Vol. 161, No. 1, 1989 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 30, 1989 Pages 113-120

Thiyl Radical Attack on Polyunsaturated Fatty Acids: A Possible Route to Lipid Peroxidation

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Received March 15, 1989

Absolute rate constants have been measured for the reaction of cysteinyl free radicals, $CysS^\bullet$, with linoleic (18:2), linolenic (18:3) and arachidonic acid (20:4) in water/alcohol mixtures using the radiation chemical technique of pulse radiolysis. They are in the order of 10^6 - 10^7 M^{-1} s^{-1} and increase with the number of biallylic functions, and with the polarity of the solvent. The reaction is shown to be a hydrogen atom abstraction from biallylic C-H bonds and yields pentadienyl radicals. The thiol mediated repair of the latter is considerably slower. Thiyl free radicals must consequently be considered as a potential source of lipid peroxidation.

Membranes contain relatively large amounts of polyunsaturated fatty acids (PUFA's), which are vulnerable to oxidative attack. Reactive species such as carbon-centered or, in particular, oxygen-centered radicals are thus considered to be a cause of lipid peroxidation (1,2). There are, however, cellular defense mechanisms against the damage initiated by free radicals (3,4). An important chemical mechanism involves thiols. This class of compounds is known to scavenge free radicals, $X^\bullet$, in the general hydrogen transfer reaction (1) (in radiation biology referred to as "repair" reaction)

$$X^\bullet + RSH \longrightarrow RS^\bullet + X\text{-}H \qquad (1)$$

It is still widely accepted that the thiyl radicals, $RS^\bullet$, formed in this process disappear essentially via diffusion controlled combination to the respective disulfide, i.e.

$$RS^\bullet + RS^\bullet \longrightarrow RSSR \qquad (2)$$

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0006-291X/89 $1.50

Since the steady state $RS^{\bullet}$ concentrations are usually very low in any biological sample the actual rate of this reaction will be extremely slow in most cases, however. Other possible reactions of $RS^{\bullet}$ must, therefore, be taken into consideration in order to assess the entire chemical role of RSH and $RS^{\bullet}$. Peroxidase-catalysed co-oxidation studies have shown, for example, that glutathione enhances oxigenation of arachidonic acid under formation of ESR-detectable glutathionyl radicals in certain cases (**5**). Chemically, thiyl radicals have been identified as reasonably good oxidants (**6,7**), and they are frequently involved in reversible addition reactions, e.g. with molecular oxygen ($RS^{\bullet} + O_2 \rightleftharpoons RSOO^{\bullet}$) (**8,9**) or thiolate ($RS^{\bullet} + RS^{-} \rightleftharpoons RS\therefore SR^{-}$) (**10**). A most interesting process in this respect is H-atom abstraction by $RS^{\bullet}$ from activated C-H bonds, i.e. essentially the reverse of the repair reaction. Absolute rate constants have been determined for a number of such reactions (**11-13**), with $k(PenS^{\bullet} + 2\text{-propanol}) = 1.2 \times 10^4\ M^{-1}\ s^{-1}$ demonstrating the order of magnitude for abstraction from an α-hydroxy hydrogen (**13**).

In view of the low C-H bond energy in biallylic functions [82 kcal/mol (**2**)] which is, in fact, lower than that of an S-H bond in thiols [91±1.5 kcal/mol (**14**)], a corresponding H-atom abstraction by $RS^{\bullet}$ from PUFA´s can be anticipated. It is reasonable to assume that rate constants for this process are even higher than the above mentioned value for alcoholic substrates. In the present communication we provide a representative example for the occurance of the general reaction

$$RS^{\bullet} + PUFA \longrightarrow PUFA(-H)^{\bullet} + RSH \qquad (3)$$

and the measurement of absolute rate constants by the radiation chemical technique of pulse radiolysis.

METHODS

All chemicals used were of highest purity available from commercial sources and used without further purification; PUFA´s (linoleic acid, 18:2, linolenic acid, 18:3, and arachidonic acid, 20:4) were obtained from Sigma, ABTS [2,2´- azino-bis-(3-ethylbenzthiazoline sulfonic acid)] from Sigma , cysteine from Riedel de Haen and Sigma and glutathione, reduced form (GSH), ethanol, acetone and t-butanol from Merck (p.a.). The experiments were performed at room temperature. Solutions were prepared just before the experiment using deionized water (Millipore Q). Deaeration was achieved by bubbling with N_2 for ca 1h per dm^3 solution, followed by saturation with oxygen free N_2O. PUFA´s were dissolved in ethanol. This stock solution was gently saturated with argon and then added in required aliquots to the samples. Because of slight light sensitivity of ABTS a 360 nm filter was posed between the UV lamp and the pulse radiolytic cell for all experiments with this compound.

Pulse radiolysis experiments were carried out at with a van de Graaf accelerator supplying short pulses of ca. 300 ns duration of 1,55 MeV electrons. Doses per pulse were in the order of 2-3 Gy, corresponding to an average concentration of radicals of (1.0-1.7) x 10^{-6} M. Detection of the radical species was done by optical absorption. Further details on this technique, the dosimetry, and the evaluation of data have been described elsewhere (**15**).

RESULTS and DISCUSSION

Direct observation of pentadienyl radicals

Pulse irradiation of N_2O-saturated, pH 5.1, ethanol / water mixtures (1:1 v/v) containing 1 x 10^{-2} M cysteine (CysSH) leads to the formation of cysteinyl radicals, $CysS^\bullet$. Shortly after the pulse, e.g. at 4 μs, they are practically the only radical species present. Optically their generation is indicated through a weak and rather uncharacteristic absorption in the 260 - 340 nm range.

Addition of linolenic acid (18:3), e.g. at 1 x 10^{-3} M, yields a significantly different result. Upon pulse irradiation of such a solution a transient UV absorption is observed with a pronounced maximum at 280 nm, as can be seen in Fig. 1. Corresponding absorption bands are obtained if linolenic acid is replaced by linoleic (18:2) or arachidonic acid (20:4).

The spectrum with λ_{max} = 280 nm matches those of transients generated in the PUFA oxidation by other radicals, namely, $^\bullet OH$ (**16**), $(CH_3)_3CO^\bullet$ (**17**), or $SO_3^{\bullet -}$ (**18**), and

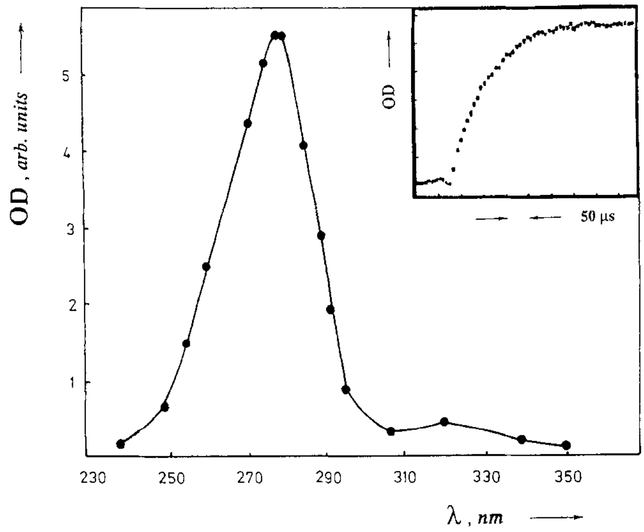
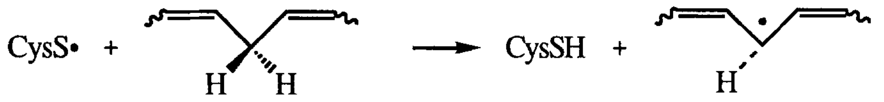


Fig. 1 Optical absorption spectrum of pentadienyl type radical obtained in $CysS^\bullet$ + linolenic acid reaction during pulse radiolysis of N_2O sat., pH 5.1, water / ethanol (1:1 v/v) mixtures containing 10^{-2} M cysteine (CysSH) and 10^{-3} M linolenic acid (18:3). *Insert*: Trace of absorption (at 280 nm) *vs.* time. Pulse length ca. 1μs. (OD: optical density)

assigned to pentadienyl type radicals. It is thus concluded that cysteinyl radicals likewise are able to oxidize PUFA's via



(4)

Mechanistically this process is considered to be a H-atom abstraction from the biallylic -CH_2- groups. This is corroborated by the fact that no 280 nm absorption, i.e. no pentadienyl radical is formed in the reaction of $CysS^•$ with oleic acid, a substrate which contains only one double bond and no biallylic hydrogen.

Since thiyl radicals are known to establish an equilibrium with alcohol radicals (13)

$$RS^• + \rangle CHOH \rightleftharpoons RSH + \rangle C^•OH \qquad (5)$$

a separate experiment has been conducted with solutions containing no cysteine. In this case no pentadienyl radical is formed which shows that any reaction of the alcohol radical with PUFA does not proceed via H-atom abstraction.

The kinetics of the pentadienyl formation follow an exponential rate law with the half-lives depending on the PUFA concentration (insert of Fig.1). Fig. 2 shows plots of the observed first order rate constants $k_{obs} = \ln 2 / t_{1/2}$ *vs.* [PUFA] for linoleic, linolenic, and arachidonic acid. From the slopes of the straight lines bimolecular rate constants k_4 were calculated. They are listed in Table 1. It is noted that k_4 increases with increasing number of biallylic C-H bonds and generally is found to be in the order of 10^6 - 10^7 M^{-1} s^{-1}.

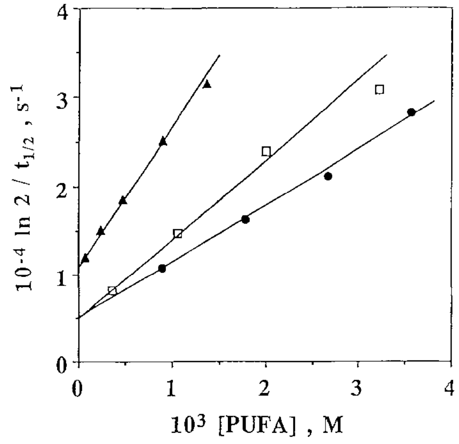


Fig.2 Plots of $k_{obs} = \ln 2 / t_{1/2}$ for the formation of pentadienyl radicals *vs.* [PUFA] in ethanolic solutions containing linoleic (18:2) (●), linolenic (18:3) (□), and arachidonic acid (20:4) (▲).

Table 1. Rate constants

co - solvent to water (1:1 v/v)	**PUFA**	**rate constant, $M^{-1}s^{-1}$**		
		direct	*competition*	
			uncorrected	corrected a)
ethanol	oleic	no reaction	$\leq 10^5$ (no reaction)	
ethanol	linoleic	5.8×10^6	3.5×10^6	4.5×10^6
ethanol	linolenic	8.6×10^6	7.4×10^6	9.1×10^6
ethanol	arachidonic	1.6×10^7	1.0×10^7	1.3×10^7
tert-butanol	oleic	no reaction	$\leq 10^5$ (no reaction)	
tert-butanol	linoleic	1.3×10^6	0.7×10^6	0.8×10^6
tert-butanol	linolenic	2.5×10^6	1.5×10^6	2.1×10^6
tert-butanol	arachidonic	4.8×10^6	d.n.p. in this system	
tert-butanol /	linoleic	d.n.p.	1.6×10^6	2.1×10^6
acetone	linolenic	"	4.3×10^6	5.6×10^6
	arachidonic	"	5.3×10^6	8.5×10^6

a) Corrections have been made for contributions by reaction (2) which is considered to be responsible for the difference between the total $CysS^{\bullet}$ yield (100%) and the maximum $ABTS^{+\bullet}$ yield (80%, 60%, and 60% in solutions with ethanol, t-butanol, and t-butanol/acetone as co-solvents, resp.). A corresponding treatment of experimental data for $RS^{\bullet}$ + alcohol reactions has demonstrated the validity of this procedure (ref. 13). (d.n.p.: determ. experimentally not possible)

For unambiguous determination of k_4 the experimental conditions were chosen to ensure that equilibrium (5) was on the left-hand-side $\{k_5 = 3.3 \times 10^3\ M^{-1}\ s^{-1}, k_{-5} \approx 1 \times 10^8\ M^{-1}\ s^{-1}$ (**19**)$\}$, and reaction (4) was the rate determining step for the pentadienyl radical formation. Variation of the cysteine concentration, accordingly, had practically no effect on the measured rates. Combination of thiyl radicals (reaction 2) which under pulse radiolysis conditions competes with reaction (4) has been taken into account in the kinetic analysis.

The half-life of the pentadienyl radical derived from linolenic acid was ca 500 µs. It did not change significantly with cysteine concentration indicating an upper limit for the rate constant $k_{-4} \leq 10^5\ M^{-1}\ s^{-1}$. The back reaction of equ. (4), i.e. the "classical repair" reaction thus occurs considerably slower than the thiyl radical induced "damage" process. The same is true for the linoleic and arachidonic acid systems.

Competition experiments

The rate constants k_4 obtained from the direct measurements have been confirmed with competition experiments which evaluate the influence of the electron transfer reaction

$$\mathrm{CysS^{\bullet}} + \mathrm{ABTS} \longrightarrow \mathrm{CysS^{-}} + \mathrm{ABTS^{+\bullet}} \qquad (6)$$

on reaction (4). The measurable yield of the strongly absorbing $\mathrm{ABTS^{+\bullet}}$ radical cation $\{\lambda_{max} = 415$ nm, $\varepsilon = 3.6 \times 10^4\ M^{-1}cm^{-1}$ (**20**)$\}$ in the absence and presence of PUFA (A_0 and A, respectively) is expressed by standard competition kinetics as

$$A_0 / A = 1 + k_4\,[\mathrm{PUFA}] / k_6\,[\mathrm{ABTS}]$$

A corresponding plot of A_0/A *vs.*[PUFA]/[ABTS] yields straight lines for all three PUFA's investigated as shown in Fig. 3. The rate constants k_4 evaluated from the slopes and using our measured k_6 (e.g. $8.3 \times 10^7\ M^{-1}s^{-1}$ in ethanol) are listed in Table 1 for comparison.

Direct observation of the pentadienyl radical accounts only for H-atom abstraction from biallylic C-H bonds while k_4 derived from the competition experiments would also include any additional pathway such as hydrogen abstraction from other sites or addition to double bonds. The fact that both methods yield practically identical rate constants suggests that reaction (4) may indeed be the main pathway in the reaction of $\mathrm{CysS^{\bullet}}$ with the PUFA's.

Solvent dependence

It must be noted that k_4 depends slightly on the polarity of the solvent. Table 1 contains the respective rate constants for water/ethanol (1:1 v/v), water/t-butanol (1:1 v/v), and water/acetone/butanol (5:3:2 v/v) solutions. Such phenomena have been observed for some H-transfer reactions and could reasonably be associated with polarity effects in the transition state. The variation of k_4 stays, however, clearly within one order of magnitude.

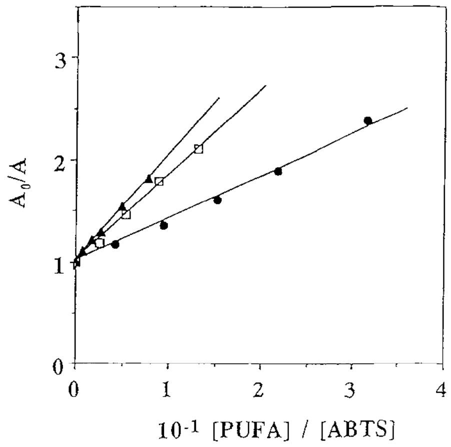


Fig.3 Plots of A_0/A *vs.* [PUFA] / [ABTS] according to competition kinetics for ethanolic solutions containing linoleic (18:2) (●), linolenic (18:3) (□), and arachidonic acid (20:4) (▲).

CONCLUSION

Thiyl free radicals are able to abstract biallylic hydrogen from PUFA in a relatively fast reaction to yield pentadienyl radicals. Thiol mediated repair of the latter is considerably slower. Thiyl free radicals must consequently be considered as a potential source of lipid peroxidation. In particular, this may shed some new light on a number of observations such as incomplete protection by thiols against radical induced damage, or on reports that the presence of thiols seemingly even enhances lipid destruction in certain cases (5,21,22).

ACKNOWLEDGMENT

Partial support of this work by the "Deutsche Forschungsgemeinschaft" (DFG) is gratefully acknowledged.

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