II-F Electron Transfer Regulation in a Tetraheme-Cytochrome, Cytochrome c_3

Cytochrome c_3 is an electron transport protein found in several spacies of sulfate-reducing bacteria. This protein is a small (M.W. ≈ 14,000) soluble protein and posses four *c*-type hemes per molecule. Cytochrome c_3 has unique properties. It shows very low oxidation-reduction (redox) potentials (typically, –240 ~ –357 mV *vs.* NHE), and the solid film of the reduced cytochrome c_3 was shown highly electro-conductive. The macroscopic and microscopic redox potentials were determined for for a variety of sulfate-reducing bacteria. The major aims of this project is to elucidate the mechanism of the regulation of the electron transfer in cytochrome c_3 on the basis of tertiary structure. For this purpose, we have established a new and efficient expression system of *c*-type multhiheme cytochromes for the first time. By the use of this expression system, structural determination of the fully reduced cytochrome c_3 from *Desulfovibrio vulgaris* Miyazaki F by NMR and analysis of oxidation-reduction properties using amino acid replacement are going on.

II-F-1 A Simple, Rapid, and Highly Efficient Gene Expression System for Multiheme-Cytochromes *c*

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It is important to establish a simple and highly efficient gene expression system of *c*-type cytochromes for the wide variety of studies such as physicochemical analyses, bioelectronics, environmental chemistry, and biotechnology. Especially, a large-scale preparation of a multiheme cytochrome *c* and its related mutants such as the amino acid replacements are very difficult. The difficulty in preparing *c*-type cytochromes is due to the complexity of the protein maturation, which needs various specific enzymes such as signal sequence peptidase and heme lyase. For this reason the heterologous expression of a *c*-type cytochrome is a special challenge. Here, we have exploited the potential of *S. oneidensis* by using the pUC-type universal vectors for *E. coli* in the transformation and have established a much more efficient gene expression system. The genes of tetraheme cytochrome c_3 and hexadecaheme high molecular weight cytochrome *c* from *Desulfovibrio vulgaris* were overexpressed in *Shewanella oneidensis* MR-1 using pUC-type vectors to yield the periplasmic holoforms. Covalent multiheme attachments were successfully performed in *S. oneidensis*. Furthermore, it was shown that *S. oneidensis* could be directly transformed by a pUC-type vector of *E. coli* through electroporation. Transcription of the heterologous gene in *S. oneidensis* could be controlled by a *lac* promotor from *E. coli*. These results indicate that *S. oneidensis* can be used as an overexpression system for *c*-type multiheme-cytochrome genes using the well established genetic techniques in *E. coli*. In conclusion, a rapid, simple, and highly efficient gene expression system of *c*-type multiheme cytochromes in a heterologous host has been established. This system would open a new horizon in various studies such as electron transfer mechanism, bioelectronics, and environmental chemistry.

II-F-2 Structure Dertemination of the Fully Reduced Cytochrome c_3 From *D. vulgaris* Miyazaki F

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Heteronuclear NMR spectroscopy was used for calculation of the solution structural ferrocytochrome c_3 from *Desulfovibrio vulgaris* Miyazaki F (*Dv*MF). From 3D TOCSY-HSQC and NOESY-HSQC experiments, all ^{15}N and ^{1}H backbone signals except N-terminus and Pro residues were assigned. To complete side chain assignment, 2D DQF-COSY, TOCSY, and NOESY experiments were performed. A total number of 633 proton signals were assigned, which correspond to 97% of the total number of expected signals. At first, NOEs from NH signals were collected using 3D NOESY-HSQC spectra. Then 2D NOESY spectra were measured. In total, 2474 NOESY constrains were obtained. Eighty four restrains of the ϕ torsion angle obtained from an HMQC-J experiment were used for structure calculation. After structure calculations in the early stage, the restrains of the χ_1 torsion angle were added for 16 residues. These residues showed single dominant conformers in early structural calculations, which were consistent with the values of $J_{\alpha\beta}$, $J_{\alpha\beta}$ obtained from E-COSY experiments. The final 20 structures showed no restraint violations greater than 0.3 Å. The RMSD of the top 20 conformers was 0.37 Å for backbone atoms, and 0.95 Å for heavy atoms respect to the mean structure. Although the major folding was similar to each other for the solution structure of ferrocytochrome c_3 and the crystal structure of ferricytochrome c_3, the region involving heme 1 and heme 2 was different. This is consist with the reported solution structure of *Desulfovibrio vulgaris* Hildenborough ferrocytochrome c_3, which is highly homologous with *Dv*MF cytochrome c_3. From the backbone dynamic analysis, the average value of the S^2, in the reduced state is larger than that in the oxidized state, suggesting that backbone of *Dv*MF cytochrome c_3 is slightly more rigid in the reduced state than in the

oxidized state. However, the region involving heme1 and heme2 shows more flexibility in the reduced state than in the oxidized state.