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Dynamics of the Enzymatic Oxidation of Methane

E. I. Karasevich
Emanuel Institute of Biochemical Physics, Russian Academy of Sciences, Moscow, 119334 Russia
e-mail: elka@sky.chph.ras.ru

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Abstract—Kinetic isotope effect data for the oxidation of deuterium-substituted methane molecules with methane monooxygenase (MMO) are analyzed in the framework of a multistep nonradical mechanism. New evidence is obtained in favor of the hypothesis of the intermediate formation of a complex containing pentacoordinated carbon. A kinetic scheme whose first step involves two hydrogen molecules of the substrate being oxidized is considered. For coincidence between the calculated and experimental distributions of the oxidation products of partially deuterated methane, the formation of the intermediate complex containing pentacoordinated carbon must be reversible and the rate of the back decomposition of this complex must be substantially higher than the rate of its formation ($w_{-1} \gg w_1$). The experimental distribution of the products of deuterated methane (CH_3D, CH_2D_2, and CHD_3) hydroxylation with MMO, which could not earlier be explained within the widely accepted oxygen rebound mechanism, is quantitatively explained for the first time in terms of the dynamics of a nonradical mechanism using parameters having a simple physical meaning and plausible values.

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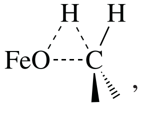
INTRODUCTION

Metalloenzymes and their chemical analogues catalyzing alkane oxidation with molecular oxygen have recently attracted great interest [1–3]. Special attention has been focused on monooxygenases, namely, cytochrome P450 and methane monooxygenase (MMO), which catalyze alkane oxidation according to the following equation:

$$RH + O_2 + 2e^- + 2H^+ \longrightarrow ROH + H_2O.$$

Cytochrome P450, which contains an iron porphyrin complex, belongs to the class of heme monooxygenases. MMO, whose active site contains a dinuclear iron complex, is classified with nonheme monooxygenases. The mechanisms of oxygen activation in the reaction cycles of these enzymes (Figs. 1, 2) are somewhat different. However, the mechanisms of C–H bond hydroxylation catalyzed by the heme and nonheme monooxygenases are very similar [4]. At present, the hidden-radical mechanism called the oxygen rebound mechanism [5] is the most popular with researchers. According to this mechanism, the intermediate formation of radicals takes place, which then recombine (without escaping into the bulk) to yield hydroxylation products. However, careful examination of experimental data concerning alkane oxidation with the heme and nonheme monooxygenases and with their chemical models demonstrates that this mechanism has not passed quantitative validation. A nonradical mechanism [6] was suggested to account for these experimental data. Its basic postulate is the intermediate formation of a complex containing pentacoordinated carbon. This complex can exist in two forms:



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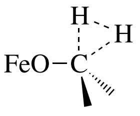
F1



F2

The form F1 can be viewed as the product of oxygen addition at the C–H bond. The form F2 is characterized by a shorter distance between the hydrogen atoms and

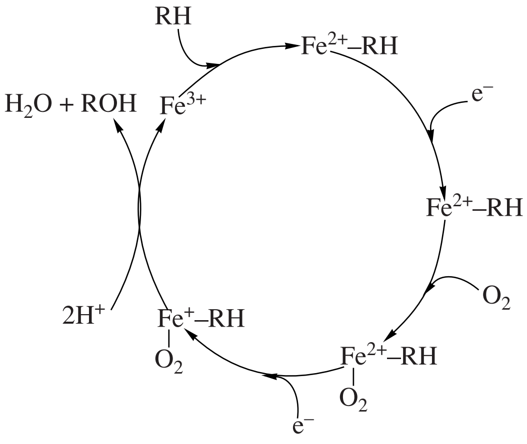


Fig. 1. Catalytic cycle of cytochrome P450.

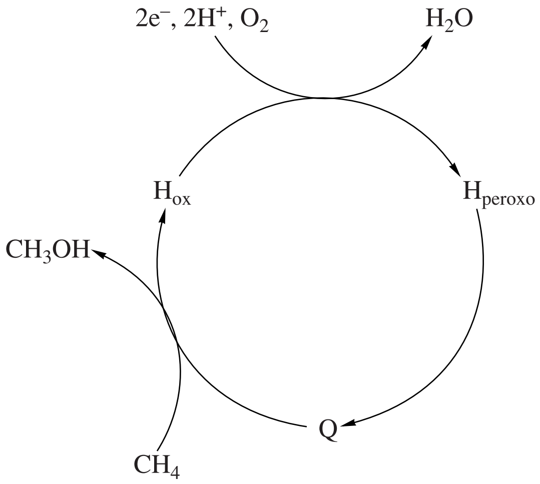
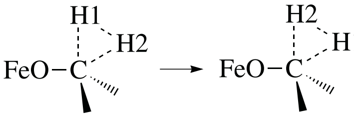


Fig. 2. Catalytic cycle of the hydroxylase component of MMO: H_{ox} is the initial form of the hydroxylase component of MMO, H_{peroxo} is one of the intermediates spectroscopically detected in the hydroxylase cycle of MMO, and Q is the reactive compound interacting with the substrate in the hydroxylase cycle of MMO.

can undergo isomerization due to the rotation of the H–H fragment:



In terms of this mechanism, we analyzed experimental product distribution data reported for the oxidation of deuterated alkanes in which the H and D atoms exchange their places during hydroxylation catalyzed by cytochrome P450 and its chemical models [7]. Calculations demonstrated that the unusual product distribution is in good agreement with the two-step nonradical mechanism. The kinetic parameters calculated in the framework of this mechanism provided, without making implausible assumptions, a self-consistent description of oxidation dynamics for all examples of compounds with one C–H bond and one C–D bond at the carbon atom being oxidized (the other two bonds are with carbon atoms).

For methane hydroxylation, it is impossible to study isomerization due to hydrogen atoms and their isotopes changing their places because there can be no chiral methane. Information concerning the enzymatic methane oxidation can be derived from hydroxylation product distribution data for deuterated methane. In our earlier works [8, 9], the nonradical mechanism involving pentacoordinated carbon was used to analyze experimental data obtained for the oxidation of deuterated methane (CH_3D, CH_2D_2, CHD_3) with the enzyme system MMO [10]. According to the results of that experiment [10], the kinetic isotope effect (KIE) calculated for the conventional oxygen rebound mechanism from product yield ratios taking into account the numbers of C–D and C–H bonds in the methane molecule increases significantly as the number of deuterium atoms is increased. An analysis demonstrated that the simple schemes of the nonradical mechanism are consistent with the nontrivial distribution of $CH_{4-n}D_n$ oxidation products, while the oxygen rebound mechanism cannot account for this product distribution. However, in order to reconcile the calculated and observed product distributions for partially deuterated methane in the framework of the nonradical kinetic schemes, it was necessary to assign values having no obvious physical meaning to the ratios of kinetic parameters. Here, we eliminate this contradiction by further analysis of the dynamics of the bacterial oxidation of methane via the nonradical mechanism.

RESULTS AND DISCUSSION

Deuterated methane, $CH_{4-n}D_n$ (CH_3D, CH_2D_2, CHD_3), is oxidized by soluble MMO from *Methylosinus trichosporium* [10] to yield $CH_{3-n}D_nOH$ and $CH_{4-n}D_{n-1}OH$ alcohols in the following ratios:

$$\begin{gathered}\text{for } CH_3D - \rho_1 = [CH_2DOH]/[CH_3OH] = 11.7 \pm 3.0,\\ \text{for } CH_2D_2 - \rho_2\\ = [CHD_2OH]/[CH_2DOH] = 9.30 \pm 0.54,\\ \text{for } CHD_3 - \rho_3 = [CD_3OH]/[CHD_2OH] = 4.00 \pm 0.33.\end{gathered} \tag{1}$$

The $CH_{4-n}D_{n-1}OH$ alcohols form, with a high rate constant, from the products of oxidation of the C–D bonds in methane ($CH_{4-n}D_{n-1}OD$ alcohols) via isotope exchange with water molecules.

In the framework of the oxygen rebound mechanism, which is accepted by many researchers, it is possible to calculate KIE values for this process from product distribution data for the oxidation of partially deuterated methane (reaction (1)) taking into account the numbers of C–D and C–H bonds in the methane molecule:

$$\sigma_n = [n/(4-n)]\rho_n = [CH_{3-n}D_nOH]/[CH_{4-n}D_{n-1}OH].$$

This calculation demonstrates that KIE increases with an increasing number of deuterium atoms (n) and takes the following values:

$$\begin{gathered}\text{for } CH_3D - \sigma_1 = (1/3)\rho_1 = 3.9 \pm 1.0,\\ \text{for } CH_2D_2 - \sigma_2 = \rho_2 = 9.30 \pm 0.54,\\ \text{for } CHD_3 - \sigma_3 = 3\rho_3 = 12.0 \pm 1.0.\end{gathered} \tag{2}$$

The variation of KIE per C–H bond is in conflict with the oxygen rebound mechanism, for which $\sigma_n = [n/(4-n)]\rho_n = k_H/k_D$, and its value is determined only by the ratio between the homolysis rate constants of the C–H and C–D bonds in the methane molecule. At the same time, the simple kinetic scheme of the nonradical mechanism of MMO-catalyzed methane oxidation, whose first step involves two hydrogen atoms [8], provides an explanation for the unusual distribution of the oxidation products of partially deuterated methane. The first step of this scheme is assumed to be the irreversible formation of F2 by the reaction between a methane molecule and the reactive intermediate Q of the catalytic cycle of MMO. In the oxidation of partially deuterated methane, $CH_{4-n}D_n$, the first step yields three types of intermediates with formation rate constants of k_1^{HH}, k_1^{HD}, and k_1^{DD}. We will designate these intermediates (HH), (HD), and (DD), according to the isotopic composition of the methylene fragment. In the analysis of the monooxygenase-catalyzed oxidation of chiral molecules in the framework of the nonradical mechanism, of great significance are the F2 isomerization reactions, which provide a quantitative explanation for experimental data. Since there can be no chiral methane molecule, the intramolecular exchanges of the H and D atoms, which are brought closer together in the methylene fragment of F2, are not considered; that is, it is accepted that the (HD) and (DH) states of this alkane are indistinguishable. The second step is the insertion of an oxygen atom into the C–H or C–D bond. Based on the kinetic scheme considered, we used quasi-steady-state treatment to determine, for each given pair of parameters ρ, the range of variation of the third parameter [8]:

$$\begin{gathered} 5.9 < \rho_2 < 7.9 \text{ for } \rho_1 = 11.7 \text{ and } \rho_3 = 4.00, \\ 5.8 < \rho_3 < 8 \text{ for } \rho_1 = 11.7 \text{ and } \rho_2 = 9.30, \\ 16.7 < \rho_1 < 25.4 \text{ for } \rho_2 = 9.30 \text{ and } \rho_3 = 4.00. \end{gathered} \tag{3}$$

Clearly, the admissible range of calculated ρ values (Eq. (3)) is at variance with the experimental distribution of methane oxidation products (Eq. (1)). Therefore, although the simple scheme is in good agreement with the dependence of KIE on the number of deuterium atoms in the methane molecule, it is in quantitative disagreement with the experiment. In order to eliminate this disagreement, we made an attempt to augment the kinetic model by including the interconversion reactions of intermediate complexes with different methylene fragments. These reactions break the hydrogen bonds between the isotopes that are initially closer together and form new hydrogen bonds by bringing other isotope atoms closer together. These reactions may be accompanied by isomerization or not—this makes no difference for methane. The introduction of the reactions (DD) $\longleftrightarrow$ (HD) $\longleftrightarrow$ (HH) into the kinetic scheme made it impossible to obtain an analytical solution and thus made it necessary to perform numerical calculations. A numerical experiment [9] demonstrated that the improved kinetic model of the oxidation of deuterated methane is in agreement with experimental data. However, as was noted in the Introduction, in order to reconcile the calculated and observed product distributions for partially deuterated methane, it was necessary to assign values with no clear physical meaning to ratios of kinetic parameters [8, 9]. As in the simple kinetic scheme, we assumed that the first step is irreversible.

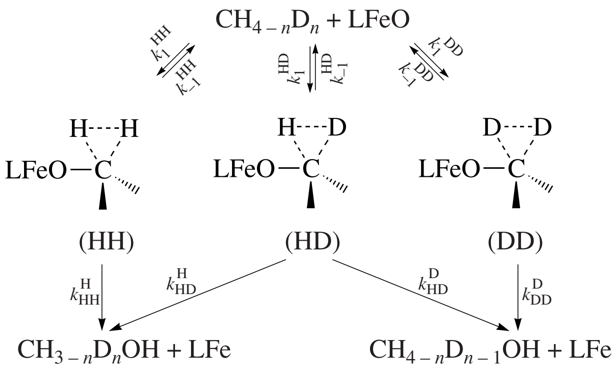


Fig. 3. Kinetic scheme of MMO-catalyzed methane oxidation via the nonradical mechanism.

Let us now analyze the simple kinetic scheme under the assumption that the first step is reversible (Fig. 3). The reaction between the reactive species LFeO (intermediate Q in the catalytic cycle of MMO) and the hydrocarbon involves two hydrogen atoms simultaneously; that is, the first step is the formation of F2. In the mechanistic model considered, F1 and F2 are kinetically indistinguishable. However, the main state of the intermediate complex, which determines the statistical coefficients at the rate constants of the reaction steps, is F2. Therefore, the following ratios of the formation rates of the intermediate complexes (HH), (HD), and (DD) can readily be obtained for partially deuterated methane, $CH_{4-n}D_n$:

$$\text{for } CH_3D\ (n = 1) - w_1^{HH} : w_1^{HD} = k_1^{HH} : k_1^{HD},\ w_1^{DD} = 0;$$

$$\text{for } CH_2D_2\ (n = 2) - w_1^{HH} : w_1^{HD} : w_1^{DD}$$

$$= k_1^{HH} : 4k_1^{HD} : k_1^{DD};$$

$$\text{for } CHD_3\ (n = 3) - w_1^{HH} = 0,\ w_1^{HD} : w_1^{DD} = k_1^{HD} : k_1^{DD}.$$

The reactions returning the compounds (HH), (HD), and (DD) to their initial state LFeO + $CH_{4-n}D_n$ have the rate constants k_{-1}^{HH}, k_{-1}^{HD}, and k_{-1}^{DD}, respectively.

The species F1 is implicitly involved in the kinetic scheme and is responsible for the formation of products from F2:

$$(HH) \xrightarrow{k_{HH}^{H}} CH_{3-n}D_nOH;$$

$$(DD) \xrightarrow{k_{DD}^{D}} CH_{4-n}D_{n-1}OH.$$

The intermediate compound containing a methylene fragment with an HD hydrogen bond can form both a C–H bond oxidation product and a C–D bond oxidation product:

$$(HD) \xrightarrow{k_{HD}^{H}} CH_{3-n}D_nOH,$$

$$(HD) \xrightarrow{k_{HD}^{D}} CH_{4-n}D_{n-1}OH.$$

The isomerization reactions (HD) $\longleftrightarrow$ (DH) are left out of consideration because, as was noted above, there can be no chiral methane. Furthermore, we will ignore the interconversion reactions of intermediate complexes with isotopically different methylene fragments: (DD) $\longleftrightarrow$ (HD) $\longleftrightarrow$ (HH). Note that, for partially deuterated camphor, ethylbenzene, and norbornane, whose monooxygenase-catalyzed oxidation is accompanied by isomerization, these reactions are impossible because the asymmetric carbon atom of these alkanes has only two bonds with hydrogen isotopes; that is, these alkanes allow only the (HD) $\longleftrightarrow$ (DH) reaction. As was noted above, the introduction of the reactions (DD) $\longleftrightarrow$ (HD) $\longleftrightarrow$ (HH) into the kinetic scheme of methane oxidation in the case of an irreversible first step does not improve the situation significantly. An ill-substantiated complication of the kinetic scheme would lead only to the impossibility of obtaining an analytical solution.

For the accepted kinetic scheme (Fig. 3), we obtain the following expressions for the variation rates of the concentrations of the intermediate compounds:

$$\mathrm{d}[(\mathrm{HH})]/\mathrm{d}t = w_1^{\mathrm{HH}} - k_{-1}^{\mathrm{HH}}[(\mathrm{HH})] - k_{\mathrm{HH}}^{\mathrm{H}}[(\mathrm{HH})],$$

$$\mathrm{d}[(\mathrm{HD})]/\mathrm{d}t = w_1^{\mathrm{HD}} - k_{-1}^{\mathrm{HD}}[(\mathrm{HD})] - k_{\mathrm{HD}}^{\mathrm{H}}[(\mathrm{HD})] - k_{\mathrm{HD}}^{\mathrm{D}}[(\mathrm{HD})], \quad (4)$$

$$\mathrm{d}[(\mathrm{DD})]/\mathrm{d}t = w_1^{\mathrm{DD}} - k_{-1}^{\mathrm{DD}}[(\mathrm{DD})] - k_{\mathrm{DD}}^{\mathrm{D}}[(\mathrm{DD})].$$

Since only the oxidation product ratios are known, the ratios rather than the absolute values of rate constants are essential for kinetic analysis. We will introduce the following designations:

$$d = k_{\mathrm{HD}}^{\mathrm{H}}/k_{\mathrm{HD}}^{\mathrm{D}}, \quad a = k_1^{\mathrm{HH}}/k_1^{\mathrm{HD}}, \quad b = k_1^{\mathrm{HD}}/k_1^{\mathrm{DD}},$$

$$c = k_{-1}^{\mathrm{HH}}/k_{-1}^{\mathrm{HD}}, \quad f = k_{-1}^{\mathrm{HD}}/k_{-1}^{\mathrm{DD}}, \quad (5)$$

$$\alpha = k_{\mathrm{HD}}^{\mathrm{H}}/k_{\mathrm{HH}}^{\mathrm{H}}, \quad \beta = k_{\mathrm{HD}}^{\mathrm{D}}/k_{\mathrm{DD}}^{\mathrm{D}}, \quad h = k_{-1}^{\mathrm{DD}}/k_{\mathrm{DD}}^{\mathrm{D}}.$$

Now we can readily obtain an expression for the ratio of the products of the oxidation of the CH and CD bonds in the methane molecule:

$$\rho = \{\alpha\beta d[(\mathrm{HH})] + \beta d[(\mathrm{HD})]\}/\{\beta[(\mathrm{HD})] + [(\mathrm{DD})]\}.$$

Solving the set of equations (4) in the quasi-steady-state approximation for the intermediates under the constraint

$$\mathrm{d}[(\mathrm{HH})]/\mathrm{d}t \approx 0, \quad \mathrm{d}[(\mathrm{HD})]/\mathrm{d}t \approx 0, \quad \mathrm{d}[(\mathrm{DD})]/\mathrm{d}t \approx 0,$$

we obtain

$$[(\mathrm{HH})] = w_1^{\mathrm{HH}}/\{k_{\mathrm{DD}}^{\mathrm{D}}(chf + \alpha\beta d)\},$$

$$[(\mathrm{HD})] = w_1^{\mathrm{HD}}/\{k_{\mathrm{DD}}^{\mathrm{D}}(hf + \beta d + \beta)\},$$

$$[(\mathrm{DD})] = w_1^{\mathrm{DD}}/\{k_{\mathrm{DD}}^{\mathrm{D}}(h + 1)\}.$$

Next, we take into account the following expressions for the formation rates of the intermediate complexes:

$$\text{for } \mathrm{CH_3D}\ (n = 1) - w_1^{\mathrm{HH}} = 3k_1^{\mathrm{HH}}[\mathrm{S}],$$

$$w_1^{\mathrm{HD}} = 3k_1^{\mathrm{HD}}[\mathrm{S}], \quad w_1^{\mathrm{DD}} = 0,$$

$$\text{for } \mathrm{CH_2D_2}\ (n = 2) - w_1^{\mathrm{HH}} = k_1^{\mathrm{HH}}[\mathrm{S}],$$

$$w_1^{\mathrm{HD}} = 4k_1^{\mathrm{HD}}[\mathrm{S}], \quad w_1^{\mathrm{DD}} = k_1^{\mathrm{DD}}[\mathrm{S}],$$

$$\text{for } \mathrm{CHD_3}\ (n = 3) - w_1^{\mathrm{HH}} = 0;$$

$$w_1^{\mathrm{HD}} = 3k_1^{\mathrm{HD}}[\mathrm{S}], \quad w_1^{\mathrm{DD}} = 3k_1^{\mathrm{DD}}[\mathrm{S}],$$

and, after simple rearrangements, we arrive at the following equations for the ratios of the oxidation products of partially deuterated methane:

$$\rho_1 = d(1 + g),$$
$$\rho_2 = d(4 + g)/(4 + e), \quad (6)$$
$$\rho_3 = d/(1 + e),$$

where

$$g = \alpha a[hf + \beta(d + 1)]/(chf + \alpha\beta d),$$
$$e = [hf + \beta(d + 1)]/[\beta b(h + 1)]. \quad (7)$$

In view of expressions (1) for experimental ρ values, Eq. (6) takes the form of a set of three equations in three variables:

$$d(1 + g) = 11.7,$$
$$d(4 + g)/(4 + e) = 9.3, \quad (8)$$
$$d/(1 + e) = 4.0.$$

Solving this set of equations, we obtain $d = 24$, $e = 5$, and $g = -0.5$. According to definitions (5) and (7), g can be only positive; therefore, the set of equations (8) has no physically meaningful solution. Note, however, that the ρ values (Eq. (1)) are experimental and their admissible ranges are determined by the respective experimental errors:

$$8.7 \le \rho_1 \le 14.7,$$
$$8.76 \le \rho_2 \le 9.84, \quad (9)$$
$$3.67 \le \rho_3 \le 4.33.$$

From expression (6), one can obtain

$$e = (d - \rho_3)/\rho_3,$$
$$g = (\rho_1 - d)/d. \quad (10)$$

In the particular case of $e = g$, expressions (6) and (10) lead to

$$d = \sqrt{\rho_1\rho_3},$$

$$\rho_2 = d(4 + g)/(4 + e) = d = \sqrt{\rho_1\rho_3}.$$

In view of the admissible ranges of ρ_1 and ρ_3 given by Eq. (9), the range of ρ_2 values in this case is $\rho_2 \le 8$.

Since e and g cannot be negative, the admissible range of d is

$$3.67 \le \rho_3 \le d \le \rho_1 \le 14.7. \qquad (11)$$

Taking $d = \rho_3 = 3.67$, for $\rho_1 = 14.7$ we obtain $e = 0$, $g = 3.0$, and $\rho_2 = 6.42$; for $\rho_1 = 8.7$ we obtain $e = 0$, $g = 1.37$, and $\rho_2 = 4.93$. Taking $d = \rho_1 = 14.7$, for $\rho_3 = 3.67$ we obtain $e = 3.0$, $g = 0$, and $\rho_2 = 8.40$; for $\rho_3 = 4.33$ we obtain $e = 2.39$, $g = 0$, and $\rho_2 = 9.20$. Clearly, the last set of parameters is in best agreement with the experimental ρ data. Thus, the experimental data are consistent with the constraint $g < e$. Since e is nonzero, it is pertinent to assume that g is also nonzero. For example, for

$$e = 2.1, \quad g = 0.1, \quad d = 13.3, \qquad (12)$$

the set of equations (6) yields $\rho_1 = 14.63$, $\rho_2 = 8.94$, and $\rho_3 = 4.29$. Within the experimental error, these values coincide with corresponding experimental data. Decreasing the parameter d or increasing the parameter e or g results in a greater deviation of the ρ values from the corresponding average experimental data.

The parameters e and g are derivatives of the parameters introduced by Eq. (5), which define the ratios of the rate constants of the reactions steps. Of particular significance is the parameter h, which defines the ratio of the rates at which the intermediate complex is converted into alcohols and back into the initial compounds.

In the particular case of $h = 1$, for the e, g, and d values selected (Eq. (12)), which imply the methane oxidation product distribution of $\rho_1 = 14.63$, $\rho_2 = 8.94$, and $\rho_3 = 4.29$, Eq. (7) reduces to the equations

$$\begin{aligned} f &= 4.2\beta(b - 3.4), \\ c &= 10\alpha(ab - 0.3)/(b - 3.4). \end{aligned} \qquad (13)$$

The parameters a, b, c, f, α, and β by their physical meaning cannot be smaller than unity. Moreover, since $f > 0$, the admissible range of the parameter b is $b > 3.4$, as follows from Eq. (13); that is, the formation rate constant of the species (HD) must be well above that of the species (DD).

In the case of $h = 0.1$ and the same e, g, and d (Eq. (12)), we arrive at the equations

$$\begin{aligned} f &= 23\beta(b - 6.2), \\ c &= 10\alpha(ab - 0.6)/(b - 6.2). \end{aligned} \qquad (14)$$

The parameter b will then be subject to a still more stringent constraint: its admissible values will lie within the interval $b > 6.2$.

For $h = 10$, we likewise obtain the equations

$$\begin{aligned} f &= 2.3\beta(b - 0.6), \\ c &= 10\alpha(ab - 0.06)/(b - 0.6). \end{aligned} \qquad (15)$$

In this case, $b \ge 1$ and no additional constraints are imposed on this parameter.

Thus, the values of $\rho_1 = 14.63$, $\rho_2 = 8.94$, and $\rho_3 = 4.29$, which coincide with the corresponding experimental data within the experimental error, allow an infinite number of sets of rate constants of the elementary steps (some examples are given in the table). Note that the parameters a, b, c, f, α, and β are, by their physical meaning, KIE values for reaction steps and must obey certain laws. First of all, it is necessary to minimize the set of these parameters considering that their large values (100 and above) seem to be unsubstantiated. Furthermore, if $k_1^{\mathrm{HH}}/k_1^{\mathrm{DD}} = b \ne 1$, then $a = k_1^{\mathrm{HH}}/k_1^{\mathrm{HD}}$ is apparently unequal to unity. Similar considerations are valid for $c = k_{-1}^{\mathrm{HH}}/k_{-1}^{\mathrm{HD}}$ and $f = k_{-1}^{\mathrm{HD}}/k_{-1}^{\mathrm{DD}}$. Raising the parameter b causes an increase in f and a decrease in c. The $a - b$ minimization condition sets a limit on the decrease of c. It is clear from the table that the parameters a, b, c, f, α, and β are minimized at large h values. The parameter h, which is determined by the ratio between the rate constants of intermediate complex conversions into alcohols and into initial compounds ($k_{-1}^{\mathrm{DD}}/k_{\mathrm{D}}^{\mathrm{DD}}$), does not require minimization. For $h = 10$–10^3, the forward reaction of the first step may have some KIE (table, the 2nd and 3rd rows from bottom) or be independent of the isotopic composition of the substrate (see the last row in the table). Note that, for all h values, the reverse reaction of the first step has a larger KIE than the forward reaction. Since the analysis was carried out using the quasi-steady-state approximation, the formation rate of the intermediate complexes must be substantially lower than their disappearance rate. Hence, the equilibrium in the first step of methane hydroxylation with MMO is shifted to the initial compounds and the condition $w_1 < w_2 \ll w_{-1}$ is satisfied.

Thus, we determined the kinetic parameters that ensure that the calculated distribution of methane oxidation products coincides with experimental data within the experimental error. These parameters have a clear physical meaning, and their values at sufficiently high h values of 10–10^3 seem quite natural. For example, as can be seen from the table, experimental data (1) can readily be obtained at the following ratios of the kinetic parameters:

(1) $h = k_{-1}^{\mathrm{DD}}/k_{\mathrm{DD}}^{\mathrm{D}} = 10$, $k_{\mathrm{HD}}^{\mathrm{H}}/k_{\mathrm{HD}}^{\mathrm{D}} = 13.3$, $k_1^{\mathrm{HD}}/k_1^{\mathrm{DD}} = 3.0$, $k_1^{\mathrm{HH}}/k_1^{\mathrm{HD}} = 1.5$, $k_{\mathrm{HD}}^{\mathrm{H}}/k_{\mathrm{HH}}^{\mathrm{H}} = 1.5$, $k_{\mathrm{HD}}^{\mathrm{D}}/k_{\mathrm{DD}}^{\mathrm{D}} = 3.0$, $k_{-1}^{\mathrm{HD}}/k_{-1}^{\mathrm{DD}} = 28.0$, $k_{-1}^{\mathrm{HH}}/k_{-1}^{\mathrm{HD}} = 16.5$;

(2) $h = k_{-1}^{\mathrm{DD}}/k_{\mathrm{DD}}^{\mathrm{D}} = 10^3$, $k_{\mathrm{HD}}^{\mathrm{H}}/k_{\mathrm{HD}}^{\mathrm{D}} = 13.3$, $k_1^{\mathrm{HD}}/k_1^{\mathrm{DD}} = 4.0$, $k_1^{\mathrm{HH}}/k_1^{\mathrm{DD}} = 2.0$, $k_{\mathrm{HD}}^{\mathrm{H}}/k_{\mathrm{HH}}^{\mathrm{H}} = 1.0$, $k_{\mathrm{HD}}^{\mathrm{D}}/k_{\mathrm{DD}}^{\mathrm{D}} = 1.0$, $k_{-1}^{\mathrm{HD}}/k_{-1}^{\mathrm{DD}} = 8.4$, $k_{-1}^{\mathrm{HH}}/k_{-1}^{\mathrm{HD}} = 20.0$;

(3) $h = k_{-1}^{\mathrm{DD}}/k_{\mathrm{DD}}^{\mathrm{D}} = 10^3$, $k_{\mathrm{HD}}^{\mathrm{H}}/k_{\mathrm{HD}}^{\mathrm{D}} = 13.3$, $k_1^{\mathrm{HD}}/k_1^{\mathrm{DD}} = 1.0$, $k_1^{\mathrm{HH}}/k_1^{\mathrm{DD}} = 1.0$, $k_{\mathrm{HD}}^{\mathrm{H}}/k_{\mathrm{HH}}^{\mathrm{H}} = 2.0$, $k_{\mathrm{HD}}^{\mathrm{D}}/k_{\mathrm{DD}}^{\mathrm{D}} = 9.5$, $k_{-1}^{\mathrm{HD}}/k_{-1}^{\mathrm{DD}} = 20.0$, $k_{-1}^{\mathrm{HH}}/k_{-1}^{\mathrm{HD}} = 20.0$.

Rate constant ratios fitted to methane oxidation product distribution data of $\rho_1 = 14.63$, $\rho_2 = 8.94$, and $\rho_3 = 4.29$

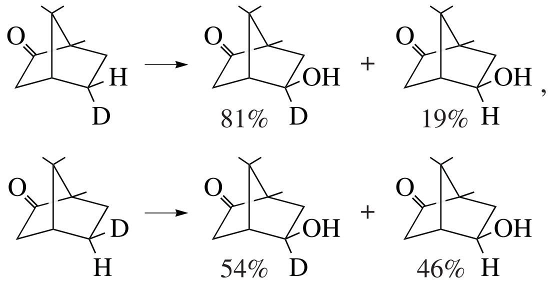
$d = k^{\mathrm{H}}_{\mathrm{HD}}/k^{\mathrm{D}}_{\mathrm{HD}}$	$h = k^{\mathrm{DD}}_{-1}/k^{\mathrm{D}}_{\mathrm{DD}}$	$b = k^{\mathrm{HD}}_{1}/k^{\mathrm{DD}}_{1}$	$a = k^{\mathrm{HH}}_{1}/k^{\mathrm{HD}}_{1}$	$\alpha = k^{\mathrm{H}}_{\mathrm{HD}}/k^{\mathrm{H}}_{\mathrm{HH}}$	$\beta = k^{\mathrm{D}}_{\mathrm{HD}}/k^{\mathrm{D}}_{\mathrm{DD}}$	$f = k^{\mathrm{HD}}_{-1}/k^{\mathrm{DD}}_{-1}$	$c = k^{\mathrm{HH}}_{-1}/k^{\mathrm{HD}}_{-1}$
13.3	1	3.5	1	1	1	0.4	334
		3.5	1	1	3	1.2	334
		3.5	1	2	3	1.2	668
		10	1	1	1	28	15
		10	2	1	1	28	51
		10	10	1	1	28	151
		10	10	1	5.5	151	151
	0.1	10	1	1	1	88	25
		20	1	1	1	317	14
		10	10	1	1	88	261
	10	1	1	1	1	0.9	25
		1	1	1	28	25	25
		10	1	1	1	22	11
		10	10	1	1	22	106
		10	10	1	5	106	106
		3	1.5	1.5	3	28	16.5
	1000	4	2	1	1	8.4	20
		1	1	2	9.5	20	20

Note: $d = 13.3$, $e = 2.1$, and $g = 0$.

For the calculated and experimental ρ data to coincide in the framework of the mechanism suggested here, it is necessary that the reaction between the active species and the substrate be reversible and the rate of the reverse reaction (w_{-1}) be much higher than the rate of the forward reaction (w_1). Furthermore, for the condition $h = k^{\mathrm{DD}}_{-1}/k^{\mathrm{D}}_{\mathrm{DD}} \geq 10$ to be satisfied, the rate of the conversion of the intermediate complexes into the reaction products must be much lower than the rate of the conversion of the complexes into the initial compounds. The rate and KIE are the highest for the reverse reaction (w_{-1}) of the first step of the kinetic scheme suggested.

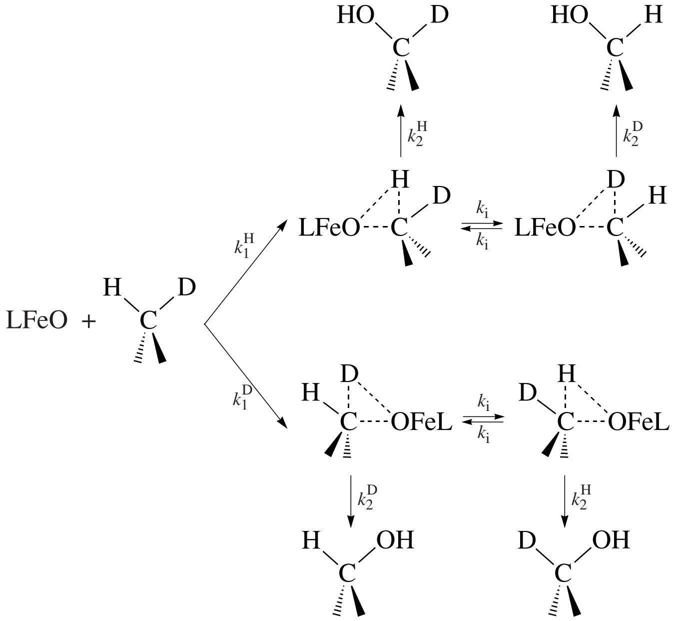
The above results provide evidence in favor of the hypothesis that the intermediate complex of the reaction contains pentacoordinated carbon. This hypothesis is particularly attractive because it accounts for the distribution of reaction products observed in the oxidation of partially deuterated alkanes with natural monooxygenases (cytochrome P450 and MMO) and its chemical models [10–15] in terms of a unified multistep nonradical mechanism. The analysis reported here indicates that, in the oxidation of methane and, possibly, ethane catalyzed by the enzyme system MMO, the formation of F2 via the bimolecular reaction between the reactive intermediate Q and the substrate is a reversible process. As distinct from the first step in the catalytic cycle of MMO (Fig. 2), the first step in the catalytic cycle of cytochrome P450 (Fig. 1) is binding between the substrate and the enzyme. In this case, the reactive intermediate results from redox transformations of the enzyme–substrate complex. Accordingly, in the reaction catalyzed by cytochrome P450, the formation of the intermediate complex is likely irreversible, particularly for alkanes in which the C–H bond energy is much higher than the C–H bond energy in the methane molecule. The formation of the intermediate complex, whose F2 form is responsible for the isomerization of the alkane oxidation products, provides a quantitative explanation for the data available on the distribution reaction products for the oxidation of deuterium-substituted alkanes catalyzed by cytochrome P450 and its chemical models [7]. In this system, hydroxylation is accompanied by H and D exchanging their places, a process not described quantitatively by the oxygen rebound mechanism [11–14].

For example, in the enzymatic hydroxylation of deuterated camphor with cytochrome $\mathrm{P450_{cam}}$, part of the deuterium atoms pass from the *exo* position to the *endo* position and vice versa [11]:



This yields 5-*exo*-hydroxycamphor because the camphor molecule is fixed in the active site of the enzyme by a hydrogen bond and cannot rotate. Therefore, isomerization in this case cannot be due to molecular rotation and cannot be explained within the oxygen rebound mechanism. The remarkable stereoselectivity of camphor oxidation was given a plausible explanation in terms of the intermediate formation of pentacoordinated carbon [7]. As distinct from the first step of MMO-catalyzed methane oxidation, the first step in this reaction is the irreversible interaction of LFeO with a C–H or C–D bond of camphor. This is dictated by the spatial structure of the active site of the enzyme.

Another example is the hydroxylation of (R)- and (S)-enantiomers of monodeuteroethylbenzene with a donor of active oxygen (iodosobenzene) catalyzed by a chiral iron porphyrin complex [14]. This process yields all possible isomers of 1-phenylethanol. The main (R)- or (S)-enantiomer hydroxylation product forms stereoselectively and is almost pure monodeuterophenylethanol, while the minor product is only half deuterated. This experimental finding was not explained in the framework of the hidden-radical abstraction–recombination mechanism. At the same time, these data can be accounted for in terms of the simple kinetic scheme of the nonradical mechanism:



This scheme takes into account the fact that, in the formation of an OD group, after the insertion of an O atom into the C–D bond, the D atom is replaced with an H atom via rapid hydrogen exchange with the solvent. The form F2 is implicitly involved in this scheme and is responsible for the isomerization of the intermediate complex.

The oxidation of chiral ethylbenzene is an example in which the change of the hydroxylation rate constant (in this case, due to the large KIE value) leads to the almost complete retaining of the initial configuration (for the C–H bond and, at the same time, to considerable isomerization (for the C–D bond) in one molecule [7]. This example helps understand how the complete retaining of the initial configuration or its inversion (i.e., racemization) can take place in similar systems. If the lifetime of the intermediate complex is short (i.e., an O atom is inserted rapidly into the C–H bond), no isomerization will take place; if the isomerization rate is comparable with the oxygen insertion rate, an appreciable amount of isomerization products will form. Note that the kinetic parameters calculated from experimental data for (R)-deuteroethylbenzene using the above scheme enables one to predict exactly the product distribution observed for the (S)-isomer (and vice versa) [7, 16]. This fact is a convincing argument in favor of the mechanism involving the intermediate formation of pentacoordinated carbon.

Along with the product distribution data for the oxidation of partially deuterated alkanes with monooxygenases and their chemical models, there are extensive experimental KIE data derived by various methods from the rates of these processes. Different authors report different isotope effects derived from the ratio of the hydroxylation rates of the individual substrates CH_4 and CD_4, namely, KIE = 5 [17] and KIE = 12 [18]. The difference between the KIE values measured by this method can readily be explained in terms of a multistep mechanism in which the reaction rate is determined by the slowest, rate-limiting step. Varying the reaction conditions can change the rate-limiting step. For example, it was demonstrated that the KIE in methane hydroxylation catalyzed by pMMO from *Methylococcus capsulatus (M)* increases dramatically with increasing temperature [19]. This seems to be quite natural because the contributions from reaction steps to the overall reaction rate are temperature-dependent.

In the series of works devoted to KIE in MMO-catalyzed methane oxidation, an impressive result was obtained in a study of the intermediate Q disappearance rate in the catalytic cycle of MMO in the oxidation of the individual substrates CH_4 and CD_4 [20]. It was found that the thus-measured isotope effect is abnormally strong and its value is $k^H/k^D \sim 50–100$. This result also indicates that MMO-catalyzed methane oxidation is a multistep process. It is likely that the rate-limiting step of the disappearance of the intermediate Q, which is characterized by a high KIE value, is other than the rate-limiting step of the formation of the oxidation products. The disappearance of Q is followed by slower steps with a smaller KIE value, and it these steps that determine the observed formation rate of the products.

The competition method of KIE determination is the most informative. This method was used to derive KIE from the ratio of product accumulation rates in the MMO-catalyzed oxidation of a $CH_4 + CD_4$ mixture [10], and the following value was obtained:

$$\rho_4 = [CH_3OH]/[CD_3OH] = 19.3 \pm 3.9. \tag{16}$$

Thus, for methane oxidation in the presence of MMO, KIE values measured by different methods differ significantly. As was noted above, the KIE value derived from the formation or disappearance rate of a chemical compound is governed by the rate-limiting step of the process.

Consider the experimental data obtained for the oxidation of a $CH_4 + CD_4$ mixture in the framework of the nonradical mechanism involving the formation of pentacoordinated carbon. The accepted kinetic scheme, whose first step is reversible (Fig. 3), lead to the following expressions for the disappearance rates of the substrates:

$$\begin{aligned} -d[CH_4]/dt &= k_1^{HH}[CH_4] - k_{-1}^{HH}[(HH)], \\ -d[CD_4]/dt &= k_1^{DD}[CD_4] - k_{-1}^{DD}[(DD)]. \end{aligned} \tag{17}$$

The rates of change of the concentrations of the intermediate compounds appear as

$$\begin{aligned} d[(HH)]/dt &= k_1^{HH}[CH_4] - k_{-1}^{HH}[(HH)] - k_{HH}^{H}[(HH)], \\ d[(DD)]/dt &= k_1^{DD}[CD_4] - k_{-1}^{DD}[(DD)] - k_{DD}^{D}[(DD)]. \end{aligned} \tag{18}$$

By solving the set of equations (18) in the steady-state approximation, so that the conditions

$$d[(HH)]/dt \approx 0, \quad d[(DD)]/dt \approx 0$$

are satisfied, one readily obtains

$$\begin{aligned} [(HH)] &= k_1^{HH}[CH_4]/\{k_{DD}^{D}(chf + \alpha\beta d)\}, \\ [(DD)] &= k_1^{DD}[CD_4]/\{k_{DD}^{D}(h + 1)\}. \end{aligned}$$

Hence,

$$\begin{aligned} -d[CH_4]/dt &= k_1^{HH}[CH_4]/\{1 + (chf/\alpha\beta d)\}, \\ -d[CD_4]/dt &= k_1^{DD}[CD_4]/(1 + h). \end{aligned} \tag{19}$$

In the accepted scheme, the slowest, rate-determining step is the first step of the formation of the intermediate complexes. Therefore, under steady-state conditions, when the formation rate of the oxidation products is constant, we have

$$\begin{aligned} d[CH_3OH]/dt &= -d[CH_4]/dt = \mathrm{const1}, \\ d[CD_3OH]/dt &= -d[CD_4]/dt = \mathrm{const2}, \\ \rho_4 &= [CH_3OH]/[CD_3OH] = \mathrm{const1}/\mathrm{const2} \\ &= \alpha ad\beta b(1 + h)/(\alpha\beta d + chf) = dg/e. \end{aligned} \tag{20}$$

For the kinetic parameters given by Eq. (12), which ensure coincidence between the calculated and observed product distributions for partially deuterated methane (within the experimental error), Eq. (20) yields $\rho_4 = 0.63$. This value falls outside the range of experimental KIE data for $CH_4 + CD_4$ oxidation:

$$15.4 \le \rho_4 \le 23.2. \tag{21}$$

Let us consider the ranges of d, g, and e in which condition (21) is satisfied. According to expression (11), the range of d is given by $d < \rho_1 \le 14.7$. For the admissible ρ_4 range (expression (21)),

$$g \ge 15.4e/d > e.$$

Therefore, condition (21) is satisfied for $g > e$. At the same time, the above analysis demonstrates that the observed distribution of oxidation products for partially deuterated methane implies $g < e$.

Thus, in the framework of the simple kinetic scheme, whose first step is reversible, there is no set of parameters ensuring the simultaneous quantitative coincidence of calculated data with experimental data (1) and (16). The range of kinetic parameters satisfying the observed distribution of the hydroxylation products of partially deuterated methane (Eq. (1)) has no intersection with the range of these parameters that lead to the KIE value observed in the oxidation of the $CH_4 + CD_4$ mixture (Eq. (16)).

In the case of intermediate complex formation being irreversible, it is pertinent to assume that ρ_4 is the KIE value of the first, slowest step. The range of the rate constants of the first step will then be limited only by the stationarity condition ($k_1 \ll k_i$), and there will be no conflict between data (1) and (16) [8, 9]. Imposing the constraint $w_1 < w_2 \ll w_{-1}$ causes the incompatibility between the sets of parameters bringing the calculated data into quantitative coincidence with experimental data (1) and (16).

In order to eliminate this conflict, it was necessary to further complicate the kinetic model of the mechanism considered. We carried out a special-purpose numerical kinetic simulation of the dynamics of methane hydroxylation in the presence of MMO. This simulation, which enabled us to explain, in terms of the dynamics of a single nonradical mechanism, the experimental data available on the oxidation of structurally different alkanes with heme and nonheme monooxygenases and their chemical models, will be reported in a forthcoming publication [21].

CONCLUSIONS

Thus, the product distribution data for the oxidation of deuterium-substituted methane molecules with MMO have been analyzed in the framework of a multistep nonradical mechanism. A kinetic scheme is suggested, whose first step involves two hydrogen atoms of the substrate being oxidized. The experimental distribution of the products of deuterated methane (CH_3D, CH_2D_2, and CHD_3) hydroxylation with MMO (data (1)), which cannot be explained within the widely accepted oxygen rebound mechanism, can be quantitatively accounted for in terms of the dynamics of a nonradical mechanism using parameters having a simple physical meaning and plausible values. For example, for $h = k_{-1}^{DD}/k_{DD}^{D} = 10$, the simplest kinetic scheme examined can readily be fitted to experimental data (1) by using the following ratios of kinetic parameters: $k_{HD}^{H}/k_{HD}^{D} = 13.3$, $k_1^{HD}/k_1^{DD} = 3.0$, $k_1^{HH}/k_1^{HD} = 1.5$, $k_{HD}^{H}/k_{HH}^{H} = 1.5$, $k_{HD}^{D}/k_{DD}^{D} = 3.0$, $k_{-1}^{HD}/k_{-1}^{DD} = 28.0$, and $k_{-1}^{HH}/k_{-1}^{HD} = 16.5$. Raising the parameter h will provide better opportunities to fit the kinetic parameters to detailed variants of the mechanism of methane oxidation in the presence of MMO.

For coincidence between calculated and experimental distributions of the oxidation products of partially deuterated methane, the formation of the intermediate complex containing pentacoordinated carbon must be reversible and the rate of the back decomposition of this complex must be well above the rate of its formation ($w_{-1} \gg w_1$). However, with these constraints, the sets of kinetic parameters of the simple scheme that afford quantitative fit between the calculated and observed products distributions for the oxidation of partially deuterated methane are incompatible with the sets of kinetic parameters that ensure fit between the calculated and observed KIE values for the competitive oxidation of CH_4 and CD_4 with the same enzyme system.

Nevertheless, the simple scheme of the nonradical mechanism for the first time provided a quantitative explanation for the unusual distribution of the oxidation products of partially deuterated methane. Furthermore, an analytical solution was obtained for the kinetic problem, and it showed the line of further investigation of the dynamics of MMO-catalyzed methane oxidation. Complication of the simple kinetic model of the mechanism examined is required to eliminate the inconsistency that emerges in the simultaneous analysis of the experimental product distributions for the oxidation of individual (partially deuterated) compounds and the product formation rates for the competing oxidations of the fully deuterated and nondeuterated substrates. This makes it impossible to obtain an analytical solution and makes it necessary to employ numerical methods.

REFERENCES

1. Groves, J.T., *J. Inorg. Biochem.*, 2006, vol. 100, no. 4, p. 434.
2. Poulos, T.L., *Biochem. Biophys. Res. Commun.*, 2005, vol. 338, no. 1, p. 337.
3. Newcomb, M. and Chandrasena, R.E.P., *Biochem. Biophys. Res. Commun.*, 2005, vol. 338, no. 1, p. 394.
4. Karasevich, E.I., Kulikova, V.S., Shilov, A.E., and Shteinman, A.A., *Usp. Khim.,* 1998, vol. 67, no. 4, p. 376.
5. Groves, J.T., *J. Chem. Educ.*, 1985, vol. 62, no. 1, p. 928.
6. Shestakov, A.F. and Shilov, A.E., *J. Mol. Catal.*, 1996, vol. 105, no. 1, p. 1.
7. Karasevich, E.I., Shestakov, A.F., and Shilov, A.E., *Kinet. Katal.,* 1997, vol. 38, no. 6, p. 852 [*Kinet. Catal.* (Engl. Transl.), vol. 38, no. 6, p. 782].
8. Karasevich, E.I., Karasevich, Yu.K., Shestakov, A.F., and Shilov, A.E., *Kinet. Katal.,* 2003, vol. 44, no. 1, p. 122 [*Kinet. Catal.* (Engl. Transl.), vol. 44, no. 1, p. 112].
9. Karasevich, E.I., Karasevich, Yu.K., Shestakov, A.F., and Shilov, A.E., *Kinet. Katal.,* 2003, vol. 44, no. 2, p. 266 [*Kinet. Catal.* (Engl. Transl.), vol. 44, no. 2, p. 247].
10. Nesheim, J.C. and Lipscomb, J.D., *Biochemistry,* 1996, vol. 35, no. 31, p. 10240.
11. Gelb, M.H., Heimbrook, D.C., Malkonen, P., et al., *Biochemistry,* 1982, vol. 21, no. 2, p. 370.
12. Traylor, T.G., Hill, K.W., Fann, W.-P., et al., *J. Am. Chem. Soc.,* 1992, vol. 114, no. 4, p. 1308.
13. White, R.E., Miller, J.P., Favreau, L.V., et al., *J. Am. Chem. Soc.*, 1986, vol. 104, no. 19, p. 6024.
14. Groves, J.T. and Viski, P., *J. Am. Chem. Soc.*, 1989, vol. 111, no. 22, p. 8537.
15. Priestley, N.D., Floss, H.G., Froland, W.A., et al., *J. Am. Chem. Soc.*, 1992, vol. 114, no. 19, p. 7561.
16. Karasevich, E.I., Shestakov, A.F., and Shilov, A.E., *Exp. Toxicol. Pathol.*, 1999, vol. 51, nos. 4–5, p. 335.
17. Gvozdev, R.I., Shushenacheva, E.V., Pylyashenko-Novochatnii, A.A., et al., *Oxid. Commun.,* 1984, vol. 7, nos. 3–4, p. 249.
18. Green, J. and Dalton, H., *J. Biol. Chem.*, 1989, vol. 264, no. 30, p. 17698.
19. Korshunova, L.A., Gvozdev, R.I., and Dalton, G., *Dokl. Akad. Nauk*, 1997, vol. 352, no. 4, p. 545 [*Dokl. Biochem. Biophys.* (Engl. Transl.), vols. 352–354, p. 4].
20. Nesheim, J.C. and Lipscomb, J.D., *J. Inorg. Biochem.*, 1995, vol. 59, no. 1, p. 369.
21. Karasevich, E.I., Karasevich, Yu.K., and Shilov, A.E., *Kinet. Katal.,* 2008, vol. 49, no. 2 [*Kinet. Catal.* (Engl. Transl.), vol. 49, no. 2] (in press).