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Evidence for an Essential Arginine in the Flavoprotein Nitroalkane Oxidase

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The flavoprotein nitroalkane oxidase from the fungus *Fusarium oxysporum* catalyzes the oxidative denitrification of primary or secondary nitroalkanes to yield the respective aldehydes or ketones, hydrogen peroxide and nitrite. The enzyme is inactivated in a time-dependent fashion upon treatment with the arginine-directed reagents phenylglyoxal, 2,3-butanedione, and cyclohexanedione. The inactivation shows first order kinetics with all reagents. Valerate, a competitive inhibitor of the enzyme, fully protects the enzyme from inactivation, indicating that modification is active site directed. The most rapid inactivation is seen with phenylglyoxal, with a k_{inact} of 14.3 ± 1.1 M^{-1} min^{-1} in phosphate buffer at pH 7.3 and 30 °C. The lack of increase in the enzymatic activity of the phenylglyoxal-inactivated enzyme after removing the unreacted reagent by gel filtration is consistent with inactivation being due to covalent modification of the enzyme. A possible role for an active site arginine in substrate binding is discussed.

Keywords: Nitroalkanes, Flavoprotein, Chemical modification, Active site, Arginine, Phenylglyoxal, Flavoprotein nitroalkane oxidase

INTRODUCTION

Biological systems able to catalyze the removal of a nitro group from nitroalkanes are of considerable interest both for applied and fundamental reasons. Due to their chemical properties, nitroalkanes are widely used as organic solvents, industrial intermediates, fuel additives, and explosives.[1] No recent production figures appear to be available but estimates run as high as 30 million pounds annually,[1] with consequent concerns of environmental pollution. Nitroalkanes are carcinogenic and toxic.[1] Therefore, an enzymatic activity able to degrade these compounds is of significant interest for bioremediation. From a chemical standpoint, the deprotonation of nitroalkanes in solution is a well-characterized chemical reaction[2] that provides the basis for the understanding of the enzyme-catalyzed

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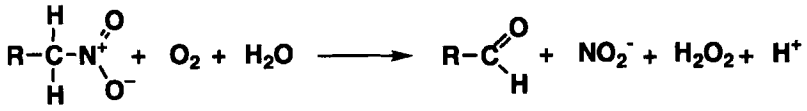
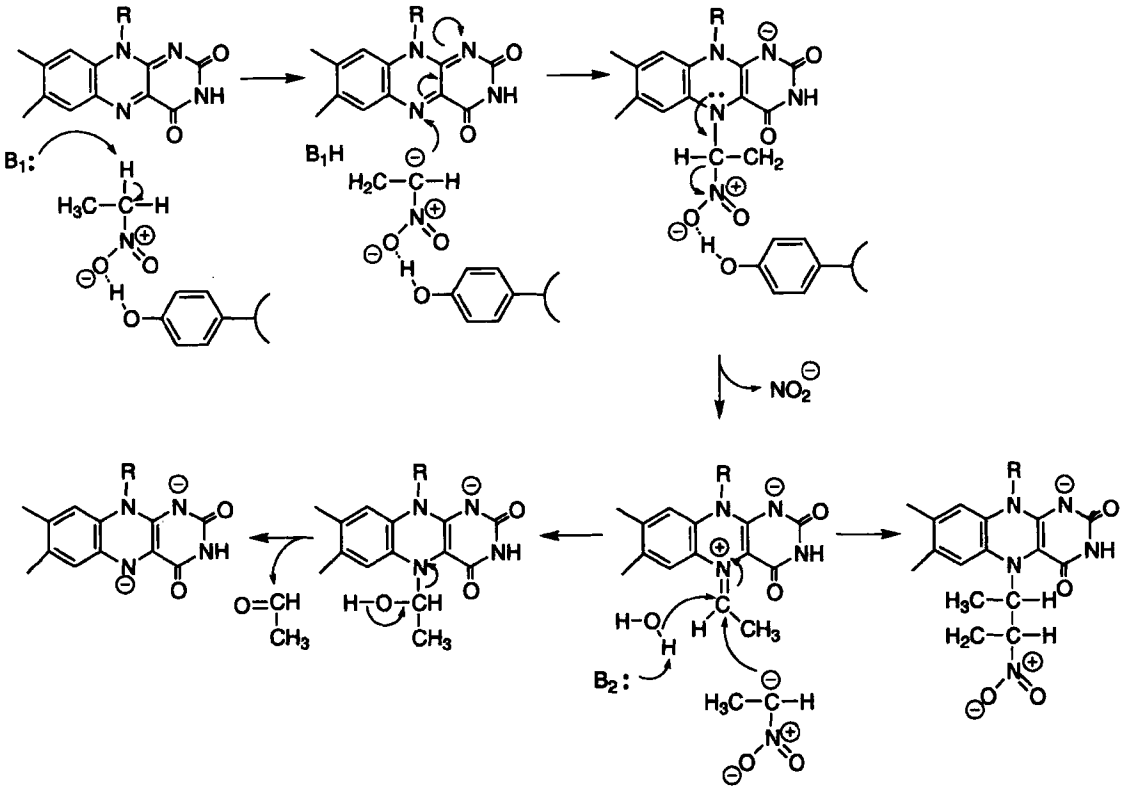
formation of carbanions. Consequently, the study of an enzymatic activity able to catalyze the cleavage of the carbon–hydrogen bond of nitroalkanes provides the unique opportunity to compare the enzyme-catalyzed formation of nitronates with the reaction in solution.

The flavoprotein nitroalkane oxidase from the plant pathogenic fungus *Fusarium oxysporum* (ATCC 695) catalyzes the oxidative denitrification of primary or secondary nitroalkanes to the corresponding aldehydes or ketones, with formation of nitrite and hydrogen peroxide (Scheme 1).[3] The enzyme is active on a broad range of nitroalkanes[4,5] and requires the neutral form of the substrate for catalysis.[6,7] Recent findings that nitro compounds with antifungal activity, such as 2-(4-methoxyphenyl)-1-nitroethane, are stress metabolites in cabbage leaves[8] suggest that the physiological role for nitroalkane oxidase is the conversion of nitroalkanes into less harmful compounds, thereby inactivating the natural defenses of the host organisms. Consistent with this conclusion, the



SCHEME 1 Reaction catalysed by nitroalkane oxidase.



SCHEME 2 Mechanism of action of nitroalkane oxidase.

enzyme is produced in large amounts when *F. oxysporum* is grown on nitroethane as the sole carbon source.[3] Mechanistic studies of nitroalkane oxidase support a chemical mechanism in which an active site base with a pK_a value of 7 abstracts a proton from the α-carbon of the nitroalkane substrate.[9,10] The resulting carbanion attacks the N(5)-position of FAD to form a covalent adduct that subsequently decays to form nitrite and the aldehyde or ketone product (Scheme 2). This covalent adduct can be trapped by nitroethane anion during turnover of the enzyme with nitroethane, resulting in the formation of 5-[3-nitrobut-2-yl]-1,5-dihydroflavin adenine dinucleotide.[7,11] Recently, the steady state kinetic mechanism of nitroalkane oxidase has been determined with nitroethane.[9,10] Despite these advances in the biochemical and mechanistic characterization of the enzyme, no structural information is available beyond the sequence of part of the gene coding for the N-terminal half of the protein.[(a)]

The known structures of several flavoproteins whose substrates contain a carboxylate moiety, such as D-amino acid oxidase,[12] glycolate oxidase,[13] flavocytochrome b_2,[14] and *p*-hydroxybenzoate hydroxylase,[15] show at least one tyrosine and one arginine residue in the active site. Based on the structural similarity of the nitro and carboxylate groups, it is reasonable that in nitroalkane oxidase the same amino acid residues participating in substrate binding are also involved in binding carboxylate-containing inhibitors. Lacking crystallographic data, an effective strategy to identify active site residues has been the use of irreversible inhibitors. In the present report, we have used the arginine-directed reagents phenylglyoxal,[16] 2,3-butandione,[17] and cyclohexandione[18] to obtain insight as to whether arginine residues are present in the active site of nitroalkane oxidase.

MATERIALS AND METHODS

Materials

Nitroethane, FAD, phenylglyoxal, 2,3-butanedione, cyclohexanedione, and valerate were from Sigma-Aldrich. Nitroalkane oxidase was purified from *Fusarium oxysporum* (ATCC 695) as previously described.[4] The activated FAD-containing form of the enzyme was prepared according to Gadda *et al.*[7,19] and was stored at $-70\,^{\circ}C$ in the presence of 0.5 mM FAD to prevent formation of the less stable apoprotein. The concentration of nitroalkane oxidase was determined by the method of Bradford[20] using bovine serum albumin as standard. All other reagents were of the highest purity commercially available.

Methods

Enzyme activities were measured with 20 mM nitroethane as substrate in air-saturated 0.5 mM FAD, 50 mM potassium phosphate, pH 7, by monitoring the rate of oxygen consumption with a computer interfaced Hansatech Clark oxygen electrode at $30\,^{\circ}C$ as previously described.[4] Stock solutions of phenylglyoxal, 2,3-butanedione, or cyclohexanedione were prepared in buffer just prior to use. Unless otherwise stated, nitroalkane oxidase (10–40 μM) was incubated with phenylglyoxal, 2,3-butandione, or cyclohexanedione in 0.5 mM FAD, 36 mM potassium phosphate, pH 7.3, at $30\,^{\circ}C$. At different times, aliquots were withdrawn and assayed for enzymatic activity. For experiments in which the effect of valerate on the rate of inactivation was measured, the enzyme was incubated for 5 min with this compound before addition of the reagent of interest. To stop the inactivation by phenylglyoxal, the unreacted reagent was

[(a)] Gadda, G. and Fitzpatrick, P.F.; unpublished results.

removed by gel filtration on a Sephadex G-25 column equilibrated with 25 mM potassium phosphate, pH 7, at 25 °C. The irreversibility of phenylglyoxal inactivation was determined by incubating the inactivated enzyme isolated by gel filtration for 3 h in 0.5 mM FAD, 25 mM potassium phosphate, pH 7, at 30 °C, monitoring changes in enzymatic activity.

Data analysis

The time course of inactivation of nitroalkane oxidase by the reagent of interest was analyzed by fitting the residual activity (A) at a given time (t) to Equation (1), where A_0 is the initial activity and k_{obs} is the observed rate of inactivation.

$$A = A_0 e^{-k_{obs}t}. \quad (1)$$

RESULTS

Treatment of nitroalkane oxidase with the arginine-directed reagents cyclohexanedione, 2,3-butanedione or phenylglyoxal, at pH 7.3 and 30 °C resulted in a time-dependent loss of enzymatic activity (Figure 1). Due to the low affinity of the enzyme for FAD, with a K_d value of 1 μM at pH 7.5,[19] a saturating concentration of FAD was present during the incubations to prevent formation of apoprotein. The observed rate of inactivation was significantly faster with phenylglyoxal than with the other reagents tested. This is in keeping with previous results showing that the enzyme has a hydrophobic binding site that can bind bulky aromatic substrates or inhibitors.[4,6,19] In order to test whether the inactivation is active site-directed, the kinetics of inactivation by phenylglyoxal were studied in the presence of valerate, a competitive inhibitor of nitroalkane oxidase with a K_i value of 0.6 mM.[10] No loss of enzymatic activity was observed in the presence of 25 mM valerate (Figure 2), indicating that inactivation was active site-directed. Similar results were obtained when the enzyme was treated in the presence of valerate with 2,3-butanedione and cyclohexanedione (Table I).

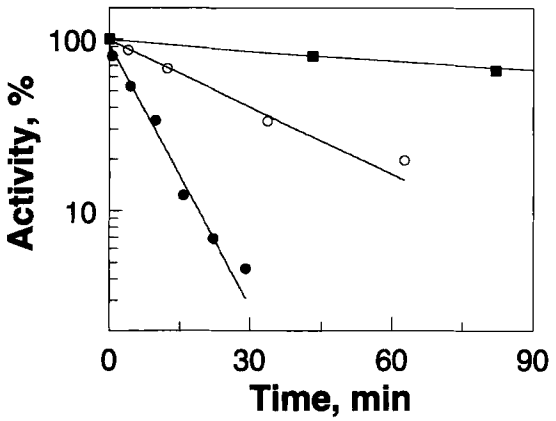


FIGURE 1 Time-dependent inactivation of nitroalkane oxidase by arginine-directed reagents. Nitroalkane oxidase (20–40 μM) was incubated with (●)10 mM phenylglyoxal, (○)100 mM 2,3-butanedione or (■)25 mM cyclohexanedione in 0.5 mM FAD, 36 mM potassium phosphate, pH 7.3, 30 °C. At different times, aliquots were withdrawn and assayed for enzymatic activity with 20 mM nitroethane as substrate as described under Materials and Methods. The lines are fits of the data to Equation (1).

Since lower concentrations of phenylglyoxal were required to inactivate the enzyme as com-

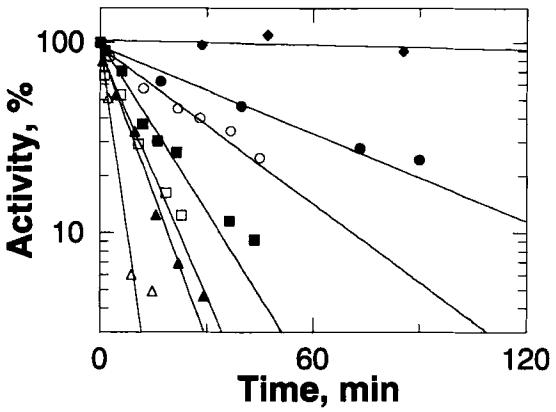


FIGURE 2 Time-dependent inactivation of nitroalkane oxidase by phenylglyoxal. Nitroalkane oxidase (10 μM) was incubated with phenylglyoxal in the presence and absence of 25 mM valerate in 0.5 mM FAD, 36 mM potassium phosphate, pH 7.3, 30 °C. At different times, aliquots were withdrawn and assayed for enzymatic activity with 20 mM nitroethane as substrate as described under Materials and Methods. Phenylglyoxal concentrations were (●) 1.2 mM, (○) 2 mM, (■) 3.8 mM, (□) 5.7 mM, (▲)10 mM, (△) 20 mM, and (◆) 3 mM in the presence of valerate. The lines are fits of the data to Equation (1).

TABLE I Effect of valerate on inactivation of nitroalkane oxidase by arginine-directed reagents

Reagent[a]	No Valerate	25 mM Valerate
	k_{obs} (min^{-1})	
Phenylglyoxal (3 mM)	0.033 ± 0.003	0.001 ± 0.001
2,3-Butanedione (100 mM)	0.030 ± 0.002	0.001 ± 0.001
Cyclohexanedione (25 mM)	0.015 ± 0.004	0.0002 ± 0.0001

[a]Nitroalkane oxidase concentration was between 10 and 40 μM.

pared to the other two reagents, a kinetic characterization of the inactivation of nitroalkane oxidase with phenylglyoxal was carried out. As shown in Figure 2, the rate of inactivation was dependent on the concentration of reagent, following first-order kinetics for at least four half-lives. The observed rate of inactivation was linearly dependent on the concentration of phenylglyoxal used (Figure 3), indicating that no reversible complex between the reagent and the enzyme was detectable prior to irreversible inactivation. A second-order rate constant for inactivation (k_{inact}) of 14.3 ± 1.1 M^{-1} min^{-1} was calculated for the reaction carried out in potassium phosphate at pH 7.3 and 30 °C, which is in the same range as values previously reported for the inactivation of other flavoprotein oxidases by phenylglyoxal.[21,22] Model studies in solution showed that the reaction of phenylglyoxal with

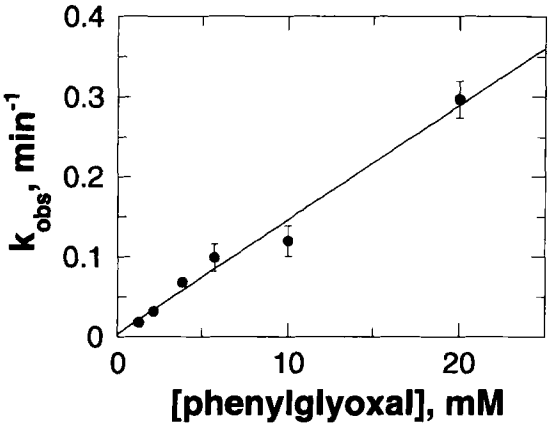


FIGURE 3 Dependence of the observed rate of inactivation on the concentration of phenylglyoxal. The data are from Figure 2. The line is a fit of the data to $k_{obs} = k_{inact}$[phenylglyoxal].

arginine residues has a pH optimum between 8 and 9 when the reaction is carried out in borate buffer.[16] Consequently, the inactivation of nitroalkane oxidase by phenylglyoxal was also studied in 100 mM sodium borate, 10% glycerol, 1 mM EDTA, pH 8 and 25 °C. The choice of pH and temperature was dictated by the necessity of preventing denaturation of the enzyme which could ensue independently of the action of phenylglyoxal upon incubation at high pH and temperature. Under these conditions the k_{inact} value is 7.5 ± 0.6 M^{-1} min^{-1}.

To determine whether nitroalkane oxidase inactivated by phenylglyoxal retained bound FAD, inactivated enzyme with 17% the residual activity of the untreated enzyme was isolated by gel filtration after 6 hours incubation with 1.2 mM phenylglyoxal. The UV-visible absorbance spectrum of the resulting enzyme showed the typical flavin peaks in the 450 and 380 nm regions, indicating that FAD was not lost as a result of phenylglyoxal modification (data not shown). Whether the inactivation was irreversible was tested by following the changes in enzymatic activity of the inactivated enzyme after removing the excess reagent by gel filtration. No recovery of activity was detected when the inactivated enzyme with 10% the residual activity of the untreated enzyme was incubated for 3 hours with 0.5 mM FAD at 30 °C, indicating that the modification is irreversible.

DISCUSSION

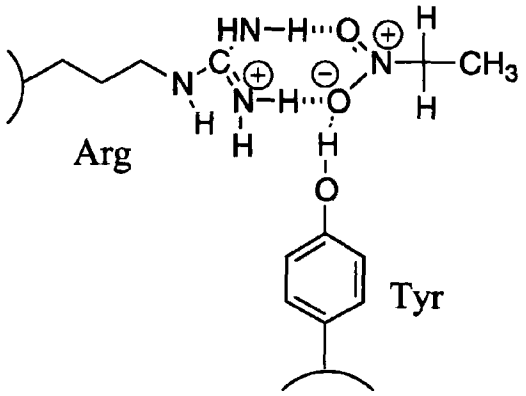
Nitroalkane oxidase is a novel FAD-containing enzyme that catalyzes the oxidative denitrification of primary and secondary nitroalkanes to yield the corresponding aldehydes or ketones, utilizing molecular oxygen as final electron acceptor. The enzyme was first described by the group of Soda in 1978 as a colorless flavoprotein which requires exogenous FAD for catalysis.[3] Recently, our group has shown that nitroalkane oxidase is isolated with the flavin

in the form of an N(5)-nitrobutyl dihydroflavin adenine dinucleotide and is consequently not active.[7,11] Conversion of the nitrobutyl-flavin adduct to FAD yields active enzyme.[7,19] A number of biochemical and mechanistic properties of the FAD-containing enzyme have been characterized.[4–7,9–11,19] However, little is known at a structural level in that the sequence of only part of the gene encoding for the N-terminal half of the protein is available.[(a)] Lacking structural data, we have used chemical modification to study the active site of the FAD-containing form of nitroalkane oxidase. With this approach, a tyrosine and a cysteine residue have been recently identified in the active site.[23,24] In the present report we have further studied the active site of nitroalkane oxidase with arginine-directed irreversible inhibitors. The rationale for targeting arginine residues was previous results showing that carboxylate-containing compounds bind to the active site of nitroalkane oxidaese[4,10,19] and the observation that the structures of a number of flavoproteins with substrates containing a carboxylate group show at least one arginine residue in the active site.[12–15]

The results presented here show that nitroalkane oxidase is irreversibly inactivated in a time-dependent fashion by treatment with arginine-directed reagents. Inactivation is active site-directed, as indicated by the complete protection from inactivation afforded by valerate. The most rapid inactivation is observed with phenylglyoxal, in agreement with previous results showing that the enzyme has a hydrophobic binding site able to accommodate aromatic substrates or inhibitors.[4,19]

Althought the identification of the residue whose modification results in the inactivation of the enzyme has not been attempted in this study, an arginine residue is the most likely candidate. Model studies on the reactivity of phenylglyoxal, cyclohexanedione, and 2,3-butanedione in solution show that these compounds preferentially react with the guanidino group of arginine residues but can also react in a much slower fashion with the amino group of lysine residues.[16–17] The rapid inactivation of nitroalkane oxidase argues against a lysine residue being the target of modification.

A reasonable role for the active site arginine residue of nitroalkane oxidase is to bind the negatively charged nitro group of the substrate. The active sites of a number of flavoproteins with carboxylate-containing substrates, such as D-amino acid oxidase,[12] glycolate oxidase,[13] flavocytochrome b_2,[14] and *p*-hydroxybenzoate hydroxylase,[15] contain arginine and tyrosine residues which bind the carboxylate group of the substrate. A tyrosine residue has been previously identified in the active site of nitroalkane oxidase by treatment with tetranitromethane.[23] Also, nitroalkane oxidase can bind carboxylic acids.[4,10,19] Therefore, it is possible that the same arginine-tyrosine pattern seen in the structures of flavoproteins with carboxylate-containing substrates is also present in nitroalkane oxidase. This is illustrated in Scheme 3. The active site arginine of nitroalkane oxidase could be involved in the binding of the coenzyme by providing a positive charge that neutralizes the negatively charged ribityl or adenosyl diphosphate portion of FAD. However, the observation that no loss of FAD is observed in the enzyme upon modification with phenylglyoxal is not consistent with this conclusion.



SCHEME 3 Proposed binding of nitroalkanes at active site of nitroalkane oxidase.

In summary, the chemical modification studies with arginine-directed reagents presented here are consistent with the presence of at least one arginine residue, likely to be essential for substrate binding, in the active site of nitroalkane oxidase. These results represent a prerequisite to future structural studies aimed at a better understanding of the catalytic mechanism of this enzyme.

Acknowledgment

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