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Roles of active site residues in LodA, a cysteine tryptophylquinone dependent ε-lysine oxidase

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ABSTRACT

Site-directed mutagenesis identified residues in the substrate channel of LodA that play multiple roles in regulating K_m values of substrates, k_{cat} and the extent of biosynthesis of the protein-derived cysteine tryptophylquinone (CTQ) cofactor. Mutations of Cys448 increase K_m values for lysine and O_2, with the larger effect on K_{lysine}. Tyr211 resides within a mobile loop and is seen in the crystal structure of LodA to form a hydrogen bond with Lys530 that appears to stabilize its position in the channel. Y211F LodA had reduced levels of CTQ but near normal levels of k_{cat}. K530A and K530R variants exhibited diminished levels of CTQ but significantly increased k_{cat}. The Y211F, K530A and K530R mutations each caused large increases in the K_m values for lysine and O_2. These effects of the mutations of Tyr211 and Lys530 suggest that when these residues are hydrogen-bonded they may form a gate that controls entry and exit of substrates and products from the active site. Y211A and Y211E variants had the highest level of CTQ but exhibited no activity. These results highlight the different evolutionary factors that must be considered for enzymes which possess protein-derived cofactors, in which the catalytic cofactor must be generated by posttranslational modifications.

Introduction

LodA (previously called marinocine) is a ε-lysine oxidase that was first discovered in *Marinomonas mediterranea*, a melanogenic marine bacterium [1–3]. It catalyzes the oxidative deamination of lysine by removing the ε-amino group and releasing the aldehyde product plus hydrogen peroxide (Eq. (1)). When secreted out into the biofilm in which the bacterium resides, LodA exerts antimicrobial activity as a consequence of the production of H_2O_2. This is beneficial because causing death of a subpopulation of cells facilitates biofilm differentiation and dispersal of surviving cells with phenotypic variation among dispersed cells [4]. Wild type (WT) LodA has been recombinantly expressed in *Escherichia coli* [5] and isolated. LodA contains a protein-derived cofactor [6] which is generated by posttranslational modifications. The cofactor is cysteine tryptophylquinone (CTQ[1]) which is formed by the di-oxygenation of Trp581 and the crosslinking of this residue with Cys516 (Fig. 1). Expression of active LodA with correctly synthesized CTQ requires the co-expression of the *lodB* gene [5], which encodes a flavoprotein that is required for the posttranslational modifications that generate CTQ.

$$\text{Lysine} + O_2 + H_2O \rightarrow \text{2-aminoadipate 6-semialdehyde} + NH_3 + H_2O_2 \quad (1)$$

LodA is the first enzyme that was shown to use a tryptophylquinone cofactor that functions as an oxidase. All previously described enzymes that use CTQ or tryptophan tryptophylquinone (TTQ) cofactors are dehydrogenases that use other redox proteins or small molecules other than $NAD(P)^+$ as electron acceptors [7]. LodA is distinct from the other known tryptophylquinone enzymes in using an amino acid as a substrate. LodA is also unusual among amino acid oxidases since the other enzymes previously described in this group utilize a flavin cofactor. Three crystal structures of LodA have been determined, LodA isolated from *M. mediterranea* alone (PDB 3WEU) and with the lysine substrate covalently-bound to CTQ (PDB 3WEV) [8], and recombinant LodA isolated from *E. coli* (PDB 2YMW). Inspection of these structures identified the residues which are modified to form CTQ, Trp581 and Cys516. It was subsequently shown that mutation of either of these two residues to Ala

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[1] *Abbreviations:* CTQ, cysteine tryptophylquinone; TTQ, tryptophan tryptophylquinone; WT, wild-type; MADH, methylamine dehydrogenase; QHNDH, quinohemoprotein amine dehydrogenase.

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0003-9861/

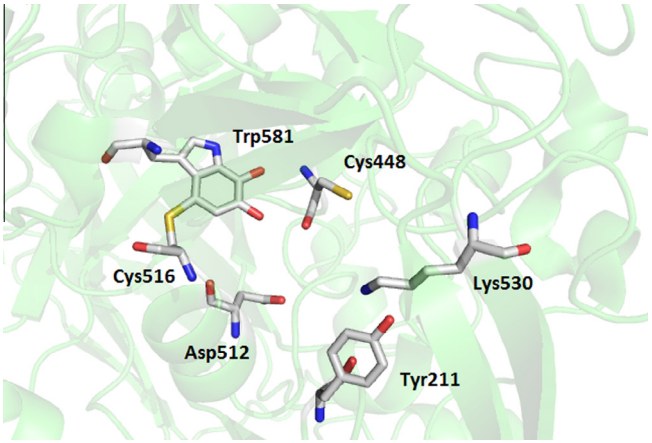

Fig. 1. Residues of interest in the active site of LodA. A portion of the structure of LodA (PDB entry 2YMW) is shown with the residues that form CTQ and other residues of interest in the active site shown as sticks.

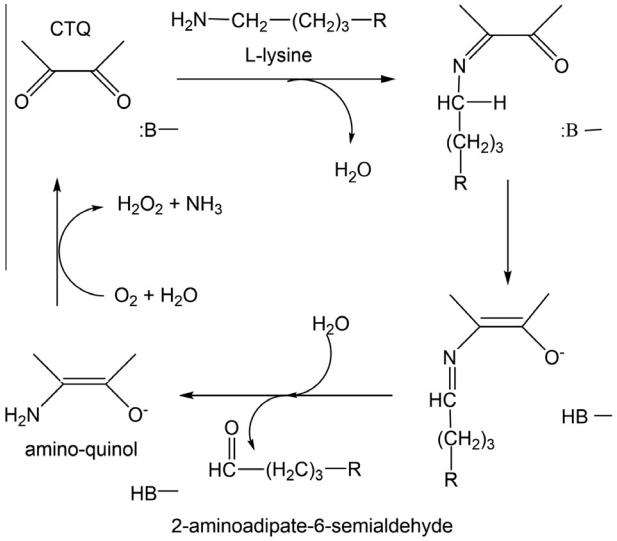

Fig. 2. Reaction mechanism for the LodA-catalyzed oxidative deamination of lysine. B represents an active-site residue which participates in acid–base chemistry.

resulted in no CTQ biosynthesis [9]. It was also shown that conversion of Asp512, which is in close proximity to CTQ, to Ala led to expression of a precursor of LodA without CTQ [9]. A similar effect was reported when the structurally analogous Asp was mutated in methylamine dehydrogenase (MADH). That led to expression of a precursor of MADH without TTQ [10].

A steady-state kinetic study showed that LodA follows a ping-pong mechanism in which it first interacts with lysine forming a Schiff base intermediate with CTQ, then releases the aldehyde product, and then binds O_2 to finally release NH_3 and H_2O_2 [11] (Fig. 2). From the structure of LodA it was also possible to identify other residues of interest in the active site of LodA which could participate in binding of one or both substrates, or catalysis, as well as CTQ biosynthesis (Fig. 1). These residues are the topic of this study. Cys448 is located in close proximity to the quinone oxygens of CTQ. Two other quinoproteins, the CTQ-containing quinohemoprotein amine dehydrogenase (QHNDH) [12] and the TTQ-containing MADH [13], have a structurally conserved Asp residue in the position corresponding to Cys448 in the LodA structure in the active site. Mutation of the corresponding Asp in MADH affected the efficiency of MauG-dependent TTQ biosynthesis but had no effect on the catalytic activity of the population of the isolated MADH with fully formed TTQ [10]. As such, Cys448 in LodA was converted to Asp, as well as Ala, to ascertain its role in CTQ biosynthesis or catalysis or both. Tyr211 resides in a loop comprised of residues 206–215, and it is flanked by one and three Gly residues on either side. This loop which is located close to the entrance of the active site was not visible in the crystals structures of the free and substrate bound native enzymes. In the structure of recombinant LodA, this loop was defined in the structure of one of the two molecules in the asymmetric unit but not in the other. These observations are consistent with mobility of this loop and a possible role in controlling substrate binding or product release, or both, from the active site. To probe the role of Tyr211 it was converted to Phe, Ala and Glu. Lys530 is of interest because it appears to form a hydrogen bond (2.7 Å) between its ε-amino N and the phenolic O of Tyr211. It was converted to Ala and Arg. The results of this study demonstrate that these residues in the active site of LodA play multiple roles in binding of lysine and O_2, catalysis and CTQ biosynthesis. Analysis of the Integrated Microbial Genomes database of genome sequences identified 168 genes from 144 different bacterial sequences that encoded LodA-like proteins [14]. LodA from *M. mediterranea* was classified as a member of Group IA LodA-like proteins. The residues which were mutated in this study were highly conserved in these sequences. These results are discussed in the context of the different evolutionary factors that must be considered for enzymes which possess protein-derived cofactors, in which the catalytic cofactor must be generated by posttranslational modifications.

Experimental

Construction, expression and purification of recombinant proteins

Wild-type (WT) LodA and LodA variants that were generated by site-directed mutagenesis were expressed in *E. coli* Rosetta cells as described previously with the *lodB* gene co-expressed [5,11]. The recombinant LodA possessed a 6XHis tag to facilitate purification as previously described [11]. The following LodA variants were generated by site directed mutagenesis: C448A, C448D, Y211A, Y211E, Y211F, K530A and K530R. The forward and reverse primers that were used are shown in Table 1. Most of the mutations were made using the QuikChange Lightning kit. The Y211A and C448A mutations were constructed using the overlapping extension method. In the latter case, pETLODAB15 was used as a template for the mutagenesis PCR [9] using *Pfu* DNA polymerase (Promega). The outer primers used to perform the last PCR step containing the mutations were MARDIRsac1 (5′-CTCTGGTGAGCTCCTACAG-3′)

Table 1
Primers used to generate site-directed mutations.

Mutation	Primers
C488A	Forward: 5′-GTTGGATTGCCTATGGCTCCAGGAATAGAAATG-3′ Reverse: 5′-CATTTCTATTCCTGGAGCCATAGGCAATCCACC-3′
C448D	Forward: 5′ GATTGCCTATGGATCCAGGAAT 3′ Reverse: 5′ ATTCCTGGATCCATAGGCAATC 3′
Y211A	Forward: 5′-CAGATCTCAGCGGTGCTGGTGGTGGAGATGA-3′ Reverse: 5′-TCATCTCCACCACCAGCACCGCTGAGATCTG-3′
Y211E	Forward: 5′ GATCTCAGCGGTGAAGGTGGT 3′ Reverse: 5′ ACCACCTTCACCGCTGAGATC 3′
Y211F	Forward: 5′ GATCTCAGCGGTTTTGGTGGT 3′ Reverse: 5′ ACCACCAAAACCGCTGAGATC 3′
K530A	Forward: 5′ AGCGTCAATGCAGCAAGTCAG 3′ Reverse: 5′ CTGACTTGCTGCATTGACGCT 3′
K530R	Forward: 5′ AGCGTCAATCGAGCAAGTCAG 3′ Reverse: 5′ CTGACTTGCTCGATTGACGCT 3′