Mechanism of Nitroalkane Oxidase: 2. pH and Kinetic Isotope Effects†

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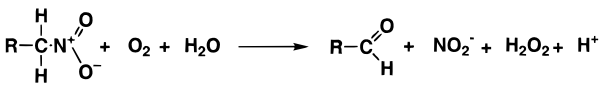
ABSTRACT: Nitroalkane oxidase catalyzes the oxidation of nitroalkanes to aldehydes or ketones with production of nitrite and hydrogen peroxide. pH and kinetic isotope effects with [1,1-$^2H_2$]nitroethane have been used to study the mechanism of this enzyme. The V/K_{ne} pH profile is bell-shaped. A group with a pK_a value of about 7 must be unprotonated and one with a pK_a value of 9.5 must be protonated for catalysis. The lower pK_a value is seen also in the pK_{is} profile for the competitive inhibitor valerate, indicating that nitroethane has no significant external commitments to catalysis. The $^D(V/K)_{ne}$ value is pH-independent with a value of 7.5, whereas the $^DV_{max}$ value increases from 1.4 at pH 8.2 to a limiting value of 7.4 below pH 5. The V_{max} pH profile decreases at low and high pH, with pK_a values of 6.6 and 9.5, respectively. Imidazole, which activates the enzyme, affects the V_{max} but not the V/K_{ne} pH profile. In the presence of imidazole at pH 7 the $^DV_{max}$ value increases to a value close to the intrinsic value, consistent with cleavage of the carbon−hydrogen bond of the substrate being fully rate-limiting for catalysis in the presence of imidazole.

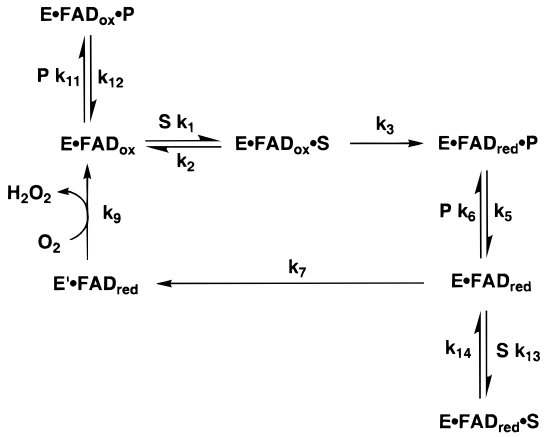
The flavin adenine dinucleotide- (FAD-)containing nitroalkane oxidase from *Fusarium oxysporum* (ATCC 695) catalyzes the oxidation of primary and secondary nitroalkanes to the corresponding aldehydes and ketones, respectively, with production of nitrite and hydrogen peroxide (Scheme 1). The enzyme is unique among the flavoproteins able to catalyze this reaction in that it requires the neutral form of the substrate for catalysis (*1*, *2*). The partial sequence of the gene encoding the N-terminal half of nitroalkane oxidase[1] and the amino acid composition of the enzyme do not match any known protein or open reading frame, suggesting that this enzyme has not been previously described (*3*). The enzyme is isolated from the fungus with the cofactor in the form of 5-nitrobutyl-1,5-dihydroflavin adenine dinucleotide and is not active (*3*−*4*). The mechanism of formation of the 5-nitrobutyl-FAD has been determined and is consistent with a carbanion mechanism for catalysis (*2*).

The steady-state mechanism of the FAD-containing enzyme has been determined with nitroethane (*5*). The data are consistent with the isomechanism of Scheme 2. After formation of E·FAD_{ox}·S, nitroethane is oxidized to form E·FAD_{red}·P. Following product release, E·FAD_{red} isomerizes to form E′·FAD_{red}, the species that reacts with oxygen. Alternatively, E·FAD_{red} can bind nitroethane to form the dead-end E·FAD_{red}·S complex. A dead-end complex between the oxidized enzyme and the aldehyde product can also be formed (E·FAD_{ox}·P). In this paper, we describe the use of pH and of kinetic isotope effects to study further the mechanism of nitroalkane oxidase.

Scheme 1



Scheme 2



MATERIALS AND METHODS

Materials. Valerate and FAD were from Sigma. Nitroethane and [1,1-$^2H_2$]nitroethane were from Aldrich. Nitroalkane oxidase was purified from *Fusarium oxysporum* (ATCC 695) as described in the accompanying paper (*5*).

Enzyme Assays. Enzyme activity was measured as described in the accompanying paper (*5*). The concentration of nitroethane used was in the range 1−25 mM to minimize substrate inhibition. When the pH was varied, 50 mM sodium pyrophosphate was used over the pH ranges pH 5.4−7 and 8−10.2, and 50 mM potassium phosphate was used between pH 7 and 8. No differences in enzyme activity were observed at the regions where a transition was made from one buffer to another. The pH dependence of valerate inhibition was

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[1] G. Gadda and P. F. Fitzpatrick, unpublished observations.

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determined by measuring the enzyme activity with 1 mM nitroethane in the presence of varied amounts of valerate. The concentration of nitroethane was less than 3 times the K_m value at all the pH values used. The apparent inhibition constant (K_{is}') determined from a Dixon plot was then used to calculate the inhibition constant (K_{is}):

$$K_{is}' = K_{is}[1 + (A/K_a)] \quad (1)$$

Data Analysis. Data were fit with the KaleidaGraph software (Adelbeck Software, Reading, PA) or the KinetAsyst software (IntelliKinetics, State College, PA). The steady-state kinetic parameters of nitroalkane oxidase were determined by fitting the data to the Michaelis−Menten equation for one substrate. When the initial rates were determined by varying the concentrations of both nitroethane and oxygen, the data were fit to eq 2, which describes a ping-pong mechanism; K_a and K_b are the Michaelis constants for nitroethane (A) and oxygen (B), respectively, and V is the maximal velocity:

$$v = \frac{VAB}{K_aB + K_bA + AB} \quad (2)$$

The pH dependences of steady-state kinetic parameters were determined by fitting initial rate data to eqs 3−5. Equation 3 was used to fit data from pH profiles that showed decreases with unit slopes at both high and low pH. Equation 4 was used to fit data that decreased with a unit slope at high pH. The pH dependence of $^{D}V_{max}$ was determined by fitting the data to eq 5, which describes a curve with a slope of −1 and plateau regions at both low and high pH values. K_1 and K_2 are the dissociation constants for the ionization of groups that must be unprotonated and protonated for catalysis, respectively. Y_L and Y_H are the limiting values at low and high pH, respectively. C is the pH-independent value of the kinetic parameter of interest.

$$\log Y = \log \frac{C}{1 + (K_2/H) + (H/K_1)} \quad (3)$$

$$\log Y = \log \frac{C}{1 + (K_2/H)} \quad (4)$$

$$\log Y = \log \frac{Y_L + Y_H[1 + (K_2/H)]}{1 + (K_2/H)} \quad (5)$$

Kinetic isotope effects with [1,1-$^2H_2$]nitroethane as substrate were determined by fitting the data to eq 6, which describes separate isotope effects on V_{max} and V/K_{ne}. F_i is the atom fraction of deuterium label in the substrate. E_{VK} and E_V are the isotope effects minus 1 on V/K and V_{max}, respectively:

$$v = \frac{VA}{K_a[1 + F_i(E_{VK})] + A[1 + F_i(E_v)]} \quad (6)$$

RESULTS

pH Dependence of the Kinetic Parameters for Nitroethane. The pH dependence of the V/K value for nitroethane was determined by measuring initial rates at different concentrations of nitroethane. This was done at 30 °C in air-saturated buffer, since the V/K_{ne} value is independent of the concentration of oxygen (*5*). As shown in Figure 1A, the V/K_{ne} pH profile is bell-shaped, consistent with the involvement of two ionizable groups. One group with an apparent pK_a value of 6.9 ± 0.1 must be unprotonated and one with an apparent pK_a value of 9.5 ± 0.1 must be protonated for catalysis. The V_{max} pH profile for nitroethane is also bell-shaped, consistent with the involvement of two ionizable groups for catalysis (Figure 1B). One group with a pK_a value of 6.6 ± 0.1 must be unprotonated and one with a pK_a value of 9.5 ± 0.1 must be protonated for catalysis. The pH dependences of the kinetic parameters were also studied in the presence of imidazole, which activates the enzyme (*5*). The V_{max} pH profile was significantly different, whereas no changes were observed in the V/K_{ne} pH profile (Figure 1). The pK_a values determined from these studies are summarized in Table 1.

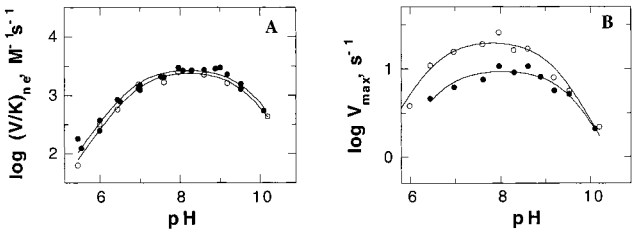


FIGURE 1: pH dependence of steady-state kinetic parameters of nitroalkane oxidase with nitroethane as substrate. Nitroalkane oxidase activity was measured in the presence and absence of imidazole in air-saturated buffer at 30 °C. (●) Values determined with no imidazole; (○) values with 12 mM unprotonated imidazole. (A) pH dependence of the V/K value for nitroethane; (B) pH dependence of the V_{max} value. The lines are fits of the data to eq 3.

Table 1: pH Dependence of the Kinetic Parameters of Nitroalkane Oxidase

kinetic parameter	pK_{a1}	pK_{a2}	eq
V/K_{ne}	6.9 ± 0.1	9.5 ± 0.1	3
V/K_{ne} with imidazole[a]	7.0 ± 0.1	9.5 ± 0.1	3
V_{max}	6.6 ± 0.1	9.5 ± 0.1	3
V_{max} with imidazole[a]	6.5 ± 0.1	9.1 ± 0.1	3
$K_{is(valerate)}$		7.2 ± 0.1	4

[a] Imidazole concentration was 12 mM in the unprotonated form.

A V/K_{O_2} value of 0.2 ± 0.06 μM^{-1} s^{-1} was previously reported for the FAD-containing nitroalkane oxidase with nitroethane at pH 7 (*5*). To test whether the ionization state of amino acid residues affects the rate of the reaction with oxygen, the V/K_{O_2} values were determined at low and high pH. The V/K_{O_2} value was 0.1 ± 0.05 μM^{-1} s^{-1} at pH 5.4, 0.1 ± 0.02 μM^{-1} s^{-1} at pH 9.2, and 0.04 ± 0.02 μM^{-1} s^{-1} at pH 10.2 (data not shown).

pH Dependence of Inhibition by Valerate. Carboxylic acids such as benzoate, phenylacetic acid, and 3-nitro-1-propionate are competitive inhibitors of nitroalkane oxidase (*3*, *6*). Thus, a compound such as valerate is expected to be a good inhibitor of the enzyme. Initial rates were measured in the presence of varying concentrations of both nitroethane and valerate at pH 7. A typical competitive inhibition pattern with lines intersecting on the *y*-axis was observed in the double-reciprocal plot of the rates versus the concentration of nitroethane, with a K_{is} value of 0.6 ± 0.1 mM (data not shown). The inhibition by valerate was also studied as a function of the pH. The data were best fit by eq 6, consistent with a single ionizable group with a pK_a value of 7.2 ± 0.1 that must be protonated for inhibition (Figure 2).

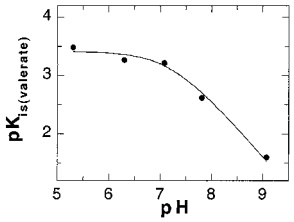


FIGURE 2: pH Dependence of inhibition by valerate. Nitroalkane oxidase activity was measured with nitroethane as substrate in the presence of valerate in air-saturated buffer at 30 °C. The K_{is} values for valerate were determined by fitting the data to eq 1. The line is a fit of the data to eq 4.

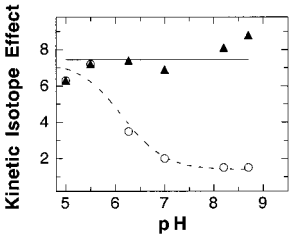


FIGURE 3: pH Dependence of the $^{D}V_{max}$ and $^{D}(V/K)_{ne}$ values for nitroethane. Nitroalkane oxidase activity was measured with [1,1-$^{2}H_2$]nitroethane or nitroethane as substrate at 30 °C. The $^{D}V_{max}$ and $^{D}(V/K)_{ne}$ values were determined by fitting the initial rate data to eq 6. (○) pH profile of the $^{D}V_{max}$ value; (▲) pH profile of the $^{D}(V/K)_{ne}$ value. The solid line represents the mean value of the $^{D}(V/K)_{ne}$ values; the dashed line is a fit of the data to eq 5.

Kinetic Isotope Effects with [1,1-$^{2}H_2$]Nitroethane. Deuterium kinetic isotope effects were measured with [1,1-$^{2}H_2$]-nitroethane between pH 5 and 8.7 (Figure 3). The $^{D}(V/K)_{ne}$ value was pH-independent with an average value of 7.5 ± 0.9. The $^{D}V_{max}$ value decreased from a limiting value of 7.4 ± 0.8 at low pH to a limiting value of 1.4 ± 0.4 between pH 8 and 9. In the presence of imidazole at pH 7, the $^{D}V_{max}$ and $^{D}(V/K)_{ne}$ values were 6.9 ± 0.9 and 6.8 ± 0.6, respectively (data not shown).

DISCUSSION

The mechanisms by which carbon−hydrogen bonds are cleaved by enzymes represent a fundamental problem in enzymology. Particularly intriguing are systems in which the bond is broken by removing a proton, because of the high energetic barrier associated with this process. In many instances, such as with thiamin- and pyridoxal-dependent enzymes, this energetic barrier is lowered as a consequence of the derivatization of the substrate. Flavoprotein oxidases must necessarily use a different strategy, since the oxidation is carried out on the underivatized substrate (*7*). The mechanisms by which the substrate pK_a is decreased sufficiently for bond cleavage are still not yet understood, although recent models involving strong hydrogen bonds provide an attractive explanation (*8*, *9*). In contrast to the situation with α-amino or α-hydroxy acids, the pK_a values for nitroalkanes are below 12 (*10*), so that little activation of the substrate is required for proton removal. For this reason, nitroalkanes have been extensively studied as model systems for the formation of carbanions in solution (*11*). These studies provide the basis for understanding the enzyme-catalyzed formation of carbanions involving much weaker carbon acids. However, caution should be exerted in extrapolating results obtained in solution with nitroalkanes to enzyme-catalyzed reactions involving amino acids and hydroxy acids. An enzyme that catalyzes the cleavage of a carbon−hydrogen bond in nitroalkanes would allow direct study of the formation of carbanions by an enzyme and comparison with model solution studies.

The analyses of the steady-state kinetic mechanism of nitroalkane oxidase with nitroethane as substrate described in the previous paper (*5*) were consistent with the mechanism of Scheme 2. The effects of imidazole were consistent with imidazole increasing the rate of isomerization of the free reduced enzyme (*5*). In the present study, pH and isotope effects have been used to probe further the mechanism of nitroalkane oxidase.

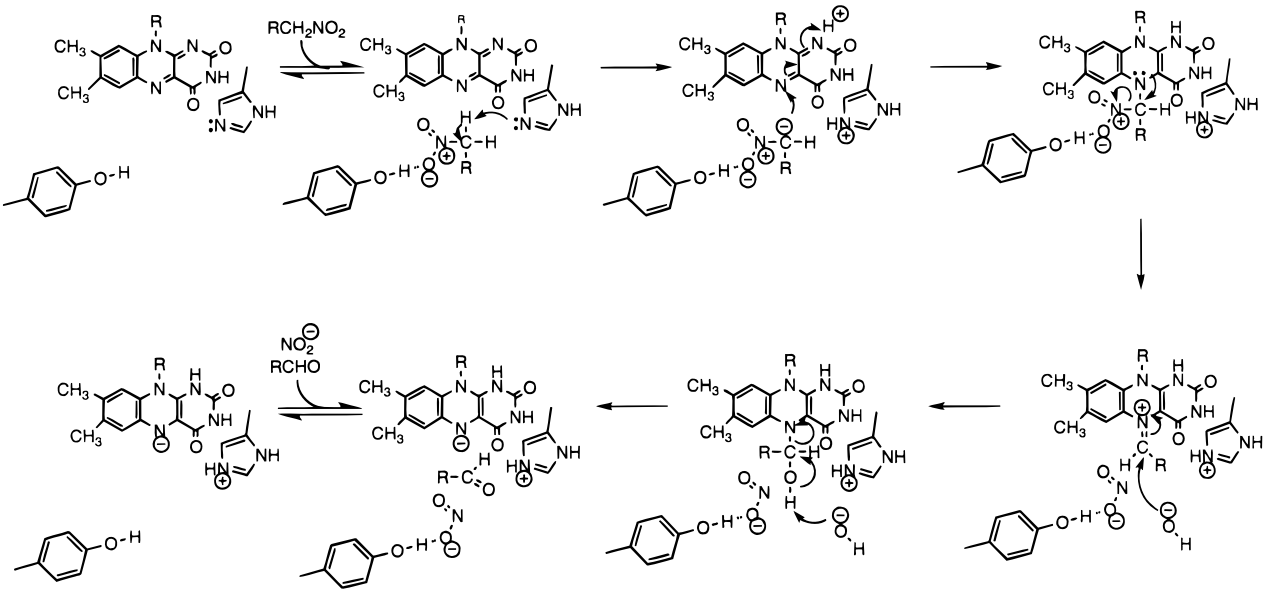
Although nitroethane has a pK_a value of 8.5 (*10*), the pK_a values determined in this study can be assigned to amino acid residues on the enzyme rather than nitroethane. Previous studies of the pH dependence by Heasley and Fitzpatrick (*1*) showed that the anionic nitroalkanes are not substrates for nitroalkane oxidase. The second-order rate constant for the deprotonation of nitroethane in solution is about 5 M^{-1} s^{-1} (*12*). Since the assays were initiated with fully protonated nitroethane, the concentration of anionic nitroethane was negligible over the time required to determine initial rates (30−40 s).

The V/K_{ne} value decreases at both low and high pH. While pK_a values determined from V/K pH profiles can be perturbed if the substrate has a significant commitment to catalysis (*13*), the $^{D}(V/K)_{ne}$ value is pH-independent, establishing that there are no external commitments to catalysis with this substrate. In addition, the pK_a value of about 7 is seen in both the V/K_{ne} and the valerate pK_{is} profiles; pK_a values determined with competitive inhibitors are expected to reflect intrinsic values (*13*). The amino acid residue responsible for the pK_a value of 7 must be unprotonated for catalysis and protonated for inhibition. This is the expected result if the net charge in the active site is to be conserved, since binding a negatively charged carboxylate would require a residue to be protonated, while binding of a formally neutral nitroalkane would require it to be unprotonated. This pK_a value is in the expected range for a histidine residue. Diethyl pyrocarbonate inactivates nitroalkane oxidase in the absence but not in the presence of valerate,[2] providing further evidence for a histidine residue in the active site. Since the catalytic mechanism of nitroalkane oxidase has previously been proposed to involve a carbanion (*2*), a reasonable role for the group with the pK_a value of about 7 is as the base that abstracts the proton from the α-carbon of the substrate.

Cleavage of the carbon−hydrogen bond of nitroethane is fully rate-limiting for catalysis at low pH and must be nearly irreversible. The deprotonation of [1,1-$^{2}H_2$]nitroethane in solution is a well-characterized process with a maximal value for the kinetic isotope effects of 9.3, depending on the base (*12*). The observed isotope effect on the V/K_{ne} value is given by eq 7 where $^{D}k_3$ is the intrinsic isotope effect for the deprotonation of nitroethane, C_f and C_r are the forward and reverse commitments to catalysis, and ^{D}Eq is the value for the equilibrium isotope effect. The forward commitment is very close to zero, so that any decrease from the intrinsic value must be due to a reverse commitment. If $^{D}k_3$ is 9.3

[2] G. Gadda, A. Banerjee, and P. F. Fitzpatrick, unpublished observations.

Scheme 3



and ^{D}Eq is 1.01 (*14*), a value of 0.28 can be calculated as an upper limit for the ratio of the rate for the reversal of carbon−hydrogen bond cleavage to the net rate constant for subsequent steps.

$$^{D}\left(\frac{k_{cat}}{K}\right) = \frac{^{D}k_3 + C_f + ^{D}EqC_r}{1 + C_f + C_r} \qquad (7)$$

The pK_a value of 9.5 seen in the V/K_{ne} pH profile is due to an amino acid residue that must be protonated for substrate binding. Chemical modification studies of nitroalkane oxidase with tetranitromethane indicate that the active site contains a tyrosine residue (*15*), which would have the pK_a value in the expected range. A reasonable role for this residue is as a hydrogen-bond donor to the substrate nitro moiety.

The V_{max} value also decreases at both high and low pH. In the mechanism of Scheme 2, the V_{max} value will reflect the relative magnitudes of the individual rate constants k_3, k_5, and k_7. The ^{D}V value increases with decreasing pH, establishing that the later steps are significantly slower than carbon−hydrogen bond cleavage at neutral pH. At pH 8 and above, where the ^{D}V value is pH-independent at about 1.4, carbon−hydrogen bond cleavage must be about 20 times as fast as subsequent steps.

The pK_a value of 6.6 seen in the V_{max} pH profile is due to a residue that must be unprotonated for catalysis in the enzyme−substrate complex. This residue is likely to be the same residue responsible for the pK_a value of 7 in the free enzyme. Its pK_a value would be perturbed in the V_{max} pH profile by the presence of slower steps occurring after the cleavage of the carbon−hydrogen bond. The upper pK_a value seen in the V_{max} pH profile is due to a residue that must be protonated for catalysis. This group is likely to be the same residue responsible for the pK_a value of 9.5 in the free enzyme. The pK_a value of 9.1 seen in the presence of imidazole must be close to the true pK_a value in the enzyme−substrate complex, since no steps other than proton abstraction are rate-limiting for catalysis.

The previous paper (*5*) showed that imidazole activates nitroalkane oxidase by increasing the V_{max} value without altering the V/K value for either substrate and that the uncharged form of imidazole is required for activation. In agreement with this model, the pK_a values in the V/K profile are not affected by imidazole. In contrast, the upper pK_a value in the V_{max} pH profile is shifted by 0.4 pH unit when imidazole is present. In the presence of imidazole the $^{D}V_{max}$ value increases to a value close to the intrinsic value. Since k_5 is fast (*5*), the effect on $^{D}V_{max}$ is most readily explained if imidazole increases the value of k_7 without altering the rate of k_3. The increase in the $^{D}V_{max}$ effect due to imidazole is consistent with k_7 being about 70% rate-limiting for catalysis in the absence of imidazole at pH 7. A similar conclusion was reached based upon the alleviation of substrate and product inhibition by imidazole (*5*).

The V/K value for oxygen is much larger than the rate of the reaction of free reduced flavins with molecular oxygen (*16*). Thus, as in the case of other flavoprotein oxidases, nitroalkane oxidase activates the reduced flavin for reaction with oxygen. The pK_a value of reduced FAD in solution is about 6.7 (*17*). This pK_a value must be shifted at least to a value close to pH 10 when the reduced flavin is bound to nitroalkane oxidase.

Scheme 3 shows a detailed proposal for the chemical mechanism of catalysis by nitroalkane oxidase that is consistent with the present and earlier studies (*18*−*21*). Binding of nitroethane to the oxidized enzyme involves an active-site tyrosine residue with a pK_a value of 9.5 that interacts with the substrate nitro moiety. A similar role for a tyrosine residue in substrate binding has been established for a number of flavoproteins with carboxylate-containing substrates, such as D-amino acid oxidase (*22*), flavocytochrome b_2 (*23*), *p*-hydroxybenzoate hydroxylase (*24*), glycolate oxidase (*25*), and vanillyl-alcohol oxidase (*26*). Catalysis by nitroalkane oxidase would be initiated when an active-site histidine with a pK_a of 7 abstracts a proton from the α-carbon of the substrate. The resulting carbanion can attack the N(5)-position of FAD to form a covalent adduct; this step must be significantly faster than carbon−hydrogen bond cleavage, given the magnitude of the kinetic isotope effect. Elimination of nitrite by cleavage of the

carbon−nitrogen bond of the nitroalkane results in formation of a cationic electrophilic imine that can be attacked by hydroxide. This is the species that can be trapped by nitroalkane anion to form the previously described 5-nitrobutyl−flavin adduct (*2*). In the normal course of catalysis, elimination of aldehyde yields reduced FAD.

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