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Methane Hydroxylation by Methane Monooxygenase: On the Problem of the Process Dynamics

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Abstract—Published data on the kinetic isotope effects of the hydroxylation of deuterium-substituted methane molecules (CHD_3, CH_2D_2, and CH_3D) by methane monooxygenase are examined in the framework of the two-step nonradical mechanism through the intermediate formation of a complex containing pentacoordinate carbon. The kinetic schemes with the first step involving one, two, and three hydrogen atoms of the oxidized substrate are considered. Contrary to the widely accepted oxygen rebound mechanism, the experimental results obtained for the oxidation of various substrates by methane monooxygenase and cytochrome P450 can be explained from the viewpoint of the dynamics of a general nonradical mechanism.

INTRODUCTION

Enzymes belonging to the class of monooxygenases catalyze alkane oxidation by molecular oxygen according to the equation

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

Among these enzymes, cytochrome P450 and methane monooxygenase (MMO) have been studied more than others [1]. The active site of cytochrome P450 contains an iron porphyrin complex, and that of MMO includes a binuclear iron complex. The reaction cycle of these enzymes includes two main processes: oxygen activation and hydrocarbon substrate oxidation. The spectral studies enabled detailed examination of intermediate complexes formed in the sequence of steps of the reductive activation of oxygen in these systems. The catalytic cycle of MMO (Fig. 1) begins with the reduction of the hydroxylase component: its initial state $\mathbf{H}_{\mathbf{ox}}$ transforms into the active one $\mathbf{H}_{\mathbf{red}}$. The interaction of the reduced hydroxylase component with oxygen leads to the formation of an intermediate designated by O, whose structure is disputable. The protonation of the O intermediate (possibly in several steps) is completed by the formation of an intermediate designated as Q, which interacts with the substrate in a bimolecular process [2, 3]. According to the Mössbauer spectroscopic data, a diferryl or bis-μ-oxo complex structure is assumed for this species. A porphyrin complex of iron in a high oxidation state, formally PFe(V)=O or $P^{+\bullet}$ Fe(IV)=O, is usually accepted as the reactant in the catalytic cycle of cytochrome P450 [1].

Despite some distinctions in the mechanisms of oxygen activation and a substantial difference in the structures of active intermediates LFeO ($P^{+\bullet}$ Fe(IV)=O for cytochrome P450 and diferryl intermediate Q for MMO), the mechanisms of hydroxylation of the C–H bond in these enzymatic systems are evidently similar [1]. In the early papers devoted to natural monooxygenases and their chemical models, the direct insertion of an oxygen atom into the C–H bond in the interaction of an active intermediate with a substrate molecule was assumed [4]. More recently, many researchers accepted the latent radical mechanism named the “oxygen rebound mechanism” [5]. However, the scrutiny of experimental data on the oxidation of hydrocarbons by cytochrome P450 and its chemical models showed that the oxygen rebound mechanism quantitatively fails

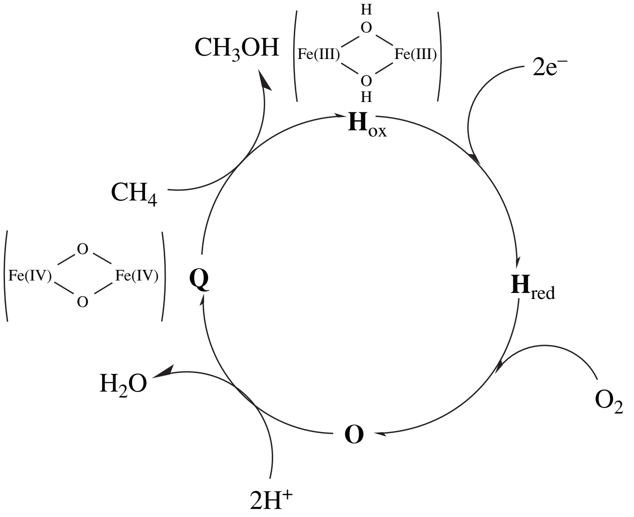
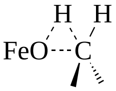
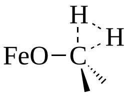


Fig. 1. Catalytic cycle of MMO.

[1, 6]. A new nonradical mechanism has been proposed [6] characterized by the formation of an intermediate complex of a substrate with an active site. This complex is assumed to contain a pentacoordinated carbon atom and can exist in two forms. One of them, the FI form, results from the addition of the O atom to the C–H bond

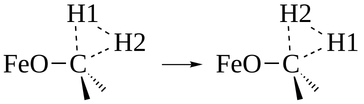


The second form (FII) is characterized by the approaching of H atoms



The step of O atom insertion into the C–H bond following complex formation leads to the hydroxylation product.

Published data on the distribution of oxidation products of deuterium-substituted alkanes (camphor, norbornane, and ethylbenzene) catalyzed by cytochrome P450 and its chemical models have been examined in the framework of the proposed mechanism [7]. In these systems, the H and D atoms exchange their positions along with hydroxylation, which quantitatively contradicts the oxygen rebound mechanism. The calculation showed that the unusual distribution of reaction products of hydroxylation of isotope-substituted molecules agrees well with the two-step nonradical mechanism. In this case, isomerization (racemization) is explained by the exchange of positions of hydrogen isotopes in the FII form



Kinetic parameters calculated in the framework of the new mechanism gave the self-consistent picture of the process dynamics without using barely probable assumptions for all examples considered for the oxidation of compounds with one C–H and one C–D bond of the oxidized carbon atom.

Methane is the most abundant and least reactive representative of the family of saturated hydrocarbons. Therefore, its catalytic oxidation under mild conditions is practically and theoretically important. It is not surprising that the mechanism of methane oxidation by enzymatic systems of methanotrophic bacteria has received special attention from researchers recently [8]. The purpose of this work is to scrutinize the published data on partially deuterated methane (CH_3D, CH_2D_2, and CHD_3) oxidation by MMO in the framework of the mechanism, including the formation of an intermediate complex with pentacoordinated carbon.

RESULTS AND DISCUSSION

Unlike examples considered earlier for the oxidation of chiral deuterium-substituted alkanes [7], the study of isomerization with exchanging positions of hydrogen atoms and its isotopes in methane hydroxylation is impossible because chiral methane does not exist. Information on the dynamics of enzymatic methane oxidation is provided by the study of kinetic isotope effects in the hydroxylation of deuterium-substituted methane molecules. The simplest and most popular method is the investigation of the influence of isotopic substitution in the substrate molecules on the rate of intermediate Q consumption during hydroxylation by MMO. A high value of the kinetic isotope effect (KIE) has been obtained by this method for CH_4 and CD_4 hydroxylation ($k_H/k_D \sim 50–100$ [9]) and accepted by many researchers as the most convincing argument for the radical mechanism of methane hydroxylation [10]. However, further studies showed that the hydroxylation of substrates by MMO does not occur in a single step [11]. Moreover, as shown by experiments using directed mutagenesis [12], the protein environment of the active site has a substantial effect on KIE calculated from the ratio of the rate constants of the reactions of the Q intermediate with CH_4 (k_H) and CD_4 (k_D). The values $k_H = 14$ l mmol^{-1} s^{-1} and $k_H/k_D = 42$ were obtained for the natural components of the MMO system, and the modified components are characterized by $k_H = 2$ l mmol^{-1} s^{-1} and $k_H/k_D = 6$ [12]. The unusually high KIE and its influence on the steric organization of the active site of MMO point to proton tunneling in methane hydroxylation. The presence of the tunneling effect makes it impossible to judge whether the mechanism is radical or molecular based on the k_H/k_D value [1].

Despite many papers devoted to the mechanism of hydroxylation of substrates by MMO on the basis of KIE values, the unusual result obtained for the oxidation of isotopically labeled methane (CH_3D, CH_2D_2, and CHD_3) by soluble MMO from *Methylosinus trichosporium* escaped notice [13]. The KIE values calculated from the ratio of the reaction products taking into account the number of C–D and C–H bonds in the $CH_{4-n}D_n$ molecule,

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

were found to increase substantially with an increase in the number of deuterium atoms (n). These are

$$\text{for } CH_3D$$
$$\sigma_1 = (1/3)[CH_2DOH]/[CH_3OH] = 3.9 \pm 1.0,$$

$$\text{for } CH_2D_2$$
$$\sigma_2 = [CHD_2OH]/[CH_2DOH] = 9.3 \pm 0.54, \quad (1)$$

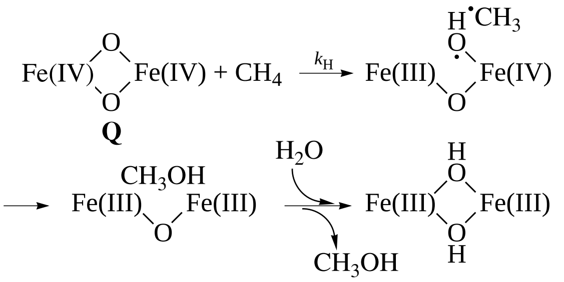


Fig. 2. Oxygen rebound mechanism of methane oxidation by MMO.

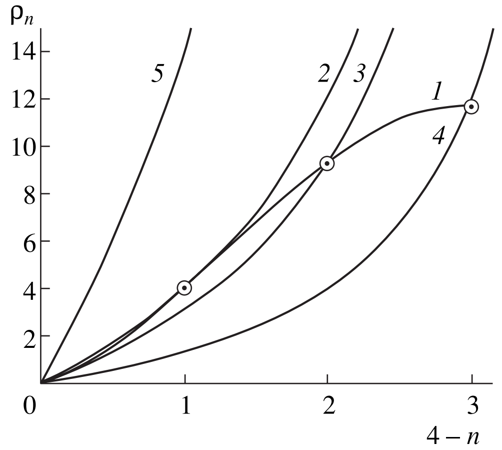


Fig. 3. Plots of the ρ_n parameter of the distribution of the products of partially deuterated methane oxidation by MMO vs. the number of H atoms in the CD_nH_{4-n} molecule: (*1*) experimental plots and those calculated according to Eq. (4) at σ_n equal to (*2*) 12, (*3*) 9.3, (*4*) 3.9, and (*5*) 42.

$$\text{for } CD_3H$$
$$\sigma_3 = 3[CD_3OH]/[CHD_2OH] = 12.0 \pm 1.0,$$

where $CH_{3-n}D_nOH$ and $CH_{4-n}D_{n-1}OH$ are the products of oxidation of the C–H and C–D bonds of the $CH_{4-n}D_n$ methane molecule. Alcohols $CH_{4-n}D_{n-1}OH$ are formed with a high rate constant from $CH_{4-n}D_{n-1}OD$ in isotope exchange with water molecules.

Note that the “overall” KIE values calculated directly from the ratio of the oxidation products,

$$\begin{gathered}\text{for } CH_3D\\ \rho_1 = [CH_2DOH]/[CH_3OH] = 11.7 \pm 3.0,\\ \text{for } CH_2D_2\\ \rho_2 = [CHD_2OH]/[CH_2DOH] = 9.3 \pm 0.54,\\ \text{for } CD_3H\\ \rho_3 = [CD_3OH]/[CHD_2OH] = 4 \pm 0.33,\end{gathered} \tag{2}$$

correspond to a descending plot of the KIE vs. number of the ^{1}H isotopes in the methane molecule

$$(\rho_2/\rho_3):(\rho_1/\rho_2) = 2.32:1.26; \quad (\rho_2/\rho_3) > (\rho_1/\rho_2). \tag{3}$$

In the framework of the oxygen rebound mechanism of methane oxidation by MMO (Fig. 2), which has been commonly accepted by now [8, 14, 15], it seems evident that $\sigma_n = k_H/k_D$ should not change with n variation. In this case,

$$\rho_n = \{(4-n)/n\}(k_H/k_D) = \{(4-n)/n\}\sigma_n. \tag{4}$$

Figure 3 presents the experimental plot (curve *1*) of the isotope effect ρ_n on the number $(4-n)$ of the ^{1}H isotopes during $CH_{4-n}D_n$ oxidation (see Eqs. (2)) [13] and similar plots (curves *2*–*5*) calculated according to Eq. (4). The following values were used in calculations: (1) σ_n obtained in [13] by the distribution of the products of $CH_{4-n}D_n$ oxidation (curves *2*–*4*) and (2) $\sigma_n = 42$ obtained [12] from the consumption rate of the Q intermediate (curve *5*). Obviously, the experimental plot of the KIE value vs. number of the ^{1}H isotopes in the methane molecule (curve *1*) differs substantially from curves *2*–*5* calculated in the framework of the oxygen rebound mechanism. Let us examine the experimental plot of the KIE in the framework of the mechanism including the formation of an intermediate with pentacoordinated carbon.

Kinetic model I (Fig. 4) was proposed for the oxidation of hydrocarbons by natural monooxygenases and their chemical models. This model is in good agreement with all experimental observations for hydrocarbons with one C–H and one C–D bonds at the oxidized carbon atom [7]. The first step in this kinetic model is the formation of isotopomers of FI form in the interaction of the oxygen atom of the active form of the enzyme (LFeO) at the C–H or C–D bond of the partially deuterated substrate with the rate constants k_1^H and k_1^D, respectively. For simplicity, this step is assumed to be irreversible. The FI and FII forms are assumed to be kinetically indistinguishable, and the FI form is taken to be the main state of the intermediate complex determining the statistical coefficients at the rate constants of the reaction steps. The FII species is present in the kinetic scheme in implicit form and responsible for the isomerization of the FI species. For all isomerization steps where the H–D fragment rotates, the rate constants of the forward and backward reactions are taken to be equal to k_i. The hydroxylation products are formed due to the direct insertion of the oxygen atom into the C–H or C–D bond. It is assumed for the insertion step that the rate constant only depends on isotopic substitution (k_2^H and k_2^D rate constants). This kinetic model can be presented as a simplified scheme (Fig. 5) for methane derivatives in which n hydrogen atoms are substituted by deuterium. According to this scheme, the expression for the kinetic iso-

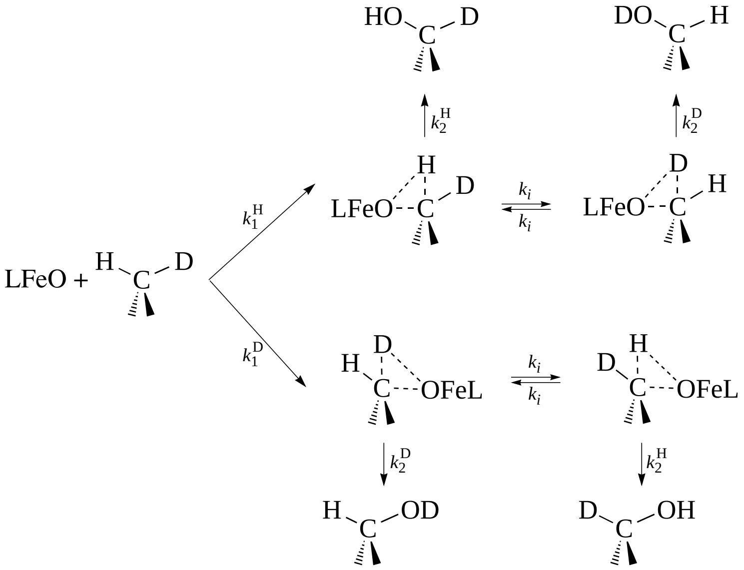


Fig. 4. Kinetic model I.

tope effects ρ_n and σ_n under stationary conditions can easily be obtained:

$$\sigma_n = \{n/(4-n)\}\rho_n$$
$$= \{ab + nd + (4-n)ad\}/\{b + n + (4-n)a\},$$

$$\text{where} \quad a = \frac{k_1^{\mathrm{H}}}{k_1^{\mathrm{D}}}, \quad b = \frac{k_2^{\mathrm{H}}}{k_i}, \quad d = \frac{k_2^{\mathrm{H}}}{k_2^{\mathrm{D}}}. \tag{5}$$

Provided that $a = d$, the expressions for ρ_n and σ_n can easily be obtained from Eq. (5) in the case of the exact equality of the kinetic isotope effects of the first and second steps:

$$\sigma_n = a = d; \quad \rho_n = \{(4-n)/n\}a = \{(4-n)/n\}d. \tag{6}$$

For this case, the σ_n value should be independent of the isotope composition of the molecule, which reliably differs from experimental values in Eqs. (1). The analysis of Eqs. (5) shows that an increase in the KIE values calculated per bond according to Eqs. (1) with an increase in the D atoms in methane ($\sigma_3 > \sigma_2 > \sigma_1$) is fulfilled when

$$(\sigma_3 - \sigma_1)$$
$$= \{2b(a-1)(a-d)\}/\{(a+b+3)(3a+b+1)\} > 0. \tag{7}$$

Since $a \geq 1$, according to the physical sense of definition (5), inequality (7) is fulfilled if $a > 1$ and $d < a$. At the same time, inequality (3) for the experimental KIE values in the framework of the accepted kinetic scheme holds under the condition

$$(c^2 - 1)b^2 + 4cb(1 + a)(c - 1) > 0, \tag{8}$$

where $c = d/a$. It follows from this that $c > 1$, that is, $d > a$, corresponds to the experimental KIE values. Therefore, conditions (7) and (8) are incompatible. Thus, the previously accepted kinetic model I does not agree with the experimental distribution of the oxidation products of partially deuterated methane. However, according to this model, the σ_n values can change with a change in the number of deuterium atoms in the methane molecule (see inequality (7)). This is an advantage of the model under consideration compared to the oxygen rebound mechanism.

Let us consider more complicated model II of the nonradical mechanism. Assume that the reaction of an

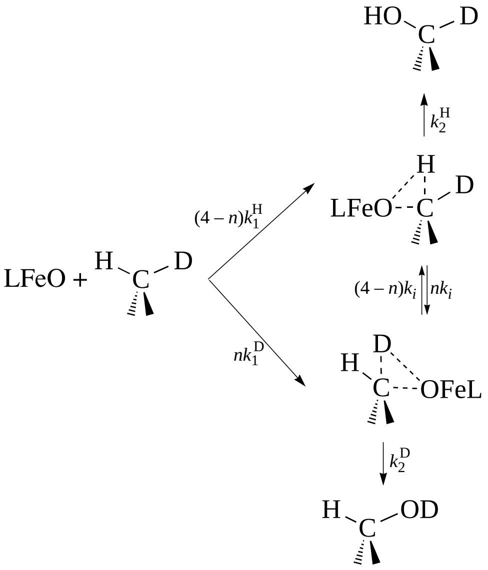


Fig. 5. Scheme of methane oxidation in the framework of kinetic model I.

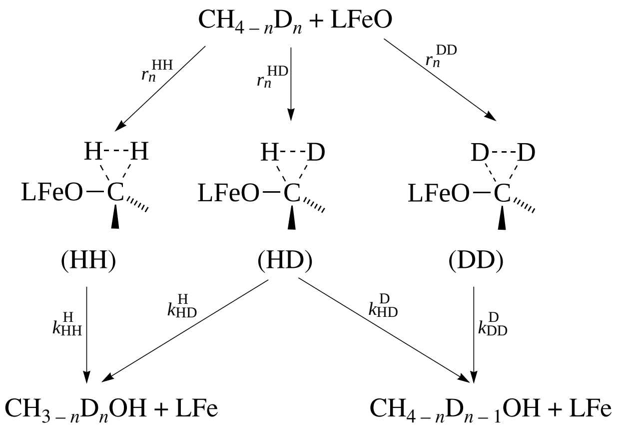


Fig. 6. Scheme of methane oxidation in the framework of kinetic model II.

active species with a hydrocarbon simultaneously involves two hydrogen atoms; that is, the first step is the formation of the FII form. The first step of partially deuterated methane oxidation leads to intermediate compounds of three types with the rate constants k^{HH}, k^{HD}, and k^{DD}. According to the composition of the methylene unit, let us denote them as (HH), (HD), and (DD), respectively (Fig. 6). Similarly to kinetic model I, in the model of the mechanism under study, the FI and FII forms are kinetically indistinguishable. However, the main state of the intermediate complex that determines the statistical coefficients at the rate constants of the reaction steps is FII. Therefore, the ratios of the formation rates of the intermediate complexes can easily be obtained:

for CH_3D ($n = 1$) $r_1^{HH} : r_1^{HD} = k^{HH} : k^{HD}$, $\quad r_1^{DD} = 0$;

for CH_2D_2 ($n = 2$) $r_2^{HH} : r_2^{HD} : r_2^{DD} = k^{HH} : 4k^{HD} : k^{DD}$;

for CHD_3 ($n = 3$) $r_2^{HH} = 0$, $r_2^{HD} : r_2^{DD} = k^{HD} : k^{DD}$.

The FI form is present in the kinetic scheme in implicit form and is responsible for the formation of products from the FII forms:

$$(HH) \xrightarrow{k^{H}_{HH}} CH_{3-n}D_nOH,$$

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

The intermediate compound containing the methylene unit with the HD hydrogen bond can form the products of oxidation of both the C–H and C–D bonds

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

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

Since chiral methane does not exist, let us exclude the isomerization of the (HD) forms from consideration.

The following expressions for the ratio of the oxidation products of deuterated methane can easily be obtained in the approximation of quasi-stationary concentrations of intermediate compounds in the framework of the adopted kinetic scheme:

$$\begin{aligned} \rho_1 &= e(1+g)+g, \\ \rho_2 &= h[e(1+g)+4g]/(1+g+4h), \\ \rho_3 &= hg/(1+h+g), \end{aligned} \qquad (9)$$

where $h = k^{HD}/k^{DD}$, $e = k^{HH}/k^{HD}$, and $g = k^{H}_{HD}/k^{D}_{HD}$.

These expressions can be transformed into the system of equations

$$\begin{aligned} h &= \rho_3(1+g)/(g-\rho_3), \\ e &= (\rho_1-g)/(g+1), \\ \rho_2 &= \rho_3(\rho_1+3g)/(3\rho_3+g). \end{aligned} \qquad (10)$$

Since the e and h values can only be positive due to their physical sense (9), according to Eqs. (10) and taking into account the ρ_1 and ρ_3 numerical values from (2), the g and ρ_2 parameters are restricted by the intervals $4 = \rho_3 < g < \rho_1 = 11.7$ and $5.9 < \rho_2 < 7.9$. The h values should be higher than 6.6, and the e values are lower than 1.5. Thus, based on the experimental values of ρ_1 and ρ_3, in the framework of the kinetic scheme considered, we determined the region of allowable values for ρ_2 to which its experimental value $\rho_2 = 9.30 \pm 0.54$ does not belong. Note that (in terms of the allowable values for this parameters) for $\rho_2 = 7.8$ we obtain $(\rho_2/\rho_3) : (\rho_1/\rho_2) = 1.95 : 1.5$; that is, $(\rho_2/\rho_3) > (\rho_1/\rho_2)$ and inequality (3) is fulfilled.

Using the experimental values for ρ_1 and ρ_2, according to the system of equations (9), we obtain the ranges of allowable values for ρ_3 and other parameters of the system: $h > 12.3$, $e < 0.3$, $8.5 < g < 11.7$, and $5.8 < \rho_3 < 8$. In this case, for $\rho_3 = 5.9$ we obtain $(\rho_2/\rho_3):(\rho_1/\rho_2) = 1.58 : 1.26$; that is, $(\rho_2/\rho_3) > (\rho_1/\rho_2)$ and inequality (3) is true. Analogously, proceeding from the experimental values for ρ_2 and ρ_3, we obtain $h > 5.6$, $e < 4.2$, and the ranges $4 < g < 16.7$, $16.7 < \rho_1 < 25.4$. For $\rho_1 = 16.8$, we obtain $(\rho_2/\rho_3) : (\rho_1/\rho_2) = 2.32 : 1.8$; that is, $(\rho_2/\rho_3) > (\rho_1/\rho_2)$ and inequality (3) is true.

Thus, simple calculations show that the set of equations (9), taking into account the experimental values of the product distribution of partially deuterated methane (2),

$$\begin{aligned} \rho_1 &= e(1+g)+g = 11.7, \\ \rho_2 &= h[e(1+g)+4g]/(1+g+4h) = 9.3, \\ \rho_3 &= hg/(1+h+g) = 4 \end{aligned} \qquad (11)$$

is incompatible for the values $e > 0$ and $h > 0$. Nevertheless, the kinetic scheme involving two hydrogen atoms in the first step makes it possible to select the values of the parameters for which inequality (3) is valid.

Note that the equations for ρ_1 and ρ_2 of this system have no common solution at $e \geq 1$, while the physical sense of this parameter ($e = k^{HH}/k^{HD}$) assumes that its value cannot be lower than unity. For $e = 1$, based on the ρ_1 and ρ_3 values, we obtain $g = 5.4$, $h = 18.3$, and $\rho_2 = 6.4$ (compare with the experimental value $\rho_2 = 4 \pm 0.33$). In this case, $(\rho_2/\rho_3) : (\rho_1/\rho_2) = 1.6 : 1.8$; that is, $(\rho_2/\rho_3) < (\rho_1/\rho_2)$ and inequality (3) is not fulfilled. Using the ρ_2 and ρ_3 values, we obtain $h = 63$, $g = 10$, and $\rho_1 = 21$ (compare with the experimental value $\rho_1 = 11.7 \pm 3.0$), and in this case inequality (3) is not fulfilled either. Note that an increase in the e parameter results in a greater deviation of the calculated ρ_1 and ρ_2 values from the experimental values.

Thus, the regions of $e = (k^{HH}/k^{HD}) \geq 1$ and $h = (k^{HD}/k^{DD}) \geq 1$ contain no set of numerical values of the parameters of kinetic model II providing the distribution of the oxidation products of partially deuterated methane, which coincides with the experimental distribution within the experimental accuracy. Moreover, inequality (3) is only fulfilled in the framework of the considered model at $e < 1$, which is unreasonable.

Let us consider model III for the nonradical mechanism of methane oxidation, which is more complicated. Assume that three hydrogen atoms are simultaneously involved in the reactions of an active species with hydrocarbon. The first step of partially deuterated methane oxidation leads to the formation of intermediate compounds of four types with the rate constants k^{HHH}, k^{HHD}, k^{HDD}, and k^{DDD} (Fig. 7). Taking into account the number of deuterium molecules in the oxidized substrate, we can easily obtain the ratio of formation rates of these compounds

for CH_3D ($n = 1$)

$$r_1^{HHH} : r_1^{HHD} = k^{HHH} : 3k^{HHD},\ r_1^{HDD} = r_1^{DDD} = 0;$$

for CH_2D_2 ($n = 2$)

$$r_2^{HHD} : r_2^{HDD} = k^{HHD} : k^{HDD},\ r_2^{HHH} = r_2^{DDD} = 0;$$

for CHD_3 ($n = 3$)

$$r_3^{HDD} : r_3^{DDD} = 3k^{HDD} : k^{DDD},\ r_3^{HHH} = r_3^{HHD} = 0.$$

According to the isotopic composition of the activated C–H (C–D) bonds, let us designate the intermediate complexes as (HHH), (HHD), (HDD), and (DDD). As in the previously considered kinetic models of the methane oxidation mechanism, let us exclude from consideration the isomerization reactions of these compounds. The oxidation products are formed in the second step by the reactions

$$(HHH) \xrightarrow{k^H_{HHH}} CH_{3-n}D_nOH,$$

$$(HHD) \xrightarrow{k^H_{HHD}} CH_{3-n}D_nOH,$$

$$(HHD) \xrightarrow{k^D_{HHD}} CH_{4-n}D_{n-1}OH,$$

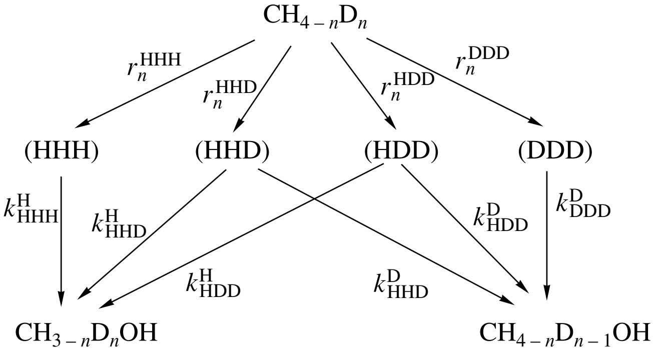


Fig. 7. Scheme of methane oxidation in the framework of kinetic model III.

$$(HDD) \xrightarrow{k^H_{HDD}} CH_{3-n}D_nOH,$$

$$(HDD) \xrightarrow{k^D_{HDD}} CH_{4-n}D_{n-1}OH,$$

$$(DDD) \xrightarrow{k^D_{DDD}} CH_{4-n}D_{n-1}OH.$$

In the framework of the kinetic scheme presented in Fig. 7, in the approximation of quasi-stationary concentrations of intermediate compounds, one can easily obtain expressions for the ratio of oxidation products of partially deuterated methane, which are transformed into the system of equations taking into account the experimental values in (2):

$$\begin{aligned} \rho_1 &= \eta\delta + (\alpha/3)(\eta\delta + 1) = 11.7, \\ \rho_2 &= [\beta\eta\delta(1+\delta) + \delta(1+\eta\delta)]/[\beta(1+\delta) \\ &\quad + (1+\eta\delta)] = 9.3, \\ \rho_3 &= 3\gamma\delta/(1+\delta+3\gamma) = 4, \end{aligned} \tag{12}$$

where $\alpha = k^{HHH}/k^{HHD}$, $\beta = k^{HHD}/k^{HDD}$, $\gamma = k^{HDD}/k^{DDD}$, $\eta = \delta_1/\delta_2$, $\delta_1 = k^H_{HHD}/k^D_{HHD}$, and $\delta = \delta_2 = k^H_{HDD}/k^D_{HDD}$.

Unlike the system of equations (11), which have no solution if the values of kinetic parameters are positive, system (12) has numerous solutions, for example, $\alpha = 1.3$, $\beta = 1.5$, $\gamma = 2.3$, $\delta = \delta_2 = 11$, $\delta_1 = 8$, and $\eta = 0.73$. The ranges of allowable values of kinetic parameters are constrained by several conditions. For brevity, we do not present all of them because only the condition $\eta < 1$, that is, $\delta_1 < \delta_2$ and $k^H_{HHD}/k^D_{HHD} < k^H_{HDD}/k^D_{HDD}$, is of fundamental significance.

Thus, we considered kinetic schemes involving one, two, and three hydrogen atoms simultaneously in the first step. The scheme involving one C–H (or C–D) bond in the reaction with the active intermediate of the catalytic cycle of MMO explains a change in the σ_n value with a change in the number of deuterium atoms in the methane molecule due to the multistep character of the process and the possibility of exchanging positions of the H and D atoms in the methylene unit of the

FII form. However, we failed to obtain descending plots of the ρ_n values vs. the number of ^{1}H isotopes in the methane molecule corresponding to inequality (3) in the framework of this scheme. The scheme involving two hydrogen atoms simultaneously in the first step provides the descending plot of the ρ_n values vs. the number of ^{1}H isotopes in the methane molecule if $e = (k^{HH}/k^{HD}) < 1$ and $h = (k^{HD}/k^{DD}) > 1$. However, for this scheme, there is no set of kinetic parameters capable of providing the calculated distribution of oxidation products of partially deuterated methane, which coincides within the error with the experimental values. The kinetic scheme involving three hydrogen atoms simultaneously in the first step agrees with the experimental results if the insertion of oxygen into the C–D bond of the intermediate (HHD) compound with one activated C–D bond occurs with a higher probability than that for the (HDD) compound with two activated C–D bonds.

Since chiral methane does not exist, the isomerization of the (HD), (HHD), and (HDD) forms was not considered for simplicity. However, these reactions are very important for explaining the racemization of the products of enzymatic oxidation (involving soluble MMO) of chiral ethane, whose molecule contains a methyl group in which two hydrogen atoms are substituted by deuterium and tritium [16]. Analysis showed [7] that both racemization [16] and complete configuration retention during the oxidation of chiral ethane by MMO can be explained in the framework of simple kinetic model I (Fig. 4). However, as follows from the aforesaid, model I does not agree with the experimental data on methane oxidation by this enzymatic system. It seemed necessary to consider more complicated kinetic models involving two and three C–H bonds in the first step for methane and ethane taking into account isomerization. Such a complication of the kinetic scheme prevented us from obtaining analytical solutions to the corresponding systems of differential equations and required numerical calculations.

A special investigation devoted to the kinetic simulation of the dynamics of methane and ethane hydroxylation by MMO was carried out using the numerical method [18]. Published data on the kinetic isotope effects of oxidation of deuterium-substituted methane molecules (CHD_3, CH_2D_2, and CH_3D) and the distribution of the products of oxidation of chiral ethane (R- and S-MeCHDT) by methane monooxygenase were examined in the framework of the mechanism including the formation of an intermediate with pentacoordinated carbon. The models were considered in which the FI and FII forms are either kinetically different (models involving one C–H or C–D bond in the first step) or similar (models involving two carbon–carbon bonds in the first step). In the former case, the kinetic schemes take into account the transformations of the FI forms into the FII forms and back. In the second case, FII is chosen as the main form determining the statistical coefficients at the rate constants of the reaction steps. All kinetic schemes take into account the possibility of the steric isomerization of molecules due to the exchange of positions of the H and D isotopes in the methylene units of the FII forms in the intermediate complex. In addition, the transformations of the FII forms with different methylene units ((CDD), (CHD), and (CHH)) into each other (with and without isomerization) were introduced into the kinetic schemes

$$\mathrm{FII_{DD}} \rightleftharpoons \mathrm{FII_{HD}} \rightleftharpoons \mathrm{FII_{HH}}.$$

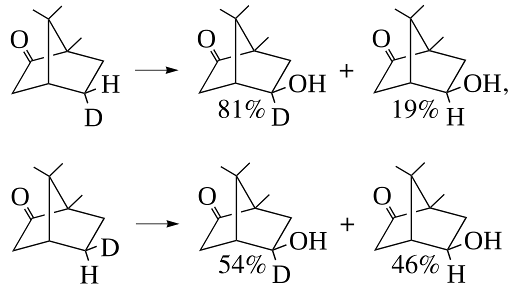
In the framework of the simple kinetic scheme II, we determined the range of allowable values $5.9 < \rho_2 < 7.9$, which does not contain the experimental value $\rho_2 = 9.3 \pm 0.54$. The introduction of the mutual transformations of the FII forms with different methylene units into the kinetic schemes allowed an increase in ρ_2 without changing the ρ_1 and ρ_3 values. In the kinetic schemes involving one C–H or C–D bond in the first step, an increase in ρ_2 is achieved by selecting the ratio of kinetic parameters that increases the contribution of the channel $CH_{4-n}D_n \longrightarrow FI_{OD} \longrightarrow FII_{DD} \longrightarrow FII_{HD} \longrightarrow FII_{HH} \longrightarrow FI_{OH} \longrightarrow CH_{3-n}D_nOH$ to the formation of the products (FI_{OD} and FI_{OH} are the intermediates formed due to the addition of the O atom to the C–D and C–H bonds, respectively). With the same purpose, the ratio of the kinetic parameters, which increases the contribution of the channel $CH_{4-n}D_n \longrightarrow FII_{DD} \longrightarrow FII_{HD} \longrightarrow FII_{HH} \longrightarrow CH_{3-n}D_nOH$ to product formation, is chosen for the kinetic schemes involving simultaneously two C–H (C–D) bonds in the first step. Since such channels are absent in the case of CHD_3 and CH_3D, their occurrence increases the KIE value only for CH_2D_2.

As is shown above, when three C–H (C–D) bonds are simultaneously involved in the first step, numerous sets of kinetic parameters corresponding to experimental data exist even in the framework of simple kinetic scheme III. The involvement of mutual transformations of intermediate complexes providing the isomerization of molecules in the kinetic scheme extended the region of acceptable values of the kinetic parameters for this case.

Thus, the kinetic schemes corresponding to the mechanism of methane oxidation through the formation of the FeO–CH_4 complex were examined by the method of quasi-stationary concentrations (this work) and a numerical method [18]. In the particular case of one C–H bond and one C–D bond at the oxidized carbon atom, the kinetic schemes considered can be reduced to kinetic scheme I proposed earlier (Fig. 4). This scheme perfectly agrees with experimental data on the hydroxylation of hydrocarbons with this structure by cytochrome P450 and its chemical models [7]. Thus, the experimental data obtained in the study of the monooxygenase oxidation of alkanes by different enzymatic systems can be explained in the framework of the general nonradical mechanism.

Note that Yoshizawa [19] proposed another modification of the two-step nonradical mechanism for the oxidation of methane by MMO on the basis of DFT calculations. This modification assumed the formation of an intermediate methane complex with the active site of MMO through the coordination of carbon to iron OFe---CH_4 (Fig. 8). The authors of [20] believe that the η^2-H,H structure is preferable for the methane complex with the metal in a low-spin state, whereas the η^3-H,H,H structure is preferable for the methane complex with the metal in a high-spin state (Fig. 9a). For the Fe^+---CH_4 complex, the calculations point to bond formation of the η^2-H,H type for both low- and high-spin states [20]. The coordination of methane to the iron-containing electrophilic site weakens the C–H bonds. The concerted mechanism of migration of methyl and hydrogen through the transition states TS1 and TS2 (Fig. 9b) produces a product containing methanol as a ligand. The specific structure of the intermediate complex was ignored in the kinetic schemes involving two and three hydrogen atoms in the first step that are considered above (Figs. 6, 7). Therefore, the calculations for these schemes are also valid for the modification of the mechanism resulting in the formation of a complex with the coordination site on the metal. The dynamics of methane isomerization was considered in the framework of this modification of the two-step nonradical mechanism [20]. Note that chiral methane does not exist, and the isomerization mechanism is not proposed for ethane and other hydrocarbons.

The nonradical mechanism assuming the intermediate formation of the FeO---CH_4 complex is still the only one that quantitatively agrees with experimental data on the distribution of products of oxidation of stereosiomers of deuterium-substituted camphor in the natural system [7]. The catalysis by cytochrome $P450_{cam}$ results in the partial transfer of deuterium atoms from the *exo* to *endo* position and back:



This transfer leads to the sole formation of 5-*exo*-hydroxycamphor, because the camphor molecule is fixed in the active site of the enzyme by the hydrogen bond and cannot rotate. This implies that isomerization due to the rotation of the molecule is impossible and cannot be explained in the framework of the oxygen rebound mechanism.

Note that the modification of the mechanism of alkane oxidation through the formation of an intermediate complex containing pentacoordinated carbon does not exclude the possibility of methane coordination to iron in the first step; that is, the nonradical mechanism can be both two- and three-step (Fig. 10). Evidence for a more complicated mechanism of transformation of the structure of intermediate complexes is also provided by the ratios of parameters without an obvious physical sense, which were obtained by the kinetic simulation of the oxidation of partially deuterated methane by MMO [18].

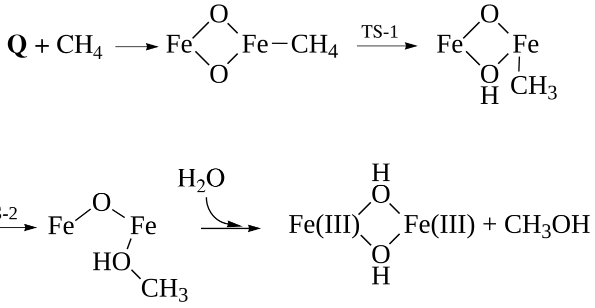


Fig. 8. Mechanism of methane oxidation by MMO through the formation of the Fe–C bond.

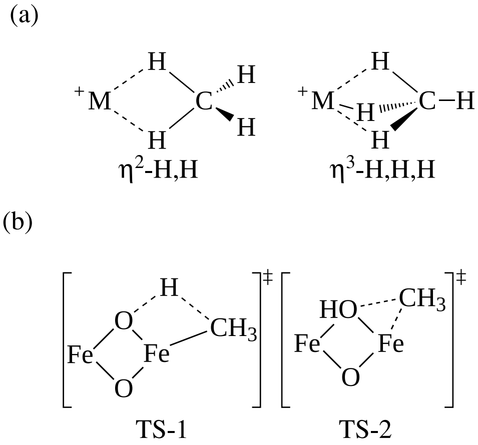


Fig. 9. Assumed structures of (a) transition metal complexes with the methane molecule and (b) transition states formed according to the mechanism of methane oxidation through the formation of the Fe–C bond.

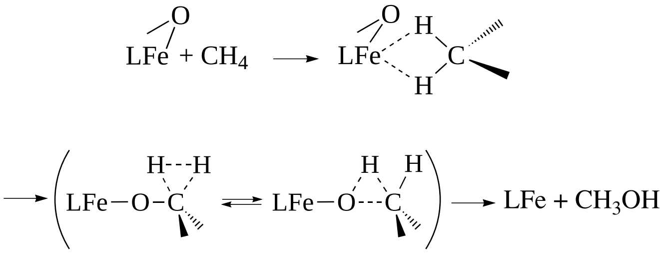


Fig. 10. Three-step mechanism of methane oxidation by MMO.

CONCLUSIONS

The consideration of the dynamics of monooxygenase alkane oxidation in natural and biomimetic systems by analytical and numerical methods ([7, 18] and this work) explained unusual experimental results in the framework of the general nonradical mechanism. The first experimental fact that finds no satisfactory explanation in the framework of other known mechanisms of alkane oxidation is the change in the steric position of the hydrogen isotopes in the absence of rotation of the alkane molecule (oxidation of camphor). Another fact is the distribution of products of partially deuterated methane oxidation, which differs from a simple correspondence with the number of C–H and C–D bonds in the substrate molecule (taking into account the KIE). These results are an indication of the complicated dynamics of the process. Analysis performed in this work demonstrates that the chosen approach is promising for mechanistic studies of saturated hydrocarbon oxidation in enzymatic systems and their chemical models.

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