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

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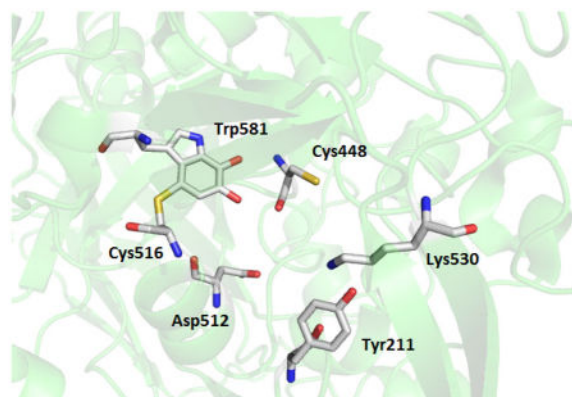
Abstract

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.

Graphical abstract

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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 *E. coli* [5] and isolated. LodA contains a protein-derived cofactor [6] which is generated by posttranslational modifications. The cofactor is cysteine tryptophylquinone (CTQ) which is formed by the di-oxygenation of Trp581 and the crosslinking of this residue with Cys516 (Figure 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.



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 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 O₂ to finally release NH₃ and H₂O₂ [11] (Figure 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 (Figure 1). These residues are the topic of this study. Cys 448 is located in close proximity to the quinone oxygens of CTQ. Two other quinoproteins, the CTQ-containing quinoxinoprotein 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 to 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 O₂, 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 (Ho *et al.*, 1989). 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) and MAREVeco2 (5'-GTGCTTGGGAGAATTCGCCTC-3'). After PCR amplification, fragments were cloned using the *SacI-SacII* sites in pETLODAB15, substituting the wild type copy of *lodA*. All mutations were confirmed by automated DNA sequencing. All cloning steps were performed using *E. coli* DH5 α and the plasmids obtained were electroporated or transformed into *E. coli* Rosetta just before induction.

The concentration of purified LodA was determined using an $\epsilon_{280} = 125,180 \text{ M}^{-1}\text{cm}^{-1}$ which was calculated from its amino acid sequence [15].

Steady-state kinetic analysis

The steady-state activity of ϵ -lysine oxidase was monitored using a coupled assay that included horseradish peroxidase (HRP), which uses the H_2O_2 product of the reaction to oxidize Amplex Red to resorufin, which has an extinction coefficient of $54000 \text{ M}^{-1}\text{cm}^{-1}$ at 570 nm [11]. The assay mixture contained 0.05 mM Amplex Red, 0.1 U/ml of HRP and 0.42 μM LodA (WT or variants) in 50 mM potassium phosphate buffer, pH 7.5, at 25 °C with the indicated amounts of lysine and O_2 included. To vary $[\text{O}_2]$ the buffer was made anaerobic by repeated cycles of vacuum and purging argon and mixed with an appropriate amount of air saturated ($[\text{O}_2]=252 \mu\text{M}$) or 100% O_2 -saturated ($[\text{O}_2]=1150 \mu\text{M}$) buffer [16]. In order to investigate the possibility that any of the mutations had converted LodA from an oxidase to a dehydrogenase, reactions were also performed under anaerobic conditions using artificial electron acceptors as described previously [11]. In these cases the reaction was initiated by addition of lysine and monitored by the spectral change associated each potential electron acceptor, phenazine ethosulfate (PES) plus 2,6-dichloroinophenol (DCIP) ($\epsilon_{600}=21,500 \text{ M}^{-1}\text{cm}^{-1}$), ferricyanide ($\epsilon_{420}=1,040 \text{ M}^{-1}\text{cm}^{-1}$), or NAD^+ ($\epsilon_{340}=6,220 \text{ M}^{-1}\text{cm}^{-1}$). The data were fit by

$$v/E = k_{\text{cat}}[\text{S}]/([\text{S}] + K_{\text{m}}) \quad [2]$$

Estimation of the extent of CTQ biosynthesis

The absorption spectrum of the purified oxidized form of active LodA exhibits characteristic features in the 350–450 nm range (Figure 3A). Inspection of the absorption spectra of the purified variant enzymes revealed the ratio of absorbance at 400 nm to absorbance at 280 nm (A_{400}/A_{280}) was affected by some of the mutations, despite the fact that proteins were pure as judged by SDS-PAGE. The features of the absorption spectra of the variants are essentially identical to that of WT LodA except for the intensity (Figure 3B). The most likely explanation for this is that some mutations affect the extent to which CTQ biosynthesis has occurred, yielding a mixed population of active LodA with mature CTQ and inactive preLodA with incompletely synthesized CTQ. Unfortunately, it was not possible to verify this by determining a molar extinction coefficient for CTQ in LodA by

performing a spectrochemical quantitative redox titration because of poor reactivity of the protein-bound CTQ towards dithionite and other reductants. For ease of comparison, the ratio of A_{400}/A_{280} for each variant is presented, normalized with the value for WT LodA set as 1.0 (Table 2). To be sure that the A_{400} was not varying due to changes in the active-site environment that were caused by mutations, the ratio of A_{400}/A_{280} was also determined for proteins after incubation in 6 M guanidine HCl. The values for these unfolded proteins were the same as for the native proteins indicating that the ratio of A_{400}/A_{280} was a valid indicator of the extent of CTQ biosynthesis. For determination of k_{cat} values, the concentration of enzyme was determined from A_{280} . As such one may wish to adjust the k_{cat} values for variants with normalized A_{400}/A_{280} values greater or less than 1.0. The K_m values will not be affected by the variation in A_{400}/A_{280} since the experimentally determined K_m is not dependent on the concentration of the enzyme.

Sequence alignments of LodA-like proteins

LodA-like proteins belonging to group IA, as defined previously [15] includes LodA and the most similar proteins. The proteins in this group were aligned using the program clustal omega [17] available at <http://www.ebi.ac.uk/Tools/msa/clustalo/>.

Results

Modification of the steady-state assay conditions to allow comparison of LodA variants

In the previous steady-state kinetic study of LodA, the maximum concentrations of lysine and O_2 that were used were 30 μM and 252 μM , respectively [11]. Those values were well above the K_m values that were determined for each substrate. Preliminary examination of the LodA variants which are the topic of the current study revealed that some of them exhibited significantly greater K_m values for these substrates. In order to obtain reasonable estimates for the K_m and k_{cat} values for these variants, it was necessary to extend the range of substrate concentrations used to 1 mM for lysine and 1150 μM for O_2 (100% O_2 saturation). In the presence of 1150 μM for O_2 , the reaction with WT LodA exhibited values of k_{cat} of $0.34 \pm 0.01 \text{ s}^{-1}$, K_m for lysine of $3.9 \pm 0.2 \mu\text{M}$. These values of k_{cat} and K_m for lysine are similar to the previous reported values of $0.22 \pm 0.04 \text{ s}^{-1}$ and $3.2 \pm 0.2 \mu\text{M}$, respectively [11]. However in the presence of 1 mM for lysine, a K_m for O_2 of $199 \pm 20 \mu\text{M}$ was obtained which is larger than the previously reported value of $37 \pm 6 \mu\text{M}$. The reason for this discrepancy is unclear. It may be an artifact caused by the very high lysine concentration which is required to study the variants. It is possible that lysine is binding non-specifically to the enzyme, or perhaps competing with the other substrate (O_2) in the ping-pong mechanism. Despite this peculiarity, in this study all LodA variants and WT LodA were analyzed at the higher concentration ranges so that the values of the kinetic parameters for each enzyme could be compared under the same conditions. Furthermore, as a consequence of some of the mutations, the apparent K_m for O_2 approached the $[O_2]$ in 100% O_2 -saturated buffer. As such it was not possible to perform experiments in which $[O_2]$ could be varied over an appropriate range so as to obtain true kinetic parameters. As a consequence of this, the K_m values that are reported here should be considered apparent K_m values. However, as can be seen in the results which follow (Table 2), the effects of these mutations on kinetic

parameters are quite evident and so the conclusions that can be drawn from these results are not compromised by these limitations imposed by the experimental system.

Effects of Cys448 mutations

Cys448 is present at the active site of LodA in close proximity (5.3 Å) to the CTQ cofactor. This residue was of particular interest because in the TTQ-dependent MADH, mutation of an Asp residue which occupies the analogous position near TTQ in the active site, to Asn caused decreased efficiency of TTQ biosynthesis [10]. C448A and C448D LodA mutations were made and each of these variant proteins was expressed and isolated at yields comparable to that of the WT LodA. The C448D LodA mutation actually increased the A_{400}/A_{280} ratio of the purified protein. This suggests that WT LodA contains a subpopulation with incompletely biosynthesized CTQ and that introducing Asp at this position in LodA has enhanced the efficiency of CTQ biosynthesis relative to WT LodA.

The C448A and C448D LodA variants each exhibited lysine oxidase activity (Figure 4, Table 2). The k_{cat} values of $0.39 \pm 0.02 \text{ s}^{-1}$ for C448A LodA and $0.29 \pm 0.03 \text{ s}^{-1}$ for C448D LodA are similar to that of WT LodA of $0.34 \pm 0.01 \text{ s}^{-1}$. Previous studies of LodA have indicated that an active site base is needed to deprotonate the lysine-CTQ adduct during catalysis [8, 11]. As the Cys448 mutations do not significantly decrease k_{cat} , this residue does not appear to function as an active-site base or play any direct role in the catalytic reaction steps. However, the K_{m} values for both substrates were increased. The K_{m} for O_2 of $544 \pm 94 \mu\text{M}$ for C448A LodA is about 3-fold greater than that for WT LodA. For C448D LodA the K_{m} of $386 \pm 70 \mu\text{M}$ is about 2-fold greater. The K_{m} for lysine of $48 \pm 4 \mu\text{M}$ for C448A LodA is 12-fold greater than that for WT LodA and for C448D LodA the K_{m} of $142 \pm 33 \text{ s}^{-1}$ is 36-fold greater. These data suggest an important role for Cys448 in lysine binding, or more specifically, facilitating the formation of the lysine-CTQ adduct.

Effects of Tyr211 mutations

Tyrosine 211 is situated on a mobile loop in proximity to the active site of LodA (discussed earlier). Tyr211 was mutated to Ala, Glu and Phe. In each case, the amount of protein expressed and isolated was similar to that of WT LodA. However, only the Y211F LodA exhibited activity. Interestingly the A_{400}/A_{280} for the purified Y211A LodA and Y211E LodA were each greater than that of WT LodA and equal to that of C448D LodA which is the highest value observed in this study. This indicates that the lack of enzymatic activity of Y211A and Y211E LodA is not due to lack of CTQ biosynthesis which was not compromised, but apparently optimized by these mutations. In contrast to those mutations, Y211F LodA exhibited an A_{400}/A_{280} less than that of WT LodA suggesting that the biosynthesis of CTQ is a bit less efficient in this variant. However, Y211F LodA was active (Figure 5, Table 2). The k_{cat} value of $0.19 \pm 0.02 \text{ s}^{-1}$ for Y211F is similar to that of WT LodA of $0.34 \pm 0.01 \text{ s}^{-1}$, particularly when one takes into consideration the lower A_{400}/A_{280} for Y211F LodA. The K_{m} for lysine is $129 \pm 22 \mu\text{M}$ which is 30-fold greater than for WT LodA. The K_{m} of O_2 is $522 \pm 162 \mu\text{M}$ which is 2.7-fold greater than for WT LodA. These results highlight the importance of an aromatic residue at position 211 for substrate binding. While Y211F LodA has a near normal k_{cat} , the affinity for each substrate is significantly compromised. Y211A and Y211E LodA are completely inactive, despite the presence of

CTQ, suggesting that binding of substrates is further compromised in these variants to the extent that activity is undetectable.

Effects of Lys530 mutations

Inspection of the one crystal structure in which Tyr211 is visible (PDB 3WEV) reveals that it forms a hydrogen bond with Lys530 (Figure 1). As such, this residue was mutated to Ala and Arg. The protein was expressed and isolated at levels comparable to WT LodA. However, the A_{400}/A_{280} was significantly decreased indicating that the presence or absence of Lys530 does affect the extent of CTQ biosynthesis. While this residue is not that close to CTQ, it is situated on the outer side of the active site and hence could be involved in CTQ biosynthesis by a direct or indirect involvement with the modifying enzyme LodB.

Interestingly, the mutations of Lys530 increased k_{cat} (Figure 5). For the reaction catalyzed by K530A LodA the k_{cat} is $1.2 \pm 0.1 \text{ s}^{-1}$ which is around 3.5-fold greater than for WT LodA and that for K530R is $0.68 \pm 0.04 \text{ s}^{-1}$ which is double that of WT LodA. Considering the lower A_{400}/A_{280} for K530A and K530R LodA, the true k_{cat} may be even greater if one considers the lower level of CTQ that is present. While the k_{cat} has been significantly enhanced by these mutations, the affinity for each substrate was greatly decreased. For K530A, the K_m value for lysine is $52 \pm 5 \mu\text{M}$ and that for O_2 is $1043 \pm 102 \mu\text{M}$. For K530R, the K_m value for lysine is $112.5 \pm 12.9 \mu\text{M}$ and that for O_2 is $706.1 \pm 94.8 \mu\text{M}$. While the affinity for O_2 is significantly decreased, because of the increase in k_{cat} , the $k_{cat}/K_m (\text{O}_2)$ values for K530 and K530R LodA of $1150 \text{ s}^{-1}\text{M}^{-1}$ and $960 \text{ s}^{-1}\text{M}^{-1}$ and not that much lower than the value for WT LodA of $1710 \text{ s}^{-1}\text{M}^{-1}$.

Conservation of Cys448, Tyr211 and Lys530 in sequences of proteins encoded by LodA-like genes

Analysis of the Integrated Microbial Genomes database of genome sequences identified 168 genes from 144 different bacterial genomes that encoded LodA-like proteins [14]. LodA from *M. mediterranea* was classified as a member of Group IA LodA-like proteins. Alignment of the sequences of the 20 members of the Group IA LodA-like proteins reveals that the residues which were mutated in this study are highly conserved in these sequences. Alignments of the regions of the sequence in which each of these three residues reside revealed that Cys448 (Supplemental Table S1) and Lys530 (Supplemental Table S2) are each conserved in each sequence. Tyr211 is conserved in 15 sequences and the other five have a Phe in this position (Supplemental Table S3).

Discussion

Site-directed mutagenesis of residues in the substrate channel of LodA have demonstrated that Cys448, Tyr211 and Lys530 play multiple roles in regulating K_m values of substrates, k_{cat} and the extent of biosynthesis of the protein-derived CTQ cofactor. It may seem intuitively odd that the WT enzyme would have less CTQ formed than some LodA variants. However, the fact that certain mutations either decrease or increase the efficiency of LodB-dependent CTQ biosynthesis is significant. This means that, in addition to furthering our understanding of the functional roles of these residues in the activity of LodA, these results

highlight the point that different evolutionary factors must be considered for enzymes which possess protein-derived cofactors. Residues present in the active site that may be optimal for catalytic efficiency may not be the best to participate in CTQ biosynthesis, and *vice versa*. While CTQ biosynthesis is catalyzed by another enzyme, the residues surrounding the nascent CTQ may play important roles in properly positioning the Cys and Trp residues for modification or substrate oxygen for insertion, or both. In this regard, it has been shown that the first modification in the biosynthesis of CTQ takes place auto-catalytically in the absence of LodB, and that some LodA residues are involved in that process [9].

The residue which corresponds in structure to Cys448 in two other quinoproteins, the CTQ-containing QHNDH [12] and the TTQ-containing MADH [13], is a structurally conserved Asp residue. Mutation of this 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]. Consistent with the results of the MADH study, it can be concluded that the residue at this position, Cys448 in LodA, is not the active-site base that is involved in deprotonation of the lysine-CTQ adduct during catalysis as the C448A mutation has no significant effect on k_{cat} . However, in contrast to the results of the MADH, mutation of Cys448 in LodA to Ala did not result in decreased levels of CTQ biosynthesis. Furthermore, the C448D LodA mutation appears to have enhanced the efficiency of CTQ biosynthesis. This suggests that the native LodA is a mixed population in which some of the protein has incompletely synthesized CTQ and is inactive. Together with the previous study that showed a role for the corresponding Asp in TTQ biosynthesis in MADH, it appears that an Asp residue in this position is preferred for tryptophylquinone cofactor biosynthesis. However, for LodA the presence of Asp at this position is not optimal for catalysis. In particular, this mutation increases the K_m for lysine 35-fold. Thus, the sub-optimal role in CTQ biosynthesis of Cys448 is more than compensated for by the greater catalytic efficiency of the enzyme.

Mutation of Tyr211 and its H-bonding partner Lys530 had significant effects on both k_{cat} and K_m values. As discussed earlier, Tyr211 is present on a mobile loop and it is possible that the H-bond to Lys530 could anchor it in place at certain times during the catalytic cycle. The aromatic character of residue 211 seems critical for activity since the Y211F LodA retains function, whereas Y211A LodA and Y211E LodA are inactive. The K530A and K530R mutations significantly increased the K_m values for the substrates, particularly O_2 . For lysine is increased 13- and 29-fold, respectively while for O_2 it increased 5- and 3,5-fold respectively. Since the loop containing Tyr211 was not observed in the structure of the lysine-CTQ adduct of LodA [8] it may not be important in stabilizing that intermediate. It is possible that Tyr211 in concert with Lys530 could aid in O_2 binding, or retention of O_2 in the active site to poise it for action. This would be consistent with the hydrophobic nature of residue 211 being important. Interestingly, the K530A mutation significantly increased k_{cat} . Since the rate-limiting step for the overall reaction catalyzed by LodA is not known, it is difficult to interpret this effect. Given the position of Lys530 in the active site, and the fact that mutations to Ala and Arg each enhance rather than eliminate activity, these results suggest that Lys530 is not playing a direct role in acid-base chemistry or nucleophilic attack during catalysis. However, it is possible that that it could control the rate of binding of one

or both of the substrates, or release of one or more of the products. If the hydrogen-bond between Lys530 and Tyr211 stabilizes the positions of these residues such that they impede the entry into or exit from the active site, then the removal of this blockage of the active site channel caused by the K530A mutation could possibly accelerate a rate-limiting product release step which could account for the increase in k_{cat} . This would also be consistent with the large increases in the K_m values that are caused by mutation of Lys530. In this sense the mobile loop which can be anchored by a hydrogen-bond between Tyr211 and Lys530 could act as a switch that regulates entry of substrates and exit of products to and from the active site.

A couple of points require consideration regarding the kinetic analysis of the LodA variants. As stated earlier, it was not possible to perform a detailed steady-state analysis in which both substrates were varied over an appropriate range of concentrations of each substrate. As such, it is an assumption that each variant obeys a ping-pong mechanism as does WT LodA. For example, with some flavoprotein oxidases it may be possible for reoxidation of the cofactor to precede or follow the release of the oxidized product. However, in the case of LodA, the substrate forms a covalent adduct with CTQ (Figure 2) and it is difficult to imagine how these mutations could alter the kinetic mechanism to allow O_2 to reoxidize the cofactor prior to release of the aldehyde product. It should also be noted that the mutations in this study involve residues with ionizable groups. It was previously shown [11] that k_{cat} for LodA exhibits a bell-shaped pH profile with a maximum at pH 7.5, implicating the involvement of at least two ionizable groups, and that the K_m for lysine also varied in this range. As such, it was not possible to compare kinetic parameters in a pH-independent region.

These results reflect the different evolutionary factors that must be considered for enzymes which possess protein-derived cofactors. Since the catalytic cofactor must be generated by posttranslational modifications, this suggests that the protein must have originally evolved for some other purpose. The modifying enzyme likely also originally evolved for a different purpose. The residues in the active site could not have evolved to optimize function as a lysine oxidase until after the evolution of the LodA that led to its specific interaction with the modifying enzyme LodB and the ability to catalyze CTQ biosynthesis. This is well-described by the observation that some Y211A and Y211E mutations maximize levels of CTQ biosynthesis but are inactive in catalysis. Also consistent with this argument is the observation that Lys530 mutations lead to decreased CTQ biosynthesis but increased k_{cat} . The fact that each of the mutations in this study increased K_m values for lysine and O_2 further indicate that the combination of residues throughout the active site channel together participate in the specificity and catalytic efficiency of the enzyme.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

CTQ	cysteine tryptophylquinone
TTQ	tryptophan tryptophylquinone
WT	wild-type
MADH	methylamine dehydrogenase
QHNDH	quinoxinohemoprotein amine dehydrogenase

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Highlights

Active site residues of LodA play multiple roles in catalysis and CTQ biogenesis

A hydrogen bond between Tyr211 and Lys530 may control access to the active site

Cys448 is a strong determinant of substrate specificity for lysine

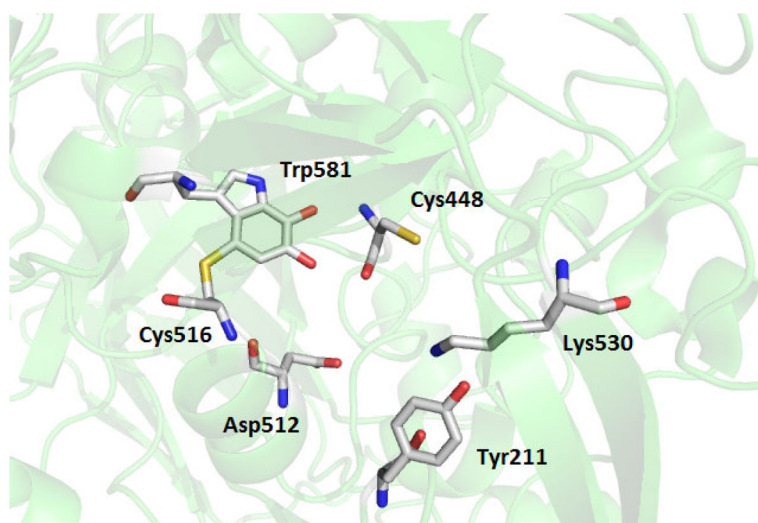


Figure 1. Residues of interest in the active site of LodA. A portion of the structure of LodA (PDB entry 2YMW) is shown with the residues that form CTQ and other residues of interest in the active site shown as sticks.

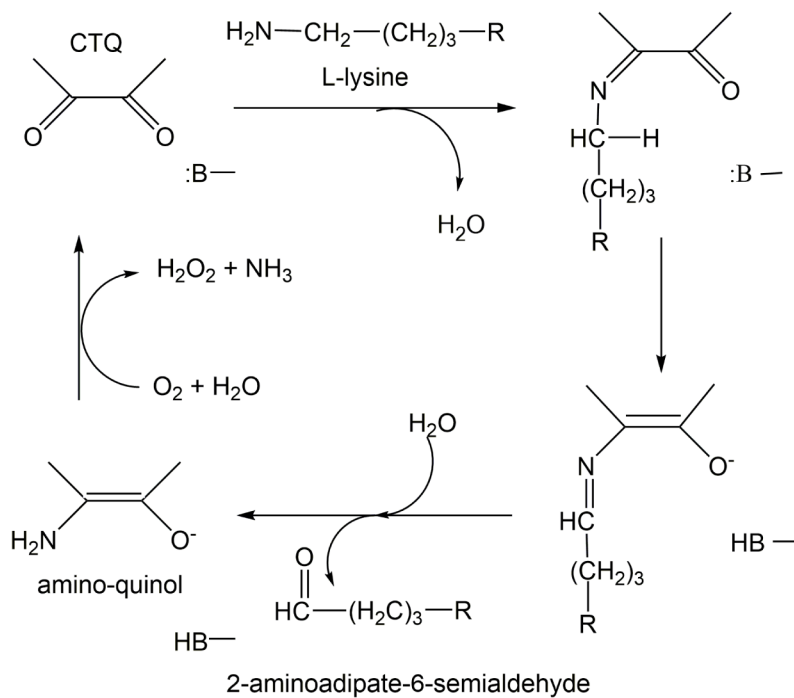


Figure 2. Reaction mechanism for the LodA-catalyzed oxidative deamination of lysine. B represents an active-site residue which participates in acid–base chemistry.

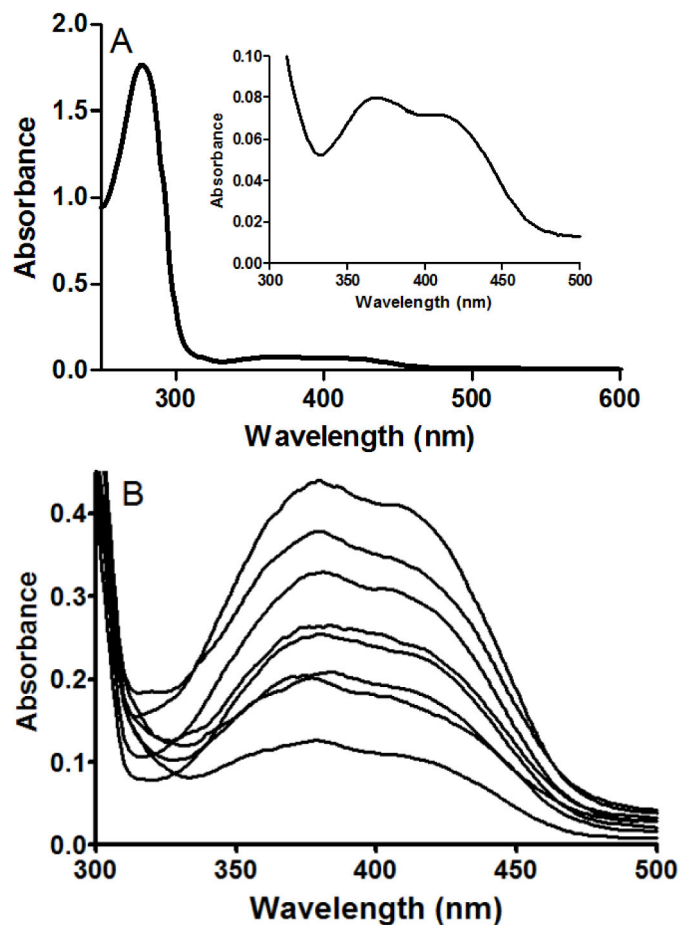


Figure 3.

The absorbance spectra of WT LodA and LodA variants. A. The absorbance spectrum of WT LodA with the visible absorbance features of CTQ magnified in the inset. B. The visible absorbance spectra of LodA variants in this study. The 280 nm absorbance in each of these spectra is identical so the observed variations in the intensity of the absorbance reflect the variation in A_{400}/A_{280} nm of these variant proteins. The spectra from top to bottom are C448D, Y211A, Y211E, WT, C448A, Y211F, K530A and K530R LodA.

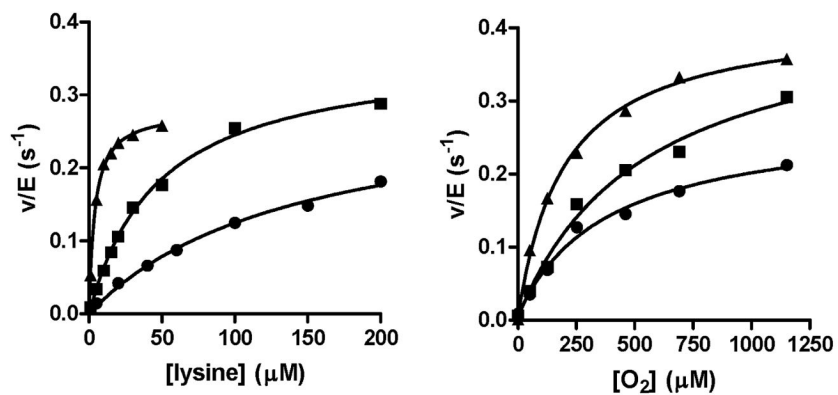


Figure 4. Effects of the C448A and C448D mutations on the steady-state activity of LodA. A. Assays were performed in the presence of a fixed concentration of 1150 O₂. B. Assays were performed in the presence of a fixed concentration of 1 mM lysine. The symbols represent WT LodA (▲), C448A LodA (■) and C448D LodA (●). The lines are fits of each data set to eq 1.

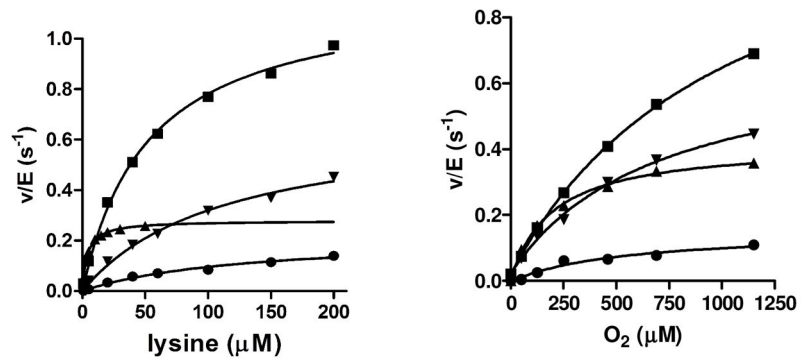


Figure 5. Effects of the Y211A, K530R and K530A mutations on the steady-state activity of LodA. A. Assays were performed in the presence of a fixed concentration of 1150 O_2 . B. Assays were performed in the presence of a fixed concentration of 1 mM lysine. The symbols represent WT LodA (▲), K530R (▼), K530A LodA (■) and Y211F LodA (●). The lines are fits of each data set to eq 1.

Table 1

Primers used to generate site-directed mutations

Mutation	Primers
C488A	Forward 5'-GTTGGATTGCCTATGGCTCCAGGAATAGAAATG-3' Reverse 5'-CATTCTATTCTGGAGCCATAGGCAATCCACC-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' ACCACCAAACCGCTGAGATC 3'
K530A	forward: 5' AGCGTCAATGCAGCAAGTCAG 3' reverse: 5' CTGACTTGCTGCATTGACGCT 3'
K530R	forward: 5' AGCGTCAATCGAGCAAGTCAG 3' reverse: 5' CTGACTTGCTCGATTGACGCT 3'

Table 2

Steady-state kinetic parameters for LodA variants

	Normalized value of A_{400}/A_{280} ^a	k_{cat} (s^{-1}) ^b	K_m lysine (μM)	K_m O ₂ (μM)
WT LodA	1.0	0.34 ± 0.01	3.9 ± 0.2	199 ± 20
C448A LodA	1.0	0.39 ± 0.02	48 ± 4	543 ± 94
C448D LodA	1.5	0.29 ± 0.03 (0.19)	142 ± 33	386 ± 70
Y211F LodA	0.8	0.19 ± 0.02 (0.23)	129 ± 22	522 ± 162
Y211A LodA	1.5	No reaction	No reaction	No reaction
Y211E LodA	1.5	No reaction	No reaction	No reaction
K530A LodA	0.7	1.2 ± 0.1 (1.7)	52 ± 5	1043 ± 102
K530R LodA	0.3	0.68 ± 0.04 (2.3)	113 ± 13	706 ± 95

^aFor comparison, this data is presented with the ratio of A_{400}/A_{280} for each variant normalized with the value for WT LodA set as 1.0.

^bFor determination of k_{cat} the concentration of enzyme was that which was determined from A_{280} . Values in parentheses are adjusted for variants with normalized A_{400}/A_{280} values greater or less than 1.0 to reflect what the k_{cat} values would be if these variants had the same level of CTQ biosynthesis as the WT LodA.