises a terminal electron acceptor with high oxygen affinity. This terminal oxidase contains *c*-type cytochromes. *c*-type cytochromes fulfil important functions in all organisms, having the main role in the respiratory electron transport chains. Previous experiments showed that *S. meliloti* mutants defective in the biogenesis pathway of *c*-type cytochromes are not able to fix nitrogen.

The *c*-type cytochromes are assembled from two subunits: the apoprotein and the heme prosthetic group. Their covalent binding is generated on the periplasmic side of the membrane by the function of a cytochrome *c* heme lyase complex (CCHL), containing the CycH, CycJ, CycK and CycL proteins. Previous experiments

demonstrated that the role of the CycH protein is to bind the apoprotein and keep it in an appropriate conformation for its assembly with the heme group. The CycJ, CycK and CycL proteins are involved in the periplasmic transport of the heme group and its binding to the apoprotein. The exact mechanism of the function of Cyc proteins is not known. Intensive research work is carried out in many laboratories on different bacterial species to better understand the molecular mechanism of the functions of Cyc proteins.

Aims

The aim of our work was to investigate the role of the Cyc proteins in the *c*-type cytochrome biogenesis under microaerobic conditions, characteristics for the symbiotic environment. Furthermore, we studied the effect of microoxic conditions on the expression of the *cycHJKL* operon. Therefore, we aimed to answer the following questions:

1. Is the heme biosynthetic pathway affected by the absence of Cyc proteins?

2. What is the explanation for the different effects of Tn5 transposons in two independent insertion mutants (AT342 and PP2982) on the downstream *cycJKL* genes?
3. Which is the shortest N-terminal CycH protein region, able to maintain the low protoporphyrin IX (PPIX) level?
4. What is the role of the C-terminal periplasmic part of the CycH protein?
5. Certain *c*-type cytochromes are produced exclusively under microoxic conditions. We examined whether these conditions induced the expression of the *cycHJKL* operon as well.
6. Which are the regulatory proteins responsible for the microaerobic transcriptional induction of *cycHJKL* operon?

Techniques applied

Molecular biology techniques: DNA, RNA and protein isolation, electrophoresis, Southern-analysis, Northern-

analysis, polymerase chain reaction, cloning procedures, sequencing analysis, homolog recombinations.

Microbiological techniques: conjugation, transformation, fage transduction.

Biochemical techniques: β -galaktosidase activity measurements, detection of *c*-type cytochromes, detection of protoporphyrin IX, „respiratory” nitrate reductase assay.

Cultivation of *Medicago sativa* under laboratory conditions and their inoculation with bacteria.

Results and discussion

Experiments with *S. meliloti* strains carrying mutations in the *cycHJKL* operon revealed that the absence of the Cyc proteins resulted in strains deficient in nitrogen fixation and nitrate reduction ability, and in addition, the late steps of the heme biosynthetic pathway were also affected in these derivatives. In the *cyc* mutants (except for the strain PP2982) the immediate heme precursor (PPIX) accumulated compared to the wild-type strain. The accumulation of PPIX was probably due to the

fact that in the absence of the Cyc proteins, heme could not be transported in the periplasm and bound to the apoprotein. The different PPIX phenotypes of the two *cycH* mutants AT342 and PP2982 could not be explained by a differential effect of the two Tn5 insertions on the transcription of downstream *cyc* genes. None of the Tn5 insertions had polar effects on the expression of the *cycJ*, *K* or *L* genes. By using a series of plasmids carrying deletion derivatives of *cycH*, we demonstrated in complementation experiments that the N-terminal 96 amino acids of CycH protein, including its first transmembrane domain and the cytoplasmic loop, were in fact sufficient to rescue the PPIX phenotype. Comparing our results with earlier data we hypothesized three possible functions of the N-terminal 96 amino acids of CycH. First, it is conceivable that this fragment is sufficient to form a complex together with the other Cyc proteins, and the formation of the complex is required for the consumption of PPIX. Another possible role of the truncated CycH protein could be to support heme transport to the periplasm, hence in its presence the PPIX can not

accumulate. The N-terminal 96 amino acids of CychH may also be involved in the interaction with the ferrochelatase enzyme, which is responsible for inserting the reduced ferric ion into the porphyrin ring of PPIX. In the absence of this CychH region, ferrochelatase may not be functional and PPIX accumulates.

Analysis of the amino acid sequence of the C-terminal periplasmic part of CychH revealed three TPR domains. The function of these motifs is to promote protein-protein interactions between the TPR-containing protein and one or more non-TPR proteins. We analysed the role of these domains with complementation experiments, using plasmids, which code for CychH proteins lacking the first, second or third TPR domain, and demonstrated that all three TPRs are essential for the function of the protein. In their absence neither the Fix⁺ nor the Rnr⁺ phenotypes could be recovered. We suppose that the TPR domains in CychH have an essential role in the interactions with the apoprotein and/or the other members of cytochrome *c* heme lyase complex during cytochrome *c* biogenesis.

The transcription of the *fixNOQP* operon, coding for the subunits of the symbiosis-specific terminal oxidase complex, is induced microaerobically. The produced FixO and FixP proteins are *c*-type cytochrome apoproteins. We investigated whether the expression of the *cycHJKL* operon is also under the control of oxygen, and we found that its expression increased under microaerobiosis. We studied whether *S. meliloti* regulatory proteins known to be involved in sensing the oxygen level may have a role in the microaerobic induction of the *cyc* operon. Our data showed that FixL, FixJ and FixK proteins are not involved in this function. The results obtained with the ActS/R two component regulatory system demonstrated that the ActR protein had a role in activating the transcription of the *cyc* operon under microaerobiosis, and under conditions of intensive nitrogen fixation.

Publications

1. **Cinege G**, Kereszt A, Kertész S, Balogh G, Dusha I (2004) The roles of different regions of the CycH protein in *c*-type cytochrome biogenesis in *Sinorhizobium meliloti*. *Mol Genet Genomics* 271:171-179
2. Oláh B, Kiss E, Györgypál Z, Borzi J, **Cinege G**, Csanádi G, Batut J, Kondorosi A, Dusha I (2001) Mutation in the *ntrR* gene, a member of the *vap* gene family increases the symbiotic efficiency of *Sinorhizobium meliloti*. *Mol Plant-Microbe Interact* 15:922-931

Posters

1. Bodogai M, **Cinege G**, Dusha I (2003) The regulatory cascade of *nif-fix* genes is under the control of *ntrR* in *Sinorhizobium meliloti*. The 5th Hungarian Genetics Congress, Siófok.
2. **Cinege G**, Kereszt A, Balogh G, Dusha I (2002) Regulation of *cycHJKL* operon and its role in *c*-type cytochrome biogenesis. 5th European Nitrogen Fixation Conference, Norwich, United Kingdom.
3. **Cinege G**, Kereszt A, Balogh G, Dusha I (2002) The role of the different domains of one cytochrome *c* heme lyase in the *c*-type cytochrome biogenesis. Annual Meeting of the Molecular Biology Section, Hungarian Society of Biochemistry, Keszthely.
4. **Cinege G**, Kereszt A, Dusha I (1999) The role and regulation of the genes responsible for the *c*-type cytochrome biogenesis in *Sinorhizobium meliloti*. Annual Meeting of the Molecular Biology Section, Hungarian Society of Biochemistry, Sopron.

Lectures

- 1. Cinege G**, Kereszt A, Balogh G, Dusha I (2003) The role of the *Sinorhizobium meliloti* CychH protein in the *c*-type cytochrome biogenesis. The 5th Hungarian Genetics Congress, Siófok.
- 2. Cinege G**, Kereszt A, Borzi J, Balogh G, Dusha I (2002) The role of the different domains of one cytochrome *c* heme lyase in the *c*-type cytochrome biogenesis. Annual Research Series of the Biological Research Center, Szeged.
- 3. Cinege G**, Kereszt A, Borzi J, Dusha I (2000) The role and regulation of *cycHJKL* operon in *Sinorhizobium meliloti*. Annual Research Series of the Biological Research Center, Szeged.
- 4. Cinege G**, Dusha I (1999) Oxygen dependent regulation of *cyc* genes in *Sinorhizobium meliloti*. Closing Symposium of the International Training Course organised by the UNESCO, ICRO and the Biological Research Center of the Hungarian Academy of Sciences, Szeged.