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Characterization of active site residues of nitroalkane oxidase

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ABSTRACT

The flavoenzyme nitroalkane oxidase catalyzes the oxidation of primary and secondary nitroalkanes to the corresponding aldehydes and ketones plus nitrite. The structure of the enzyme shows that Ser171 forms a hydrogen bond to the flavin N5, suggesting that it plays a role in catalysis. Cys397 and Tyr398 were previously identified by chemical modification as potential active site residues. To more directly probe the roles of these residues, the S171A, S171V, S171T, C397S, and Y398F enzymes have been characterized with nitroethane as substrate. The C397S and Y398 enzymes were less stable than the wild-type enzyme, and the C397S enzyme routinely contained a substoichiometric amount of FAD. Analysis of the steady-state kinetic parameters for the mutant enzymes, including deuterium isotope effects, establishes that all of the mutations result in decreases in the rate constants for removal of the substrate proton by ~5-fold and decreases in the rate constant for product release of ~2-fold. Only the S171V and S171T mutations alter the rate constant for flavin oxidation. These results establish that these residues are not involved in catalysis, but rather are required for maintaining the protein structure.

1. Introduction

The flavoenzyme nitroalkane oxidase (NAO) catalyzes the oxidation of nitroalkanes to the corresponding aldehydes or ketones with consumption of molecular oxygen and release of nitrite and hydrogen peroxide (Scheme 1) [1]. While there is growing evidence for hydride transfer mechanisms for the large family of flavoprotein oxidases [2–6], NAO is unusual in that it catalyzes nitroalkane oxidation through formation of a carbanion intermediate (Scheme 2) [3]. Based on analysis of the sequence of the cloned enzyme, NAO was identified as a homolog of the acyl-Co dehydrogenase (ACAD) family of flavoproteins [7]. This assignment is consistent with the similarities in the initial catalytic reactions of the two enzymes; in both cases a protein carboxylate abstracts an acidic proton from the substrate [1]. The subsequent determination of the structure of NAO [8] confirmed this assignment and provided insight into the use of this fold to catalyze the different reactions. Critically, while both the cofactors and the active site bases in NAO and ACAD occupy similar locations in the different proteins, the substrates access the active site from opposite sides of the protein [9], providing a structural basis for the reaction specificities. In addition to providing an opportunity to understand the structural basis by which a common structure can catalyze divergent reactions, NAO allows comparison of the enzyme-catalyzed reaction with a well-studied solution reaction. The comparable non-enzymatic reaction, formation of a nitroalkane anion from a nitroalkane, has long been studied as a model for proton abstraction from carbon [10,11]. Recently, study of the oxidation of nitroethane by NAO has allowed the evaluation of the contribution of quantum mechanical tunneling to the rate increase in the enzyme-catalyzed reaction [12–14].

As with many other flavoenzymes, the catalytic mechanism of NAO can be divided into oxidative and reductive half-reactions. In the reductive half-reaction, Asp402 abstracts the α-proton from the neutral nitroalkane substrate to form a nitroalkane anion which then attacks the N5 position of the FAD cofactor. The initial adduct rearranges to release nitrite and generate a reactive cationic imine species that reacts with hydroxide to eventually form reduced FAD and the aldehyde or ketone product. This cationic imine has been trapped with cyanide and its structure determined (Fig. 1), firmly establishing it as along the catalytic pathway [9,15]. In the more typical oxidative half-reaction, molecular oxygen attacks the reduced FAD to form hydrogen peroxide and regenerate the oxidized cofactor.

The knowledge of the structure of NAO has implicated several active site residues as potentially important for catalysis. The active site base of NAO, Asp402, is part of a catalytic triad that also contains Arg409 and Ser276 (Fig. 1). Mutation of any of these three residues decreases the rate constant for removal of the substrate proton by 2–3 orders of magnitude, confirming their importance

Abbreviations: NAO, nitroalkane oxidase; ACAD, acyl-CoA dehydrogenase; $K_{nitroethane}$, K_m value for nitroethane.

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$$RCH_2NO_2 + O_2 + H_2O \longrightarrow RCHO + NO_2^- + H_2O_2 + H^+$$

Scheme 1.

[9,16,17]. In addition, the structures of both the resting form of the enzyme and of the cyanide-trapped intermediate show that the hydroxyl of Ser171 forms a hydrogen bond with the N5 position of the flavin, suggesting it may modulate the reactivity of the flavin. A similar interaction is seen in the ACAD family, which contains a conserved threonine residue that aligns with Ser171 in NAO. Mutagenesis of this threonine to alanine in human medium-chain ACAD significantly decreases the rate constant for flavin reduction [18]. Finally, chemical modification studies have identified Cys397 [19] and Tyr398 [20] as putative active site residues in NAO. We report here the results of site-directed mutagenesis to investigate the roles these three residues play in catalysis.

2. Experimental procedures

2.1. Materials

All chemicals were purchased from Sigma–Aldrich Chemical Corp. (Milwaukee, WI). Recombinant nitroalkane oxidase was expressed and purified as previously described [7]. Mutations were generated with the QuikChange Site-Directed Mutagenesis Kit (Stratagene), and the mutant enzymes were expressed and purified following the protocol for the wild-type enzyme. DNA sequencing of the entire coding sequence of each mutant plasmid was performed at the Laboratory for Plant Genome Technologies of Texas A&M University. Protein concentrations were determined by the method of Bradford [21] with bovine serum albumin as standard; for all kinetic measurements, enzyme concentrations were determined using an ε_{446} value of 14.2 mM^{-1} cm^{-1}, as previously described [22].

2.2. Methods

Enzyme activity was measured in 200 mM HEPES, 0.1 mM FAD, pH 8.0, 30 °C, by monitoring oxygen consumption with a computer-interfaced Hansatech Clark oxygen electrode (Hansatech Instruments, Pentney King's Lynn, UK). When varying the concentration of nitroethane, the steady-state kinetic parameters were determined at ambient oxygen concentrations. (This concentration of oxygen is 5–10 times the K_m value for oxygen for each mutant.) To vary the concentration of oxygen at a constant concentration of nitroethane, oxygen and argon were combined in different ratios with a MaxBlend low flow air/oxygen blender (Maxtec Inc., Salt Lake City, Utah), and the assay buffer was equilibrated with the gas mixture. To prevent the formation of the anionic form of the substrate, stock solutions of neutral nitroethane were prepared in dimethyl sulfoxide and assays were initiated by the addition of substrate.

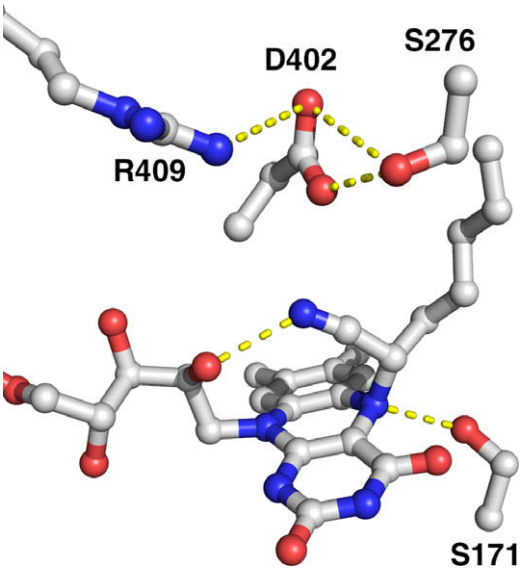
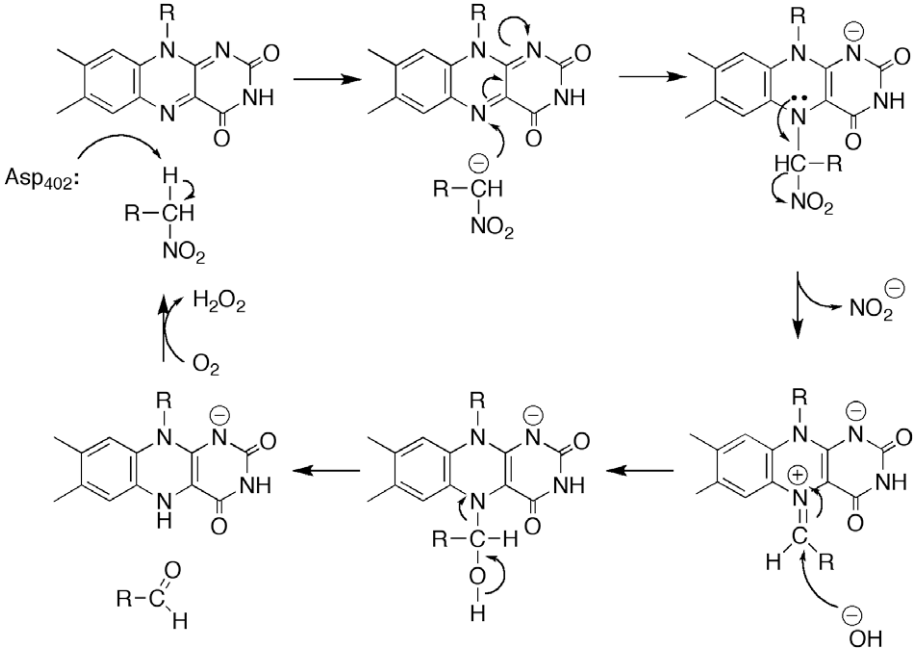


Fig. 1. Interactions in the active site of nitroalkane oxidase. The structure is that of the enzyme trapped with cyanide turning turnover with 1-nitrohexane (pdb code 3D9G) and shows the 5-cyanohexyl-FAD.



Scheme 2.