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Biochimica et Biophysica Acta 1187 (1994) 260–263

BBA Biochimica et Biophysica Acta

Bacterial genes and proteins involved in the biogenesis of *c*-type cytochromes and terminal oxidases

Linda Thöny-Meyer, Hannes Loferer, Daniel Ritz, Hauke Hennecke *

Mikrobiologisches Institut, Eidgenössische Technische Hochschule, Schmelzbergstrasse 7, CH-8092 Zürich, Switzerland

Received 7 March 1994

Abstract

A total of nine genes potentially concerned with the biosynthesis of *c*-type cytochromes have been identified recently in the bacteria *Bradyrhizobium japonicum* and *Rhodobacter capsulatus*, and homologous counterparts appear to be present also in *Escherichia coli*. Most of the respective gene products are membrane-bound, while others are located in the periplasmic space. As inferred from sequence analyses, several of these proteins may play roles in membrane transport or redox processes, both functions being consistent with the required steps in cytochrome *c* formation (membrane translocation of heme; covalent linkage of protoheme IX to cysteine thiols). Further genes of *B. japonicum*, *E. coli*, *Bacillus subtilis* and *Paracoccus denitrificans* have been studied whose products are necessary for the formation of intact heme/copper oxidases. Some of them are probably required in protein folding and assembly whereas others appear to be enzymes catalyzing steps in the biosynthesis of the heme cofactors.

Key words: Assembly; Cytochrome *c*; Heme/copper oxidase; Heme synthesis; Membrane transport; Periplasm

1. Introduction

Although much has been learned in the past about the composition of bacterial electron transport chains and the structural and functional properties of their constituent components, comparatively little information was available until recently on the pathways concerned with the biogenesis of bacterial cytochromes. To understand cytochrome biogenesis, several important questions need to be addressed. How are the apoproteins targeted to their cellular destinations (cytoplasmic membrane, periplasm)? Is correct folding of these proteins during membrane insertion or after membrane transloction assisted by specific chaperones? By which means, in which order, and in which compartments are the various cofactors synthesized and bound to their corresponding apoproteins? What are the structural requirements that govern the assembly of multi-subunit complexes in highly ordered respiratory chains? While complete answers to these questions are not yet at hand, this report presents a brief survey on some recently discovered bacterial genes and proteins that play a role specifically in the biogenesis of *c*-type cytochromes and in the formation of terminal oxidases of aerobic respiratory chains. The biogenesis of cytochromes of anaerobic respiratory chains, however interesting, is not addressed in this short review.

2. Biogenesis of *c*-type cytochromes

Type *c* cytochromes are periplasmic, soluble or membrane-anchored electron transfer proteins which contain one or several covalently bound protoheme IX molecules [1]. Covalent linkage formally occurs, in most cases, by the addition of two cysteine thiols in a characteristic Cys-X-X-Cys-His motif of the apoprotein to the double bonds in the two vinyl side-chains of protoheme IX. An enzyme called cytochrome *c* heme lyase (CCHL), as yet unidentified in bacteria, is thought to catalyze this reaction [2]. Furthermore, the periplasmic location of *c*-type cytochromes makes it likely that covalent linkage takes place in the periplasm or in the cytoplasmic membrane during or after apoprotein translocation, which suggests a requirement for addi-

* Corresponding author. Fax: +41 1 2620647.

SSDI 0005-2728(94)00069-H

tional protein factors such as specific transporters for heme or perhaps the apoprotein itself [3–5].

Two bacterial species, *Bradyrhizobium japonicum* and *Rhodobacter capsulatus*, have been studied more intensively than others with respect to a molecular analysis of genes involved in cytochrome *c* biogenesis. Genes identified thus far are listed in Table 1. They were found by studying mutations, often transposon insertions, that led to cytochrome oxidase and cytochrome *c* deficient phenotypes, and by complementation of such mutations with cosmid clones. In *B. japonicum*, the genes *cycV*, *cycW*, *ORF263*, *cycX*, *tlpB* are clustered in one chromosomal region [3,5,6], separated by a large distance from another chromosomal cluster containing the adjacent *cycHJKL* genes (Refs. [5–7]; Ritz, D., unpublished data). Similarly, homologous *helABCDX* genes and *ccl1* plus *ccl2* (Table 1) were found on separate DNA clones of *R. capsulatus* [4,8], whereas *cycH*- and *cycJ*-homologous genes have not (yet) been reported for this bacterium. According to a recent entry in the EMBL nucleotide sequence database (release 37.0; K. Robinson, 1993; accession No. Em – ba : echu49), centisome 49 of the *Escherichia coli* chromosome also contains a cluster of open reading frames that share a significant degree of similarity (~ 50–70%) with 8 of the 9 genes listed in Table 1. Knock-out mutations were constructed in most of the *B. japonicum* and *R. capsulatus* genes [3,4,7,8]. The uniform phenotype of all of the respective mutant strains was a lack of formation of most, if not all, *c*-type cytochromes in cells grown under certain defined conditions. A notable peculiarity is the *B. japonicum cycH* mutant which still synthesizes detectable amounts of cytochrome c_1, whereas all other *c*-type holocytochromes are not produced in cells grown aerobically [7]. Cytochrome *c*-deficient mutants have also been reported of many other bacteria (see Ref. [5] for a review), but the affected genes were not identified.

An inspection of the predicted amino acid sequences derived from the nucleotide sequences of the cloned genes have provided certain clues as to the properties and possible functions of the respective gene products (Table 1). It has been proposed, for example, that the CycVW/HelAB proteins are subunits of an ATP-dependent transport system which is possibly specific for heme [3–5]. HelX/TlpB is a periplasmic thioredoxin-like protein whose function might be to keep the cysteine residues of the cytochrome *c*-specific Cys-X-X-Cys-His motif in a reduced state before covalent ligation with heme takes place [8]. The CycHJ, CycK/Ccl1 and CycL/Ccl2 proteins may form part of an enzyme complex (CCHL?) that binds heme as a substrate and ligates it to the apoprotein (Refs. [4,7]; Ritz, D., unpublished results). The observation that two of these proteins have putative heme-binding motifs (see Ref. [5] for a review) would be consistent with this idea. In the future it will be necessary to establish suitable biochemical tests to assess the validity of the aforementioned speculations.

3. Biogenesis of terminal oxidases

Bacterial respiratory chains terminate either with a ubiquinol oxidase or with a cytochrome *c* oxidase, most of which are members of the large family of heme/copper oxidases. This superfamily of heme/copper oxidases encompasses three oxidase types: (i) the quinol oxidases, such as the bo_3 oxidase of *E. coli* [9], whose subunit II does not possess the Cu_A center; (ii) the

Table 1
Homologous genes which play a role in the biogenesis of *c*-type cytochromes in three Gram-negative bacterial species

Apparently equivalent genes [a]			Specific properties of gene product as derived from the predicted amino acid sequence
B. japonicum	*R. capsulatus*	*E. coli*	
cycV (200aa)	*helA* (214aa)	*yejW* (205aa)	ATP-binding subunit of an ABC-translocator
cycW (222aa)	*helB* (218aa)	*yejV* (220aa)	Membrane-integral protein
ORF263 (263aa)	*helC* (242aa)	*yejUT* (242aa) [b]	Membrane-integral protein, contains putative heme-binding motif
cycX (61aa)	*helD* (52aa)	*ORF69* (69aa)	Small membrane-bound protein
tlpB (194aa)	*helX* (176aa)	*yejQ* (185aa)	Signal sequence-directed periplasmic thioredoxin-like protein
cycH (369aa)			Membrane-anchored protein facing the periplasm
cycJ (169aa)		*yejS* (159aa)	Possibly a periplasmic protein, shares some homology with a chlorophyll-binding protein in cyanobacteria
cycK (660aa)	*ccl1* (653aa)	*yejR* (647aa)	Membrane-bound protein, contains putative heme-binding motif
cycL (160aa)	*ccl2* (149aa)	*yejP* (350aa) [c]	Possibly a periplasmic protein, contains putative heme-binding motif that is also present in eukaryotic cytochrome *c* heme lyases

[a] The number of amino acids (aa) present in the predicted gene products is given in parentheses; for references see text.
[b] The *yejU* and *yejT* coding sequences were combined to the *yejUT* gene by arbitrarily introducing one nucleotide to create amino acid 154 = X and fusing the frames of the two coding sequences. The fusion product corresponds in size and overall homology to ORF263 and HelC.
[c] The homology spans the region between amino acids 1 and 159.

aa_3-type cytochrome *c* oxidases, present not only in many aerobic bacteria but also in mitochondria [10], whose subunit II possesses the Cu_A center and receives the electrons from reduced cytochrome *c*; (iii) the recently discovered cbb_3-type cytochrome oxidases, first studied genetically in *B. japonicum* [11] and biochemically in *Rhodobacter sphaeroides* [12] and *R. capsulatus* [13], which do not contain a subunit II homolog at all (and, hence, have no Cu_A) but possess two membrane-anchored *c*-type cytochromes. The protein that is best conserved in all of these oxidases is the membrane-integral, heme/copper-binding subunit I, in which four conserved histidines are the ligands to the high-spin heme-Cu_B binuclear center, whereas two other conserved histidines provide the ligands for a low-spin heme [10,14,15]. Subunits I of terminal oxidases vary substantially with regard to the composition of heme molecules. Of the four heme types known (hemes A, B, D, O), either two of a kind or two different hemes in many possible combinations can be found bound. (The *E. coli bd*-type oxidase, which is not a member of the heme/copper oxidase superfamily [9,16], is not further considered in this article.) Hemes O and A can be derived by modifications from protoheme IX (heme B). In heme O, the vinyl group at position 2 of heme B is replaced by a farnesylhydroxyethyl group [17]. Heme A differs from heme O in having a formyl group in place of a methyl group at position 8 [18]. Accordingly, the biogenesis of certain terminal oxidases involves enzymes that catalyze the formation of either heme O or heme A from heme B. Furthermore, the existence of assembly factors can be envisaged which may assist in the binding of the cofactors (heme, Cu^{2+}). Those oxidases whose subunit II possesses the Cu_A center may require additional assembly factors, although these may be non-specific because Cu_A appeared to be correctly assembled in a mutated form of the bo_3 oxidase in the *E. coli* background, in which no native Cu_A-containing enzymes have ever been detected [19].

Genes involved in the biogenesis of heme/copper oxidases have been studied in *Paracoccus denitrificans*, *E. coli*, *B. japonicum* and several *Bacillus* species, primarily *Bacillus subtilis*. These are listed in Table 2. With the exception of the *tlpA* gene, which arose by characterizing an oxidase-negative transposon Tn*5* mutant, all other genes were discovered in the course of sequencing DNA regions that harboured the structural genes of the corresponding oxidases. The products of the five genes having a likely or known function can be classified roughly into three groups: (i) assembly factors (CtaE, CtaG); (ii) folding enzymes (TlpA); (iii) specific heme synthases (CyoE, CtaA).

Subunit III of heme/copper oxidases does not contain any of the redox centers. Deletion of the subunit III-encoding gene *ctaE* of *P. denitrificans* resulted in the production of defectively assembled cytochrome aa_3 [20]. Although cytochrome aa_3 and its Cu_A center were spectroscopically detectable at wild-type levels, membranes isolated from the *ctaE* mutant had almost no oxidase activity. This suggested that the CtaE protein is important for cytochrome aa_3 assembly at a step

Table 2
Bacterial genes involved in the biogenesis of heme/copper oxidases [a]

Gene [Reference]	Homologous genes present in other bacteria	Properties of gene product and proposed function
P. denitrificans [20]	*ctaE ctaE* (*B. subtilis*) *ctaE* (*B. firmus* OF4) *caaC* (*Bacillus* PS3) *cyoC* (*E. coli*) *coxO* (*B. japonicum*) *coxP* (*B. japonicum*)	Membrane-integral protein; involved in late step of oxidase assembly after cofactor incorporation
P. denitrificans ctaG [21]		Periplasmic protein; involved in cytochrome aa_3 assembly at a step before or during cofactor incorporation
B. japonicum tlpA [22,23]		Membrane-anchored protein facing the periplasm; acts as protein disulfide isomerase; involved in cytochrome aa_3 formation
E. coli cyoE [24–26]	*ctaB* (*P. denitrificans*) *ctaB* (*B. firmus* OF4) *ctaB* (*B. subtilis*) *caaE* (*Bacillus* PS3) *ORF*[b] (*B. japonicum*)	Membrane-integral protein; catalyzes heme O synthesis from heme B
B. subtilis ctaA [27]	*ctaA* (*B. firmus* OF4)	Membrane-integral protein; catalyzes heme A synthesis from heme O

[a] It must be recalled here that the cbb_3-type oxidases contain two different membrane-bound *c*-type cytochromes [11–13]. Hence, many – if not all – of those functions required for cytochrome *c* biosynthesis (Table 1) are probably also involved in the biogenesis of cbb_3 oxidases.
[b] This *ORF* was not sequenced completely; it is located downstream of the gene for subunit I (*coxA*) of cytochrome aa_3 [28].

after the incorporation of heme A and copper cofactors [20]. The CtaG protein of the same bacterium may also be an assembly factor, because in the absence of the *ctaG* gene cytochrome aa_3 was not spectroscopically detectable, whereas its constituent apoproteins were still present in membranes [21]. Thus, CtaG is required in an earlier assembly step than CtaE.

A cytochrome aa_3-defective *B. japonicum* mutant was described in which the mutation affected a novel gene called *tlpA*. The gene product, TlpA, is a membrane-anchored thioredoxin-like protein whose active site is exposed to the periplasm [22,23]. TlpA was recently shown to have protein disulfide isomerase activity (Loferer, H., unpublished results), which suggests that it is involved in a protein-folding step necessary for the formation of cytochrome aa_3. Whether or not one of the cytochrome aa_3 subunits is a direct target of TlpA action is presently unclear. Since *B. japonicum tlpA* mutants have a pleiotropic phenotype, it is possible that TlpA has additional target proteins which are not related to the cytochrome aa_3 biogenesis process [22].

Deletion of the *cyoE* gene in *E. coli* resulted in a disturbed binuclear center of the *bo*-type ubiquinol oxidase, in which heme O was replaced by heme B [24]. This was a first indication that the *cyoE* gene product is involved either in heme O synthesis or in its incorporation into the oxidase complex. Recent results from Anraku and coworkers proved that the first of these two interpretations is correct [25,26]. They showed that membrane preparations from a CyoE-overexpressing *E. coli* strain were able to catalyze the conversion of ferrous protoheme IX (reduced heme B) to heme O in the presence of Mg^{2+} and farnesyl diphosphate [25]. Hence, *cyoE* appears to encode a heme O synthase. An enzyme involved in heme A synthesis appears to be encoded by *ctaA*, a gene first identified in *B. subtilis*. The *ctaA* gene product was shown to be necessary for the conversion of heme O to heme A [27].

In conclusion, the results compiled in Table 2 demonstrate that significant progress has been made in the identification of genes and proteins necessary for the formation of heme/copper oxidases; yet, more genes will certainly be identified in the future. Further biochemical work such as that done with CyoE will also be required for a better understanding of mechanistic aspects.

Acknowledgements

Work done in the authors' laboratory was supported by grants from the Swiss National Foundation for Scientific Research and from the Federal Institute of Technology, Zürich. We thank H. Paul for typing the manuscript.

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