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The Formation of Compound I of Lactoperoxidase and Horseradish Peroxidase. A Comparison.

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The rate constants for the formation of compound I of lactoperoxidase and two isoenzymes of horseradish peroxidase, and various hydroperoxides have been determined. Multivariate data analysis by the partial least square models in latent variables has been used for simultaneous comparison of rate constants and hydroperoxide substituent properties. Activation energies have also been determined for compound I formation with H_2O_2 and isopropyl hydroperoxide and the three peroxidases.

These results taken together show that the rate of formation of compound I from horseradish peroxidase C2 is dominated by pK_a. There is a less pronounced effect of pK_a on horseradish peroxidase A2:2 and lactoperoxidase, where the contributions of lipophilicity and steric hindrance are stronger.

Peroxidases are known to combine with hydroperoxides at very high rates to form "compound I", two oxidizing equivalents above the native enzyme.[1,2] This intermediate is capable of oxidizing various organic and inorganic compounds.[1-3] The two-substrate mechanism according to Keilin-Theorell-Chance-George is generally written

$$\text{enzyme} + \text{ROOH} \xrightarrow{k_{1,\text{app}}} \text{compound I} + \text{ROH} \quad (1)$$

$$\text{compound I} + \text{hydrogen donor} \xrightarrow{k_{2,\text{app}}} \text{compound II} + \text{products} \quad (2)$$

$$\text{compound II} + \text{hydrogen donor} \xrightarrow{k_{3,\text{app}}} \text{enzyme} + \text{products} \quad (3)$$

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The rate limiting function in the formation of compound I varies with the properties of substituent in ROOH. Steric effects, lipophilicity, and effects of pK_a can modulate $k_{1,\text{app}}$ by about 10 orders of magnitude.[4] The reaction with *m*-chloroperoxybenzoic acid is diffusion controlled, contrary to the reactions with H_2O_2.[5] The present study compares the rates of compound I formation from mammalian lactoperoxidase and two isoenzymes, p*I* 3.9 and 8.8, from horseradish root with various hydroperoxides, with the purpose of identifying the rate limiting properties of the substituent and comparing differences in accessibility of hydroperoxides to the enzymes.

MATERIAL AND METHODS

Abbreviations. LP, Lactoperoxidase; HRP, horseradish peroxidase (E.C.1.11.1.7); DCD, dicarboxidine γ,γ'-(4,4'-diamino-3,3'-bifenylylenedioxy)-dibutyric acid; *ES*, Taft's parameter for steric effects: π, Hansch's parameter for lipophilicity; *MR*, molecular refractivity; PLS, multivariate analysis of partial least squares models in latent variables.

LP, $A_{412}/A_{280} \geqq 0.93$, was prepared from cow's milk,[6] the final DEAE-chromatography step excluded. HRP C (pI 8.8), $A_{403}/A_{280} \geqq 3.4$, and HRP A (pI 3.9) were prepared as described.[7] HRP A was further purified by repeated chromatography on DEAE-Sephadex®, 20 mM sodium acetate pH 5.85. The main component of HRP A2 that emerged from the column was used, HRP A2:2 with $A_{403}/A_{280} \geqq 4.20$.[8]

Doubly distilled water was refluxed over $KMnO_4$ for 30 min and distilled. Chemicals for buffers, *etc.* were of analytical grade. Experiments were performed in 100 mM sodium phosphate pH 7.00, 25±0.2 °C, unless otherwise stated. Spectrophotometers were equipped with thermostatted cuvette holders and routinely checked for absorbance, linearity, wavelength accuracy, and time scale. pH was measured at 25±0.1 °C with a Radiometer PHM64 and a GK 2303 B electrode. The British standard for primary buffers was used as reference buffers for calibration which gives an accuracy of ±0.01 pH units.[9]

Hydroperoxides were either commercially available (H_2O_2, "Perhydrol 30 %", Merck, Darmstadt; ethyl hydroperoxide, Ferrosan, Malmö; *t*-butyl hydroperoxide, Merck-Schuchardt, München) or else synthesized[10,11] and purified by partition between alkaline buffer (pH>pK_a) and ether/hexane.[11]

Ti(IV) in acid solution reacts instantaneously with H_2O_2 to give a yellow colour (ε_{407}=758 $M^{-1}cm^{-1}$ for this batch of Ti(IV)) whereas alkyl hydroperoxides react very slowly.[12] In *t*-butyl and α-cumenyl hydroperoxide, contaminating H_2O_2 and short chain hydroperoxides were determined from the initial fast phase(s) in the enzymatic method (*c.f.* below) for $k_{1,app}$ determination. The H_2O_2 concentration was kept ≦0.5 % of the total hydroperoxide (H_2O_2+ROOH) concentration by partitioning or by adding 10 nM beef liver catalase. Pretreatment of *t*-butyl and α-cumenyl hydroperoxide with 10 nM catalase in combination with 5 mM ethanol eliminated most of the fast phase indicating short chain hydroperoxide.[13]

Total hydroperoxide (H_2O_2+ROOH) concentration was determined enzymatically using HRP C and dicarboxidine as hydrogen donor.[14] In 50 mM phosphate buffer pH 6.9, a stable brown product is formed with ε_{440}=12.2 $mM^{-1}cm^{-1}$ as determined on the basis of added H_2O_2. *t*-Butyl hydroperoxide was assayed spectrophotometrically from ε_{250}=13 $M^{-1}cm^{-1}$ [15] and the concentration of α-cumenyl hydroperoxide was determined iodimetrically.

pK_a-values of the hydroperoxides were determined optically by using the strong increase[16] in absorbance upon deprotonation. A_{ROOH} was measured in 10 mM sodium borate pH 9.18[9] and A_{ROO^-} in 0.75 M NaOH. Partial dissociation of the hydroperoxides was brought about by titration with potassium hydroxide. A Radiometer type B glass electrode (Radiometer Copenhagen) standardized against the borate buffer was used for pH determination. H_2O_2 with pK_a=11.60 was consistently used as reference.

$k_{1,app}$ was determined by two procedures. (i) The formation of compound I from the peroxidase (0.5–0.7 μM) and a 10-fold excess of hydroperoxide. Absorbance changes were followed at wavelengths where compounds I and II have isosbestic points; LP 408 nm,[17] HRP A2:2 and HRP C2 397 nm,[18] either in a Durrum-Gibson stopped-flow instrument or in a Beckman Acta III spectrophotometer for the slower hydroperoxides. From the absorption change a pseudo first-order rate constant was calculated. (ii) The direct procedure is inappropriate for very slowly reacting hydroperoxides because of the reduction of compound I by HRP Fe(III).[19] Dicarboxidine gives very stable coloured products and shows k_3-values of 6.3×10^5 $M^{-1}s^{-1}$, 2.6×10^6 $M^{-1}s^{-1}$ and 6.3×10^6 $M^{-1}s^{-1}$ for HRP A2, HRP C2 and LP, respectively.

The expression[20]

$$\frac{dx}{dt} = \frac{[\text{Peroxidase}]}{1/k_3[\text{Dicarboxidine}]+1/k_{1,app}[\text{ROOH}]}$$

can be simplified to

$dx/dt = k_{1,app}[\text{ROOH}][\text{PO}]$ if $k_3 >> k_{1,app}$.

This is most likely the case with *t*-butyl and α-cumenyl hydroperoxides. dx/dt is the rate of increase in A_{440} which is proportional to the rate of consumption of hydroperoxide.

For the data analysis the SIMCA-3B microcomputer package was used.

RESULTS

The apparent second order rate constants for the formation of compound I of LP, HRP C2 and HRP A2:2, and various hydroperoxides are collected in Table 1. Contrary to the fast reacting hydroperoxides, the rate of reaction with *t*-butyl and α-cumenyl hydroperoxide had to be determined indirectly (Ref. 4 and Material and

Table 1. Rate constants for the formation of compound I from lactoperoxidase (LP), horseradish peroxidase (HRP) C2 and HRP A2:2 and some hydroperoxides, ROOH. Sodium phosphate (100 mM) pH 7.0. 25 °C. n=5.

R in ROOH	$k_{1,app}/M^{-1}s^{-1}$ LP	HRP C2	HRP A2:2
Hydrogen	$1.8(\pm0.04)\cdot10^7$	$1.5(\pm0.1)\cdot10^7$	$2.0(\pm0.05)\cdot10^6$
Methyl	$1.1(\pm0.05)\cdot10^7$	$1.3(\pm0.1)\cdot10^6$	$1.6(\pm0.05)\cdot10^5$
Ethyl	$1.5(\pm0.04)\cdot10^6$	$3.3(\pm0.1)\cdot10^6$	$5.7(\pm0.1)\cdot10^4$
Propyl	$1.4(\pm0.06)\cdot10^5$	$4.9(\pm0.2)\cdot10^6$	$4.6(\pm0.1)\cdot10^4$
Butyl	$5.7(\pm0.3)\cdot10^4$	$4.2(\pm0.3)\cdot10^6$	$7.5(\pm0.6)\cdot10^4$
Isopropyl	$2.5(\pm0.1)\cdot10^2$	$0.8(\pm0.03)\cdot10^5$	$4.8(\pm0.1)\cdot10^2$
t-Butyl	$6.3(\pm1.8)\cdot10^{-3}$	$7.4(\pm3)\cdot10^{-2}$	$5.9(\pm0.01)\cdot10^{-1}$
α-Cumenyl	$1.7(\pm0.4)\cdot10^{-1}$	$2.8(\pm0.6)\cdot10^{-1}$	$4.0(\pm1.4)\cdot10^{-1}$
p-Nitroperoxybenzoic acid[a]	$7.5(\pm0.4)\cdot10^6$	$4.5(\pm0.4)\cdot10^7$	$5.9(\pm1.4)\cdot10^6$

[a] pK_a 7.3, $k_{1,app}$ measured in 100 mM sodium acetate pH 4.50.

Table 2. The influence of an inert hydrogen donor analogue (see text) on the rate constants for the formation of compound I from lactoperoxidase (LP), horseradish peroxidase (HRP) C2 and A2:2 and two hydroperoxides. 25 °C. n=5.

Peroxidase	$k_{1,app}/M^{-1}s^{-1}$ H_2O_2 Sodium phosphate (100 mM) pH 7.00	*p*-Nitroperoxybenzoic acid Sodium acetate (100 mM) pH 4.50
LP	$1.8(\pm0.04)\cdot10^7$	$7.5(\pm0.4)\cdot10^6$
LP+donor	$1.6(\pm0.16)\cdot10^7$	$6.6(\pm0.3)\cdot10^6$
A2:2	$2.0(\pm0.05)\cdot10^6$	$5.9(\pm0.01)\cdot10^5$
A2:2+donor	$1.8(\pm0.07)\cdot10^6$	$3.6(\pm0.09)\cdot10^5$
C2	$1.5(\pm0.05)\cdot10^7$	$4.5(\pm0.4)\cdot10^7$
C2+donor	$7.4(\pm0.38)\cdot10^6$	$1.5(\pm0.05)\cdot10^7$

Methods), because of the possibility of reduction of compound I by native enzyme.[19]

Hydrogen donors that reduce compound I bind to the native peroxidase (enzyme) and form an 1:1 complex, with K_d depending on donor and enzyme properties. To avoid reduction and product interaction an inert hydrogen donor analogue (Fig. 1), K_d~100 μM,[21] was used to study binding effects on compound I formation. Solubility and stopped-flow realities limited the inhibitor concentration to 110 μM in the reacting mixture. Thus, the enzyme is about half saturated. Table 2 shows that significant effects arose only when HRP A2:2 was oxidized by *p*-nitroperoxybenzoic acid and C2 by both hydroperoxides.

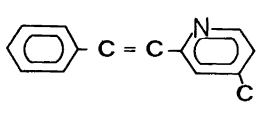

Fig. 1. The inert hydrogen donor analogue.

From the results in Table 1 we chose H_2O_2 and *i*-propyl hydroperoxide to study the effect of temperature on the formation of compound I of the three peroxidases. Because of spontaneous heme-off reactions in HRP A2:2 at temperatures above about 35 °C, compound I formation was measured in the range 5–35 °C. The temperature range for LP and HRP C2 was extended to 44 °C. The results are given as Arrhenius plots in Fig. 2. The three slopes differ significantly from each other for both H_2O_2 (p-values <0.001) and

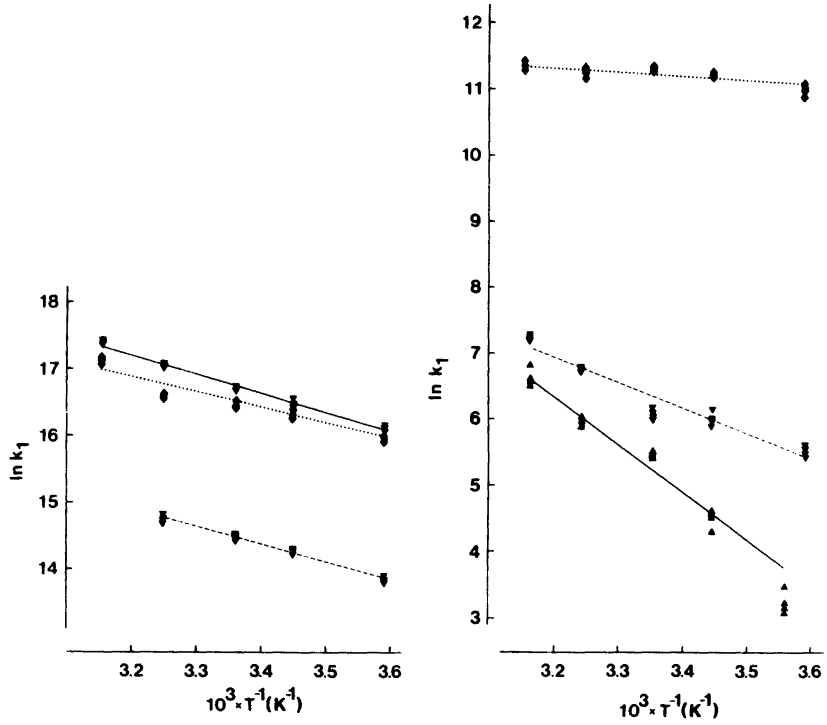

Fig. 2. The effect of temperature on the rate constant $k_{1,app}$ for the formation of compound I. Arrhenius' plot of ln k_1 *vs* T^{-1}. Left with H_2O_2, right with $(CH_3)_2CHOOH$. —— Lactoperoxidase, --- horseradish peroxidase A2:2, ····· horseradish peroxidase C2. Sodium phosphate (100 mM) pH 7.0.

i-propyl hydroperoxide (p-values <0.001). The activation energies were calculated from these slopes (Table 3).

DISCUSSION

From the collection of second order rate constants for the formation of compound I, some differences between the peroxidases can be discerned. HRP A2:2 in general reacts more slowly and is, like LP, markedly influenced by the hydroperoxide substituents. We[4] earlier correlated k_1 of A2 to pK_a of the hydroperoxides but we also found that steric effects contributed. RO–OH dissociation energies, diffusion coefficients for corresponding alcohols, and the small contribution of van der Waal's volume could be eliminated. With the present data we find that

Table 3. The effect of temperature on the rate constant for the formation of compound I from lactoperoxidase (LP), horseradish peroxidase (HRP) C2 and HRP A2:2 and two hydroperoxides. Activation energies are calculated after linear regression analysis of results in Figure 1. Sodium phosphate (100 mM) pH 7.0.

Peroxidase	E_A/kJ mol^{-1}	
	H_2O_2	$(CH_3)_2$ CH OOH
LP	23.3	59.6
HRP A2:2	21.5	31.2
HRP C2	18.4	4.6

Table 4. Some physicochemical properties of the substituent groups of the hydroperoxides. pK_a, Taft's parameter (*ES*) for steric effects,[22] Hansch's parameter (π) for lipophilicity,[23] and molecular refractivity (*MR*).[24]

R in ROOH	No	pK_a	*ES*	π	*MR*
Hydrogen	1	11.6[25]	0	0	1.03
Methyl	2	11.2[4]	−1.24	0.56	5.65
Ethyl	3	11.4[4]	−1.31	1.02	10.3
Propyl	4	11.2[4]	−1.6	1.55	14.96
Butyl	5	11.3[4]	−1.63	2.13	19.59
Isopropyl	6	11.7[4]	−1.71	1.53	14.98
t-Butyl	7	12.8[25]	−2.78	1.98	19.62
α-Cumenyl	8	12.6[26]	−3.0	3.3	39.3

pK_a, Taft's parameter (*ES*) for steric effects,[22] Hansch's parameter (π) for lipophilicity,[23] and molecular refractivity (*MR*)[24] (Table 4) are relevant parameters to correlate to $k_{1,app}$. Although related, these parameters describe different physicochemical properties of the peroxides. The attempts to correlate $k_{1,app}$ with these properties by linear regression gave no significant results, as might be expected since the properties are collinear.

To relate the two blocks of variables, *i.e.* block 1=$k_{1,app}$ (Table 1) for the formation of compound I from the three peroxidases (*y*-variables) and block 2=the selected conventional physicochemical parameters (*x*-variables) of the substituents on the hydroperoxides (Table 4), partial least squares models in latent variables (PLS)[27–30] were used. With multivariate *y*-data the model gives information about the relationships within the Y-matrix. Analogously, the relationships within the X-matrix are modelled as well as the relation between the Y-block and the X-block.

The Y-matrix (8×3) consists of $k_{1,app}$-values for the three peroxidases given in Table 1. The X-matrix (8×4) gives the substituent properties (pK_a, *ES* π and *MR*) of the hydroperoxides (Table 4). The variables were scaled to unit variance over the analyzed set of substances before the analysis.

In a PLS model with two significant dimensions the X-block explains 94 % of the Y-block. The model shows that pK_a has a strong influence on the Y-matrix. The plot in Fig. 3 shows that compounds (1–5) with small steric bulk are separated from those with large bulk (6–8). The bulky structures in *i*-propyl, *t*-butyl and *α*-cumenyl are represented by large *ES*-values.

The relations between the physicochemical properties (8×4) and $k_{1,app}$ (8×3) data matrices in the PLS model are given in Fig. 4. The rate

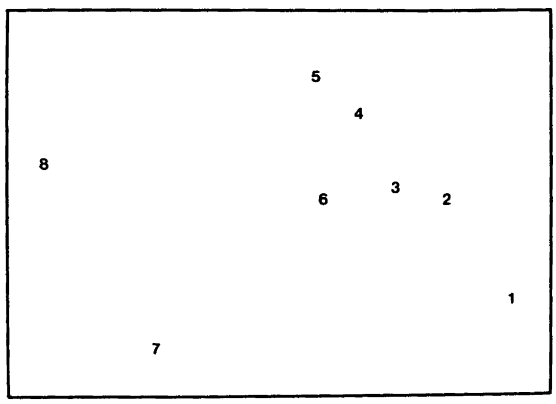

Fig. 3. PLS analysis of the physicochemical data matrix. The number code is given in Table 4.

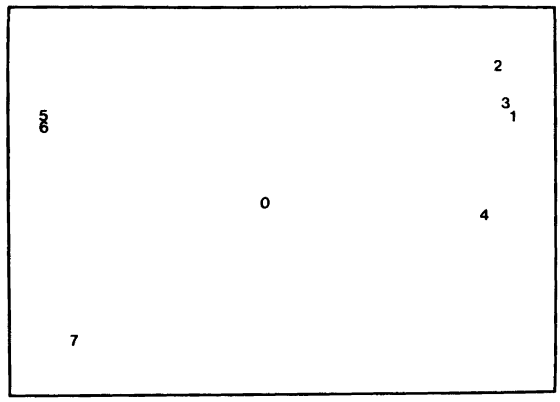

Fig. 4. PLS analysis of the relations between $k_{1,app}$ for the three peroxidases and physicochemical properties of the hydroperoxide substituent. 1 represents LP, 2 HRP C2, 3 HRP A2:2, 4 *Es*, 5 π, 6 *MR* and 7 pK_a.

constant for formation of compound I of HRP C2 (*y*-variable 2) is mainly dependent on pK_a with a minor influence of *ES*. The pK_a effect is less pronounced on $k_{1,app}$ for the formation of compound I of HRP A2:2 and LP (*y*-variables 3 and 1), where the contribution of lipophilicity and steric hindrance is stronger.

It has been shown by different techniques that native plant peroxidases form a 1:1 complex with hydrogen donors.[31] It has not yet been proven whether this complex is involved in the catalytic mechanism or not. The use of the hydrogen donor analogue[21] described in Fig. 1 is unfortunately restricted by the low solubility, which brought about a maximum of 50 % saturation. Only HRP C2 shows a significant effect of bound donor.

The rate of compound I formation with *p*-nitroperoxybenzoic acid, as compared with the likewise bulky α-cumenyl hydroperoxide, is surprisingly high. For HRP C2 it is even higher than that with H_2O_2. In HRP A2:2 and LP, $k_{1,app}$ is reduced to half of that of H_2O_2. The rate of formation of compound I with *m*-chloroperoxybenzoic acid, almost equal in size, is diffusion controlled.[5] The strong influence of pK_a points to an exceedingly higher $k_{1,app}$, had diffusion not been controlled, when pK_a is lowered from 12.6, as for α-cumenyl hydroperoxide, to 7.3 for *p*-nitroperoxybenzoic acid. Steric and/or lipophilic effects can explain the lower rate with LP and HRP A2:2 when compound I is formed with *p*-nitroperoxybenzoic acid compared with H_2O_2.

The mechanisms of formation of compound I from peroxidase and a hydroperoxide as proposed by Poulos and Kraut[32] and Jones and Dunford[2] require a minimum of two bond ruptures and the formation of two new bonds after that the involved molecules have diffused together. In agreement with Hewson and Dunford,[33] we found activation energies for this reaction with H_2O_2 at the same or at a slightly higher level than for the fluidity of water, 16.3 kJ mol^{-1}, which is the theoretical minimum activation energy for reactions in aqueous solutions.[33] Although significant, the differences between the three peroxidases are surprisingly low. With the bulky and hydrophobic isopropyl hydroperoxide with its higher pK_a a drastic difference in activation energy was obtained. The value 31.2. kJ mol^{-1} for HRP A2:2 and *i*-propyl hydroperoxide as compared to 21.5 kJ mol^{-1} with H_2O_2 indicates a more narrow entrance for the hydroperoxides. In LP the higher activation energy, 59.6 kJ mol^{-1}, may reflect not only narrowness but in addition lipophilic effects which support the PLS analysis.

In summary, it can be deduced from these results that the heme accomodating pocket of LP is more narrow than the corresponding pockets of HRP C and HRP A2:2. There is also an element of lipophilicity that affects the formation of compound I from LP. Optical and CD data also point to a narrow pocket,[34] which should prevent the heme group from being exposed to the solvent and result in a higher redox potential.[35]

$E_{m,7}$ for the peroxidases are −263,[31] −212,[31] and −190 mV[36] for HRP C2, HRP A2:2, and LP, respectively, which fits the increasing narrowness and/or lipophilicity.

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