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Characterization of recombinant biosynthetic precursors of the cysteine tryptophylquinone cofactors of L-lysine-epsilon-oxidase and glycine oxidase from *Marinomonas mediterranea*☆

María Dolores Chacón-Verdú [a], Jonatan C. Campillo-Brocal [a], Patricia Lucas-Elío [a], Victor L. Davidson [b], Antonio Sánchez-Amat [a,*]

[a] Department of Genetics and Microbiology, University of Murcia, Campus de Espinardo, Murcia 30100, Spain
[b] Burnett School of Biomedical Sciences, College of Medicine, University of Central Florida, Orlando, FL 32827, USA

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ABSTRACT

The lysine-ε-oxidase, LodA, and glycine oxidase, GoxA, from *Marinomonas mediteranea* each possesses a cysteine tryptophylquinone (CTQ) cofactor. This cofactor is derived from posttranslational modifications which are covalent crosslinking of tryptophan and cysteine residues and incorporation of two oxygen atoms into the indole ring of Trp. In this manuscript, it is shown that the recombinant synthesis of LodA and GoxA containing a fully synthesized CTQ cofactor requires coexpression of a partner flavoprotein, LodB for LodA and GoxB for GoxA, which are not interchangeable. An inactive precursor of LodA or GoxA which contained a monohydroxylated Trp residue and no crosslink to the Cys was isolated from the soluble fraction when they were expressed alone. The structure of LodA revealed an Asp residue close to the cofactor which is conserved in quinohemoprotein amine dehydrogenase (QHNDH), containing CTQ, and methylamine dehydrogenase (MADH) containing tryptophan tryptophylquinone (TTQ) as cofactor. To study the role of this residue in the synthesis of the LodA precursor, Asp-512 was mutated to Ala. When the mutant protein was coexpressed with LodB an inactive protein was isolated which was soluble and contained no modifications at all, suggesting a role for this Asp in the initial LodB-independent hydroxylation of Trp. A similar role had been proposed for this conserved Asp residue in MADH. It is noteworthy that the formation of TTQ in MADH from the precursor also requires an accessory enzyme for its biosynthesis but it is a diheme enzyme MauG and not a flavoprotein. The results presented reveal novel mechanisms of post-translational modification involved in the generation of protein-derived cofactors. This article is part of a Special Issue entitled: Cofactor-dependent proteins: evolution, chemical diversity and bio-applications.

1. Introduction

Protein-derived cofactors are catalytic or redox centers of proteins that are formed by post-translational modification of one or more amino acid residues [1]. This modification gives to the residues involved the capacity to participate in enzymatic reactions, eliminating the requirement of exogenous cofactors. It also contributes to the diversity of modified proteins detected in biological systems. Protein-derived quinone cofactors in proteins are generated through post-translational modification of either Tyr or Trp residues, within the peptide. There are two kinds of cofactors derived from Trp (Fig. 1). Tryptophan tryptophylquinone (TTQ) is a quinone cofactor in which a Trp residue is di-oxygenated and is covalently cross-linked to another Trp residue [2]. TTQ is the cofactor of tetrameric $\alpha_2\beta_2$ amine dehydrogenases, methylamine dehydrogenase (MADH) [3] and aromatic amine dehydrogenase (AADH) [4,5]. In each enzyme TTQ resides in the smaller β subunit which is approximately 130 amino acids long. Cysteine tryptophylquinone (CTQ) is a cofactor in which the di-oxygenated tryptophan residue is cross-linked to a cysteine residue. CTQ was first described in the *Paracoccus denitrificans* quinohemoprotein amine dehydrogenase (QHNDH). This is a α,β,γ heterotrimeric protein and the cofactor is located in the smallest 82 amino acid long γ subunit [6]. It has recently been shown that CTQ is also the cofactor of LodA, an enzyme with lysine-ε-oxidase activity (EC 1.4.3.20) which was isolated from the marine bacterium *Marinomonas mediterranea*[7,8]. The hydrogen peroxide that LodA generates is involved in biofilm development and differentiation in microbial biofilms [9].

Abbreviations: CTQ, cysteine tryptophylquinone; TTQ, tryptophan tryptophylquinone; MADH, methylamine dehydrogenase; QHNDH, quinohemoprotein amine dehydrogenase; LodA, Lysine-epsilon-oxidase; GoxA, Glycine oxidase; MM, molecular mass

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* Corresponding author at: Department of Genetics and Microbiology, Faculty of Biology, University of Murcia. Campus de Espinardo, Murcia 30100, Spain. Tel.: +34 868884955; fax: +34 868393963.

E-mail addresses: mcv17747@um.es (M.D. Chacón-Verdú), jonatancristian.campillo@um.es (J.C. Campillo-Brocal), patlucel@um.es (P. Lucas-Elío), victor.davidson@ucf.edu (V.L. Davidson), antonio@um.es (A. Sánchez-Amat).

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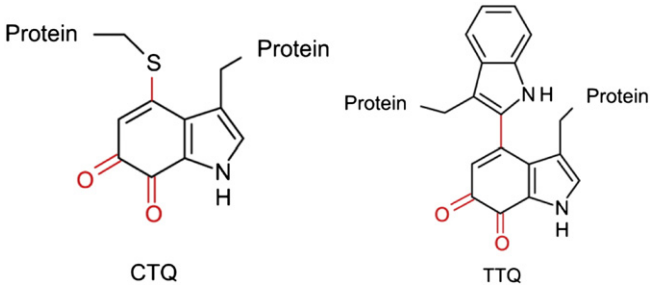

Fig. 1. Tryptophylquinone cofactors.

It has been shown that the mechanisms of post-translational modifications that are required to generate the tryptophan-derived quinone cofactors require one or more enzymes that are encoded in the same operons, or close in the genome, to the genes that encode the structural subunits. The genes encoding the two structural subunits of MADH in *P. denitrificans* are present in the methylamine utilization, *mau*, gene cluster [10,11]. Also present in this gene cluster is a gene encoding MauG [12], a diheme enzyme required for TTQ biosynthesis [13]. It was shown that inactivation of *mauG* caused production of an inactive MADH with incompletely formed TTQ. This precursor protein (preMADH) was monohydroxylated at residue βTrp-57 [14]. It was demonstrated that MauG could complete the formation of TTQ in vitro using preMADH as a substrate [15,16]. The genes encoding the three structural subunits of QHNDH in *P. denitrificans* are present in an n-butylamine-utilization operon that contains several genes and is completely different than the *mau* operon. Through mutagenesis of some of those genes, it has been possible to study the process of enzyme and CTQ maturation. *qhpD* encodes an enzyme belonging to the radical-SAM (S-Ado-Met) family, which has been proposed to participate in the formation of the intrapeptidyl thioether cross-links that are present in the γ subunit which also contains CTQ [17]. In addition, a subtilisin-like serine protease encoded by *qhpE* removes the leader peptide of the subunit containing the cofactor [18]. Recently, two more genes have been reported as essential for the synthesis of QHNDH. *qhpG* encodes a putative FAD-dependent monoxygenase and *phpF* an efflux ABC transporter [19]. In *M. mediterranea* LodA is encoded by *lodA*, a gene that forms part of the *lod* operon with only one other gene, *lodB*, that encodes a putative flavoprotein [20]. It has been shown that LodB is required for the synthesis of active LodA in the native system, although its precise role was unknown [21]. The cofactor of LodA is CTQ [8]. Unlike the other proteins containing a tryptophan derived cofactor, LodA has been successfully expressed in *Escherichia coli*. It was demonstrated that recombinant expression of active LodA in this system required the co-expression of LodB. The molecular mass (MM) of active LodA is +28 from what is predicted from the amino acid sequence [22]. This extra mass is in agreement with the post-translational modifications that generate CTQ. When *lodA* was expressed in the absence of *lodB*, a precursor of active LodA with a MM of +16 was isolated [22]. *M. mediterranea* synthesizes another enzyme with oxidase activity named GoxA with high sequence similarity to LodA, but whose substrate is glycine [23]. In the *gox* operon downstream of *goxA* is located a gene, named *goxB*, which shows a high sequence similarity to *lodB* [23].

The aim of this study has been to characterize biosynthetic intermediates in the process of the generation of the CTQ cofactors in LodA and GoxA and determine to what extent the process of cofactor biogenesis is similar and different from the biosynthesis of other protein-derived tryptophylquinone cofactors. It is demonstrated that the CTQ cofactor is correctly synthesized in the recombinant system in the presence of LodB. When *lodA* was expressed in the absence of *lodB* it was possible to isolate two precursor forms of LodA from the recombinant *lodA* expression. The extent of post-translational modification of each was determined by analysis by mass spectrometry of relevant peptides. In the soluble fraction a precursor protein monohydroxylated at the Trp in the cofactor was detected. By site directed mutagenesis a critical role in the generation of this precursor for an Asp residue conserved in LodA, MADH and QHNDH was revealed. A similar analysis was performed of precursors of GoxA that were isolated from the recombinant *goxA* expression system in the absence of *goxB*. The results indicate that while LodA and GoxA have no significant sequence or structural homology to the other known CTQ- and TTQ-dependent enzymes, and require a different type of modifying enzyme for post-translational modification, the intermediates in the process of cofactor biosynthesis are the same and the roles of amino acid residues in the cofactor site of mediating cofactor biosynthesis are similar.

2. Material and methods

2.1. Expression of recombinant proteins

The bacterial strains, plasmids and primers used in this study are listed in Supplementary Table 1. The construction of expression vectors for active LodA and LodA precursors was previously described [22]. To express GoxA, the *gox* operon, containing *goxA* and *goxB*, was cloned in the plasmid pET15-b (Novagen), generating the expression vector pETGOXAB15. To achieve that, the *gox* operon was amplified from MMB-1R genomic DNA by PCR with primers GODIRnde1-GOREVsma1. This product of the amplification was digested with *Nde*I and *Sma*I and cloned in pET15. The gene *goxA* was amplified using GODIRnde1-GOXAREVxho1 primers. In this case, the PCR product was cloned using the *Nde*I and *Xho*I restriction sites in plasmid pET15-b generating pETGOXA15. A second expression vector containing only *goxA* was generated by amplifying this gene from *M. mediterranea* genomic DNA by PCR using the primers GODIRSacI and GOXARevPst. The PCR product was cloned into pCOLADuet-1 (Novagen) using *Sac*I and *Pst*I restrictions sites to generate pGOXADuet. The expression vector pETGOXB11 was constructed by amplifying *goxB* from *M. mediterranea* genomic DNA by PCR using the oligonucleotides GODIRnde2 and GOREVsmaI. The product of the amplification was digested with *Nde*I and *Sma*I and cloned in the corresponding sites of plasmid pET11 (Novagen).

For recombinant expression of LodA *E. coli* Rosetta (DE3) cells, freshly transformed with the LodA expression vectors described in Supplementary Table 1 were inoculated in Luria–Bertani medium (LB) with the addition of 50 μg/ml ampicillin and 25 μg/ml chloramphenicol. Cultures were grown aerobically at 37 °C and 250 rpm up to an OD_{600} ~ 0.5. Then, a new culture medium with the same antibiotics was inoculated at an OD_{600} ~ 0.05 and grown in the same conditions. At an OD_{600} of ~0.6, protein expression was induced by adding 1 mM IPTG at 15 °C and 150 rpm. For recombinant expression of GoxA the *E. coli* cells freshly transformed with the GoxA expression vectors were inoculated in media LB plus 1% glucose and 50 μg/ml ampicillin and 25 μg/ml chloramphenicol. Cultures were grown aerobically at 37 °C and 250 rpm up to an OD_{600} ~ 0.5. Then, a new culture medium with the same antibiotics was inoculated at an OD_{600} ~ 0.05 and grown in the same conditions. At an OD_{600} of ~0.6, protein expression was induced by adding 0. 5 mM IPTG at 15 °C and 150 rpm. In each case cells were collected after an overnight incubation.

2.2. Site-directed mutagenesis of recombinant LodA

Single-point mutants of LodA (Cys-516-Ala, Trp-581-Ala and Asp-512-Ala) were constructed using the overlapping extension method [24]. The primers used for the amplifications are listed in Supplementary Table 1. pETLODAB15 plasmid was used as a template for the mutagenesis PCR. To perform PCRs, *Pfu* DNA polymerase (Promega) was used. The outer primers used to perform the last PCR step containing the mutations were MARDIRsac1 and MAREVeco2. After PCR amplification, fragments were cloned using the *Sac*I–*Sac*II sites in pETLODAB15, substituting the wild type copy of *lodA*. All mutations were confirmed