Development of an expression system in *E. coli* for overproduction and proper post-translational modification of multiheme cytochromes *c*

Yuri Y. Londer, P. Raj Pokkuluri, Frank R. Collart, Marianne Schiffer
Biosciences Division, Argonne National Laboratory, Argonne, IL 60439, USA

Cytochromes *c* are electron transfer proteins containing one or more hemes covalently bound to the polypeptide chain. Not only are they widely used as models to address a number of issues (including electron transfer, apoptosis and protein folding) but they are also of interest in terms of development of expression protocols for proteins containing post-translationally attached prosthetic groups. The major challenge during heterologous expression of cytochromes *c* is to provide proper post-translational modification of nascent polypeptide chains (heme attachment). In *E. coli* this can be achieved by targeting the apoprotein to the periplasm and coexpressing a group of auxiliary proteins (CcmABCDEFGH) responsible for cytochrome maturation under anaerobic conditions.

Employing this approach, we recently developed an expression system for overproduction of a triheme cytochrome (known as PpcA or cytochrome c_7) from anaerobic deltaproteobacterium *Geobacter sulfurreducens* in *E. coli*. Our expression vector featured the OmpA leader sequence fused to the cytochrome gene and expression was controlled by the *lac* promoter. Interestingly, the N-terminal His-tag was found to be detrimental for proper post-translational modification but after its removal synthesis of the mature holoprotein became possible. The yield of the mature cytochrome was optimized by reducing IPTG concentration to levels as low as 10 μM (post-translational modification appears to be a rate-limiting step and a high rate of protein synthesis results in an overload of the maturation system and a high percentage of immature species). The protein was crystallized and its structure was solved at 1.45 Å resolution.

The same system was successfully used to produce a number of PpcA point mutants (presently about 30), with yields in the same range or occasionally higher (up to 16 mg/liter), and four other PpcA homologs found in the *G. sulfurreducens* genome, with yields varying from 3 to 10 mg per liter. For all four homologs and a few mutants X-ray structures were solved.

Furthermore, the *G. sulfurreducens* genome codes for three open reading frames that appear to be oligomers of covalently linked PpcA-like triheme domains very homologous to each other. Two of the proteins consist of four such domains (total of 12 hemes) and one consists of nine (27 hemes). Interestingly, all domains lack a conserved histidine that is an axial ligand for one of the hemes in cytochromes c_7. We cloned and expressed each of four putative domains from one of the 12-heme proteins, and solved the structure of one domain at 1.7 Å resolution. The structure showed that instead of the missing histidine the corresponding heme is coordinated by a methionine. Then we cloned and expressed all three two-domain units (hexaheme proteins) from the same protein and, eventually, the whole four-domain 12-heme protein. The yield of the pure full-length cytochrome was 0.5 mg per liter of culture. The same approach was also used to produce and purify the other 12-heme cytochrome from *G. sulfurreducens*. We crystallized both proteins and their structure determination is under way.

Based on our original vector used for expression of PpcA, we also designed a family of vectors (presently three) containing ligation independent cloning (LIC) sites and specifically

intended for expression of periplasmic proteins. Two vectors contain genes for periplasmic chaperones, Skp and FkpA, expressed constitutively. Currently, we are employing these vectors for high-throughput expression of other cytochromes *c* from *G. sulfurreducens*.

Acknowledgments

We are grateful to Dr. L. Thöny-Meyer (ETH, Zürich, Switzerland) for plasmid pEC86 carrying the *ccm* genes. This work is supported by the U.S. Department of Energy, the Office of Biological and Environmental Research, Structural Biology and NABIR programs under contract No. W-31-109-Eng-38 with the U.S. Department of Energy.