Additivity rule holds in the hydrogen transfer reactivity of unsaturated fatty acids with a peroxyl radical: mechanistic insight into lipoxygenase†

Hironori Kitaguchi, Kei Ohkubo, Seiji Ogo and Shunichi Fukuzumi*

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A simple additivity rule holds in the hydrogen transfer reactivity of unsaturated fatty acids with cumylperoxyl radical, which is expressed by the additive contributions of the reactivity of active hydrogens from the 1,4-pentadiene subunit and those of the allylic subunit; the kinetic isotope effect on the hydrogen transfer reactions (KIE = 6.1) is significantly smaller than that observed for lipoxygenase (KIE = 81).

Soybean lipoxygenase-1 (SLO) has received special attention,[1–5] because the hydrogen atom abstraction step in SLO exhibits the largest H/D kinetic isotope effect (KIE), of k_H/k_D = 81 at near room temperature, so far reported for biological redox systems.[6–8] This result provides compelling evidence for tunneling effects in the SLO reaction. The abstraction of the *pro-S* hydrogen atom from the C11 position of linoleic acid by the active site (Fe^{3+}–OH) species to form the substrate radical is the rate-determining step in the catalytic mechanism.[6–8] Oxygen rapidly reacts with the substrate radical to generate the peroxyl radical which reacts with Fe^{2+}–OH_2 to yield the hydroperoxide, 13-(*S*)-hydroperoxy-9,11-(*Z*,*E*)-octadecadienoic acid (13-(*S*)-HPOD), accompanied by regeneration of the active form of the enzyme, Fe^{3+}–OH. Other origins of the large kinetic isotope effect (KIE) on catalytic rates such as magnetic field effects or reaction branching have been ruled out to conclude that the large KIE reflects an intrinsic KIE for a single hydrogen transfer step, which proceeds by a tunneling event. In such a case it would be quite interesting to examine a single hydrogen transfer step of unsaturated fatty acids and the KIE with other reactive radical species in order to know the difference in the hydrogen-abstraction step between the non-enzymatic and enzymatic systems. However, the direct determination of hydrogen transfer rates of a series of unsaturated fatty acids with reactive radical species has yet to be reported.

We report herein the direct determination of the absolute rates of hydrogen transfer from a series of unsaturated fatty acids to cumylperoxyl radical by use of electron spin resonance (ESR) at low temperatures. The absolute rates together with the direct determination of the KIE at various temperatures provide valuable insight into the tunneling hydrogen transfer mechanism of lipoxygenases.

Department of Material and Life Science, Division of Advanced Science and Biotechnology, Graduate School of Engineering, Osaka University, SORST, Japan Science and Technology Agency (JST), Suita, Osaka 565-0871, Japan. E-mail: fukuzumi@chem.eng.osaka-u.ac.jp; Fax: +81-6-6879-7370; Tel: +81-6-6879-7368

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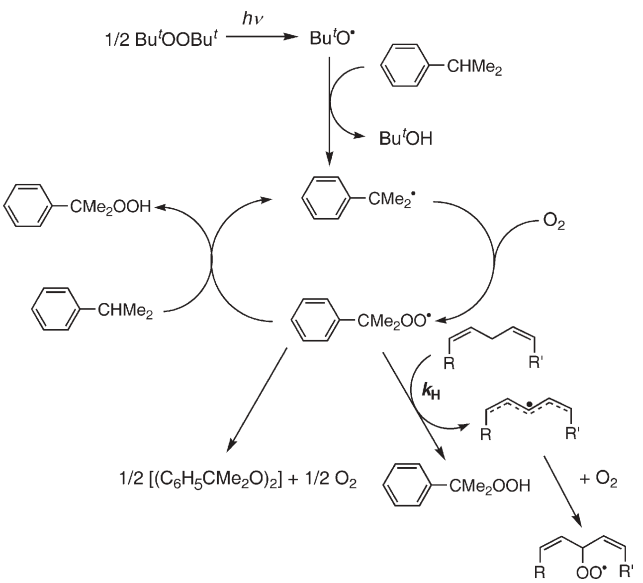
Direct measurements of the rates of hydrogen transfer from a series of unsaturated fatty acids to cumylperoxyl radical were performed in propionitrile (EtCN) at various temperatures by means of ESR. The photoirradiation of an oxygen saturated EtCN solution containing di-*t*-butylperoxide (Bu^tOOBu^t) and cumene with a 1000 W high-pressure mercury lamp results in formation of cumylperoxyl radical which is readily detected by ESR.[9] The cumylperoxyl radical is formed *via* a radical chain process started by the UV-photoirradiation of Bu^tOOBu^t as shown in Scheme 1.[10–12] In the termination step, cumylperoxyl radicals decay by a bimolecular radical coupling reaction to yield the corresponding peroxide [$(C_6H_5CMe_2O)_2$] and oxygen (Scheme 1).[10,12] When the light is cut off, the ESR signal intensity decays obeying second-order kinetics due to the bimolecular reaction (Fig. 1a).

In the presence of linoleic acid (4.0×10^{-2} mol dm^{-3}) and arachidonic acid (4.0×10^{-2} mol dm^{-3}), the decay of cumylperoxyl radical after cutting off the light becomes much faster than that in the absence of linoleic acid and arachidonic acid, as shown in Fig. 1b and 1c. The decay in the presence of linoleic acid and arachidonic acid obeys pseudo-first-order kinetics rather than second-order kinetics (see Supplementary Information S1, S2 for the first-order plots†).[13] The pseudo-first-order rate constants (k_{obs}) increase linearly with an increase in concentration of unsaturated fatty acid to exhibit a first-order dependence (S3).



Scheme 1

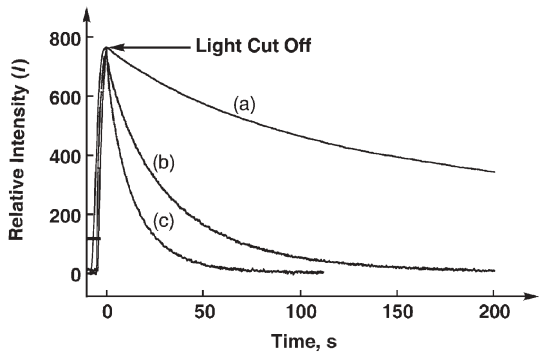


Fig. 1 The decay profile of the ESR signal intensity due to cumylperoxyl radical in the presence of (a) no unsaturated fatty acid, (b) linoleic acid (4.0×10^{-2} mol dm^{-3}) and (c) arachidonic acid (4.0×10^{-2} mol dm^{-3}) in an O_2-saturated EtCN solution of di-*t*-butyl peroxide (1.0 mol dm^{-3}) and cumene (1.0 mol dm^{-3}) with a 1000 W high-pressure mercury lamp at 203 K.

Thus, the decay process is ascribed to the hydrogen transfer from unsaturated fatty acid to cumylperoxyl radical (Scheme 1).

The rate constants (k_H) of hydrogen transfer from a series of unsaturated fatty acids to cumylperoxyl radical were determined from the slopes of linear plots of k_{obs} *vs.* the concentration of unsaturated fatty acid. The results are summarized in Fig. 2 (for the k_H values, see S4), where the k_H values at different temperatures are linearly correlated with the number of active hydrogens of the 1,4-pentadiene subunits of unsaturated fatty acids (*n*). Such linear correlations shown in Fig. 2 reveal that a simple additive rule holds in the hydrogen transfer reactions as given by eqn (1):

$$k_H = k_{H0} + nk_{H1} \quad (1)$$

where k_{H1} is the hydrogen transfer rate constant of one hydrogen of a 1,4-pentadiene subunit (linoleic acid, linolenic acid, and arachidonic acid) and k_{H0} is the hydrogen transfer rate constant of the allylic subunit (oleic acid).[14]

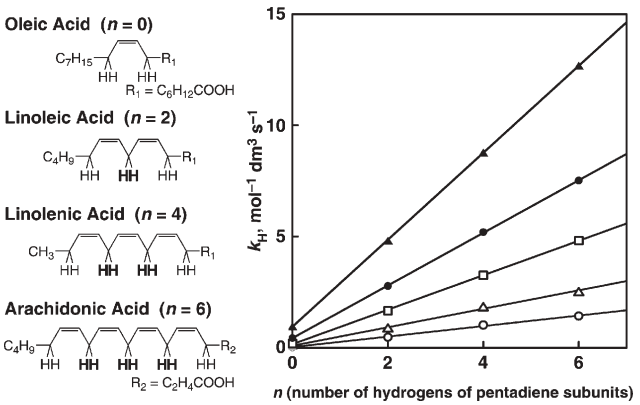


Fig. 2 Plots of rate constants (k_H) *vs.* the number of hydrogens of the 1,4-pentadiene subunits (*n*) for hydrogen transfer from unsaturated fatty acids (the *n* values are given in parentheses) to cumylperoxyl radical in an O_2-saturated EtCN solution at 203 K (○), 213 K (△), 223 K (□), 233 K (●) and 243 K (▲).

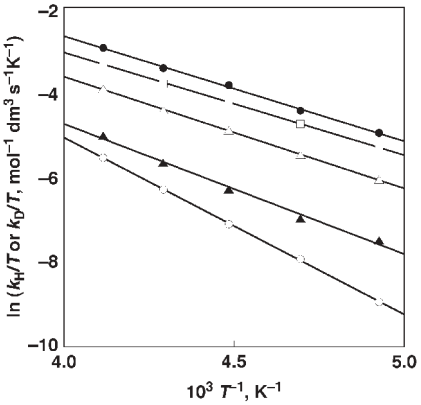


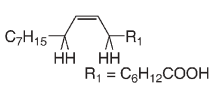
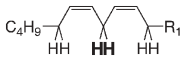
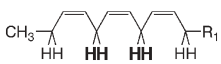
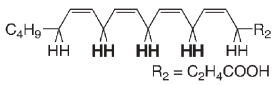
Fig. 3 Eyring plots of k_H for hydrogen transfer from oleic acid (○), linoleic acid (△), linolenic acid (□), arachidonic acid (●) and [11,11-$^2H_2$]linoleic acid (▲) to cumylperoxyl radical in O_2-saturated EtCN.

Eyring plots of the k_H and k_D values are shown in Fig. 3. The activation parameters are obtained from eqn (2):

$$\ln(k_H/T) = -\Delta H^{\ddagger}/RT + \Delta S^{\ddagger}/R + \ln(k_B/h) \quad (2)$$

where k_B is the Boltzmann constant, *h* is Planck's constant and *R* is the universal gas constant.[15] The values obtained are summarized in Table S5.[16] There is a significant difference between the activation enthalpies of the unsaturated fatty acids containing 1,4-pentadiene subunits (linoleic acid: 21.9 kJ mol^{-1}, linolenic acid: 20.1 kJ mol^{-1} and arachidonic acid: 20.6 kJ mol^{-1}) and that containing only an allylic subunit (oleic acid: 34.7 kJ mol^{-1}). This is consistent with the calculated energy differences between hydrogen-abstracted radicals and unsaturated fatty acids (ΔE), since the ΔE values of the allyl-type radicals, which are nearly the same among the unsaturated fatty acids, are larger by 50 ± 9 kJ mol^{-1} than those of the corresponding pentadienyl-type radicals derived from the same unsaturated fatty acids, which are also nearly the same among unsaturated fatty acids (see Table 1).[17]

Table 1 Energy differences (ΔE) between hydrogen-abstracted radicals and the neutral form of the unsaturated fatty acids, obtained from DFT calculations

Unsaturated fatty acid	Position of hydrogen-abstraction	ΔE^a/ kJ mol^{-1}	Structure of unsaturated fatty acid
Oleic acid	8	1689.6	
	11	1706.4	
Linoleic acid	8	1695.7	
	11	1644.8	
	14	1697.1	
Linolenic acid	8	1689.0	
	11	1638.8	
	14	1639.4	
	17	1688.6	
Arachidonic acid	4	1687.5	
	7	1641.0	
	10	1645.8	
	13	1640.7	
	16	1690.0	

[a] Determined by ROHF formalism with B3LYP/3-21G basis set.

Table 2 Rate constants (k_{H1}[a] and k_{D1}[b]) and the kinetic isotope effect (k_{H1}/k_{D1}) for hydrogen transfer from linoleic acid ([11,11-$^2H_2$]linoleic acid) to cumylperoxyl radical

T/K	k_{H1}[a]/mol^{-1} dm^3 s^{-1}	k_{D1}[b]/mol^{-1} dm^3 s^{-1}	k_{H1}/k_{D1}
203 K	$(4.5 \pm 0.3) \times 10^{-1}$	$(8.7 \pm 0.1) \times 10^{-2}$	5.2 ± 0.4
213 K	$(8.1 \pm 0.2) \times 10^{-1}$	$(1.2 \pm 0.1) \times 10^{-1}$	6.8 ± 0.6
223 K	1.5 ± 0.1	$(2.3 \pm 0.2) \times 10^{-1}$	6.5 ± 0.6
233 K	2.3 ± 0.2	$(3.7 \pm 0.5) \times 10^{-1}$	6.2 ± 1.0
243 K	3.9 ± 0.1	$(6.5 \pm 0.5) \times 10^{-1}$	6.0 ± 0.5

[a] $k_{H1} = k_H - k_{H0}$. [b] $k_{D1} = k_D - k_{H0}$.

When linoleic acid is replaced by [11,11-$^2H_2$]linoleic acid,[18] the k_D value becomes smaller than that of linoleic acid (S4). The KIE values of one active hydrogen of a pentadiene subunit are determined as 6.1 ± 3 from the k_H, k_D and k_{H0} values using the relation, $k_{H1}/k_{D1} = (k_H - k_{H0})/(k_D - k_{H0})$; see Table 2. The k_{H1}/k_{D1} value is significantly smaller than that observed for SLO (KIE = 81), exhibiting no significant tunneling effect in hydrogen transfer from linoleic acid to cumylperoxyl radical. In addition, the hydrogen transfer reactivity of oleic acid is only 5 times smaller than that of linoleic acid at 243 K (S4), whereas the SLO-catalyzed oxygenation rate of oleic acid is 10^5 times slower than that of linoleic acid.[19] On the other hand, the hydrogen transfer rate of arachidonic acid in the SLO-catalyzed oxygenation has been reported to be similar to that of linoleic acid.[20] No additivity rules hold in the hydrogen transfer reactivity of unsaturated fatty acids in the enzymatic reactions. Thus, there are significant differences with respect to the hydrogen-abstraction reactivity and the KIE of unsaturated fatty acids between the non-enzymatic and enzymatic systems.

In conclusion, we have successfully determined the absolute rates of hydrogen transfer from a series of unsaturated fatty acids to cumylperoxyl radical by the use of ESR at low temperatures. A simple additivity rule holds in the hydrogen transfer reactivity of unsaturated fatty acids, which is expressed by the additive contributions of the reactivity of active hydrogens from the 1,4-pentadiene subunit and those of allylic subunit.[19] Significant differences between the relative reactivities and the KIE values of unsaturated fatty acids in hydrogen transfer reactions with cumylperoxyl radical and those of lipoxygenases indicate that simple hydrogen transfer from unsaturated fatty acids to radical species is quite different from the tunneling hydrogen transfer (proton-coupled electron transfer) in lipoxygenases, where an electron and a proton may be transferred at the same time but separately to the Fe^{3+} site and the OH site, respectively.[21]

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