Mechanism of peroxidase-catalyzed oxidation. Substrate-substrate activation in horseradish peroxidase-catalyzed reactions

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The structure and functions of peroxidases are characterized. Attention is focused on the mechanisms of action of horseradish peroxidase in reactions with different substrates and on correlations between structure and functions of various heme-containing proteins. The phenomenon of substrate-substrate activation typical of peroxidase-catalyzed reactions is discussed.

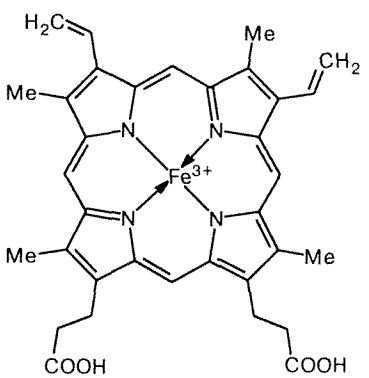
Key words: horseradish peroxidase, reaction mechanisms; substrate-substrate activation.

Heme-containing proteins and enzymes have a broad spectrum of physiological activity and take part in many processes that occur in animal and plant organisms. They can reversibly bind oxygen for transport (hemoglobin) or accumulate oxygen in a bound form (myoglobin), carry out respiratory processes (cytochromes, cytochrome oxidase), remove H_2O_2 from blood (catalase), and hydroxylate and oxidize various toxins and xenobiotics (cytochrome P-450, peroxidases).[1—3] Peroxidases also take part in lignification, photosynthesis, and regulation of plant growth.[4]

Horseradish peroxidase (EC 1.11.1.7) is the most accessible, best studied, and most stable protein among proteins of this class. A great body of information concerning the kinetics and mechanism of action of this enzyme in reactions with different substrates, relation between its structure and functions, comparative studies of peroxidases from different sources, as well as the use of peroxidase for applied purposes as a main component of various analytical reagents and systems for the detection of physiologically active substances has been accumulated by now.[5—7] At the same time, the reasons for the broad substrate specificity of peroxidase are still unclear. The phenomenon of substrate-substrate activation, typical of the reactions catalyzed by this enzyme, has also not been reviewed. The present review examines the mechanism of action of horseradish peroxidase in reactions with different substrates and the kinetics and mechanism of activation upon peroxidase-catalyzed co-oxidation of reducing substrates.

Structure of horseradish peroxidase

Horseradish peroxidase is a redox enzyme containing protoheme, a complex of trivalent iron with 1,3,5,8-tetramethyl-2,4-divinylporphin-6,7-dipropionic acid (protoporphyrin IX) as a prosthetic group.



Protoheme is non-covalently bound to the protein, apoperoxidase, by hydrophobic bonds and a salt bridge between the propionic acid residue of the heme and one of the amino groups of the protein.[5—8] Disturbance of these bonds results in a significant decrease in catalytic activity of peroxidase. Four coordination sites of heme Fe are involved in binding the N atoms of the pyrrole moieties of the porphyrin macrocycle. According to NMR spectroscopy data, the imidazole group of the histidine residue of the protein is the fifth axial ligand. The nature of the ligand in the sixth coordination position has not been established. Some authors[9] propose that Fe^{III} is pentacoordinated.

Using isoelectric focusing, about 40 isoenzymes of horseradish peroxidase have been isolated and characterized. The main isoenzyme is isoenzyme *C* whose primary structure has been determined earlier.[10] The molecular mass of its polypeptide chain is 33900 (308

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amino acid residues). Note that the molecular mass of the native enzyme is near 44000 due to the presence of eight oligosaccharide chains bound covalently to the protein and containing glucosamine, mannose, xylose, and fucose. The carbohydrate chains protect the enzyme from inactivation by radicals formed in the course of the reaction and increase the thermal stability of peroxidase. The deglycosylated enzyme whose catalytic activity is equal to that of the native enzyme is inactivated more rapidly during the reaction.[11,12] Peroxidase contains four disulfide bonds, has no free SH groups and acts as a monomer with one prosthetic group and two calcium ions.[13] The latter almost do not affect the activity of the enzyme, but they are among the factors that provide its high thermal stability.[13] The protein globule of peroxidase has an ellipsoid form.[14]

Based on literature data on the structure of horseradish peroxidase and the similarity of its properties with those of cytochrome *c* peroxidase, whose X-ray structural analysis has been carried out, a computer model of isoenzyme *C* of horseradish peroxidase has been proposed.[15,16] According to this model, the enzyme is composed of two domains (large and small), the heme being located in a hydrophobic cavity between the domains.[17] The sequence coordinating the distal region of heme, *i.e.* the fragment of the amino acid chain which is located at the sixth coordination position of iron, Phe—His—Asp—Cys—Phe—Val, belongs to the large domain and corresponds to a region responsible for the acid-base catalysis in the peroxidase molecule. The direct participation of His42 in the catalysis of oxidation of organic substrates by horseradish peroxidase has been confirmed by independent experiments using peroxidase modified with a specific reagent for histidine.[18] The proximal region, *i.e.* the fragment of the amino acid chain located near the fifth coordination position of iron, Val—Ala—Leu—Ser—Gly—Gly—(Ala)—His—Thr, belongs to the small domain. Both chain fragments (distal and proximal) are the regions of maximum homology between the amino acid sequences of plant peroxidases.

The horseradish peroxidase heme is located deep inside the protein globule. The vinyl groups of heme are directed "inward" the protein, and the heme region with substituents at positions 5 and 8 resides closer to the protein surface. The propionic acid residues at positions 6 and 7 are also directed towards the protein surface and are slightly exposed to the aqueous solution. The data[19] on the modification of carboxyl groups of peroxidase indicate that only one of the COOH groups of heme is accessible to the modifying reagent, whereas the second one is masked from the solvent. At pH values close to neutral, hemin is inaccessible even for small ions present in a solution. The dimension of the heme-binding region is different in molecules of different heme-containing proteins and is maximal for cytochrome *c* peroxidase (14 Å) and minimal for horseradish peroxidase (9 Å). The absorption spectrum of peroxidase contains a typical maximum in the Soret band (λ = 403 nm, ε_{403} 102000 L mol^{-1} cm^{-1}). The purity index, $RZ = A_{403}/A_{280}$, is 3.55 for homogeneous peroxidase preparations (see Ref. 2).

Mechanisms of peroxidase-catalyzed oxidation

The mechanism of peroxidase action was postulated by Chance back in 1949.[20] This mechanism implied the formation of enzyme intermediates, E_1 and E_2 (Scheme 1), which were previously[21] recorded by Theorell using spectral methods.

Scheme 1

$$E + H_2O_2 \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E_1 + H_2O, \quad (1)$$

$$E_1 + DH_2 \xrightarrow{k_2} E_2 + DH^{\cdot}, \quad (2)$$

$$E_2 + DH_2 \xrightarrow{k_3} E + DH, \quad (3)$$

$$2\,DH^{\cdot} \xrightarrow{k_4} P, \quad (4)$$

where E is the native enzyme; E_1 and E_2 are enzyme intermediates; DH_2 is a reducing substrate; $DH^{\cdot}$ is a semi-oxidized substrate (free radical). In the opinion of Chance, the third step limits the overall reaction rate.

The majority of the reactions of peroxidase-catalyzed oxidation involve interaction of enzyme with an oxidant (reaction (1)) such as H_2O_2, alkyl hydroperoxides, peroxybenzoic acids, as well as OCl^-, OBr^-, ClO_2^-, BrO_3^-, IO_4^-, *m*-nitrobenzoic acid, *etc.* These reagents may also form an intermediate of E_1 type with other related heme-containing enzymes (myeloperoxidase, haloperoxidase, lactoperoxidase, catalase).[22] Sometimes, however, the compound of E_1 type is not recorded, as for example, upon the action of HNO_3 on myeloperoxidase. In this case, the E_2 intermediate may be formed directly (see Ref. 22).

The reaction of peroxidase with oxidants was studied in detail by a stopped-flow method.[23] This second-order reaction is practically irreversible. The k_1 constants lie within the interval from 10^8 to 10^6 L mol^{-1} s^{-1}, depending on the structure of the oxidant. At the H_2O_2 concentration >1 mmol L^{-1}, a low-active peroxidase derivative E_3 is formed. This process inhibits the peroxidase-catalyzed oxidation of reducing substrates.[24] Compound E_1 is a direct oxidant of a wide range of organic and inorganic reductants, substrates of peroxidase, whose oxidation proceeds, as a rule, according to a one-electron mechanism with the formation of the E_2 intermediate. Only two substrates are known, iodide and hydrosulfite ions, for which intermediates of E_2 type have not been detected during oxidation.[2]

Compounds E_1 and E_2 are the oxidized forms of peroxidase. Both $E_1 \rightarrow E_2$ and $E_2 \rightarrow E$ transitions are one-electron processes. The E_1 intermediate has two oxidative equivalents with respect to the enzyme, and E_2 has one equivalent. It was shown by NMR spectroscopy[25] that iron is present in the ferryl form, *i.e.* has a formal charge of 4+ both in E_1 and E_2 intermediates. The additional oxidative equivalent in the E_1 molecule is localized either on the porphyrin macrocycle or on one of the functional groups of the protein.[26] The properties of compounds E_1 and E_2 for all heme-containing proteins studied so far do not depend on the nature of oxidizing and reducing substrates. This is a significant evidence that these compounds are not enzyme—substrate complexes.[27] The absorption spectra of intermediates E_1 and E_2 differ considerably, which allows one to use spectral methods of rapid kinetics detection for measuring the rate constants of separate steps of the peroxidase-catalyzed oxidation of different substrates. It was found that $k_2 \gg k_3$ for all the substrates studied (see Scheme 1), just as Chance has postulated. A comparative study of peroxidase-catalyzed oxidation of potassium ferrocyanide by stopped-flow and steady-state kinetic methods confirmed the Chance's suggestion on the nature of the limiting step of the process (see reaction (3) in Scheme 1).[28] The so-called catalase-like mechanism, where H_2O_2 acts simultaneously as a donor and an acceptor of electrons, is observed in some reactions of peroxidases (in particular, for myeloperoxidase, the peroxidase from *E. coli*). In this case, the E_1 intermediate converts into E_2 in the presence of H_2O_2 and in the absence of a reducing substrate.[29]

The Chance's scheme was undoubtedly an important step in understanding the mechanism of peroxidase-catalyzed oxidation. However, a great body of experimental material has been subsequently accumulated that suggested that the mechanism proposed[20] requires refinement. In particular, the Chance's scheme does not take into account complexation of peroxidase with substrates, and neither does it explain the broad substrate specificity of this enzyme.

Peroxidase substrates differ noticeably in structures, chemical properties, and molecular sizes. Their only common property is the ability to donate an electron. Numerous attempts to classify peroxidase substrates according to their reactivity,[2] character of interaction with the enzyme, or as proton donors and electron donors,[3] have been made. The classification according to the type of interaction with the enzyme is probably the most promising, since it is this property that determines, to a considerable extent, the mechanism of oxidation of a given substrate. One interesting approach is classifying the substrates into two types, the substrates interacting and not interacting with heme. In this case one may discriminate substrates that can directly contact with heme (ferrocyanide, sulfite, nitrite, and thiocyanate ions) and those for which direct contact is impossible, as determined by NMR spectroscopy (aromatic phenols, amines, and other organic compounds).[8] The substrates of the first group are electron donors and those of the second group are hydrogen atom donors.

The results obtained by NMR spectroscopy are confirmed by data of numerous kinetic studies.[30—33] No correlations between the kinetic constants of peroxidase-catalyzed oxidation and hydrophobic and steric parameters of substrate structure are observed for phenols, amines, and phenothiazines. On the other hand, peroxidase-catalyzed oxidation of these substrates demonstrates the effect of substituents, *i.e.* an increase in electron-donating properties of a substituent increases the rate constant (the Hammett relation is fulfilled).[33] However, the Okamoto—Brown relations are not fulfilled, which indicates that the proton transfer occurs simultaneously with the electron transfer. The activation of peroxidase-catalyzed oxidation of hydrogen donors (*o*-dianisidine, *p*-phenylenediamine, and luminol) with nucleophiles also points to the existence of a step of proton transfer.[7,34—39] Nucleophiles do not act as activators in reactions of peroxidase oxidation of electron donors.[3,36]

Thus, the mechanism of peroxidase-catalyzed oxidation of organic compounds can be explained by assuming that the electron transfer, accompanied by the proton transfer, occurs with the participation of functional groups of the protein. The following literature data confirm this assumption. The modification of peroxidase with diethyl pyrocarbonate, a specific reagent for histidine, results in enzyme inactivation. It was shown that in this case the inactivation was due to modification of only one of the two histidine residues capable of modification. The pH-dependence of k_{in} revealed a group with pK_a 6.2, which is close to pK_a of histidine, and this group was proposed to be His42. Upon the action of oxidants, the modified peroxidase transforms into compound E_1, but the $E_1 \rightarrow E_2$ transition does not occur in the presence of guaiacol and *p*-cresol, the organic substrates of peroxidase. Independent experiments showed that the modified amino acid residue does not take part in binding of these substrates. Thus, modification of His42 inhibits the electron transport from a substrate to E_1, hence, this residue directly participates in the oxidation process.[18]

Previously, we revealed an ionogenic group in the protein with pK_a 6.5 whose deprotonation decreases dramatically the rate of oxidation of an other organic substrate, *o*-dianisidine.[40] Similar results were obtained for phenothiazine derivatives. However, in this case it was proposed that a tyrosine residue takes part in the electron transport.[32] Modification of the three surface COOH groups of peroxidase does not affect the kinetic parameters of ferrocyanide oxidation but decreases tenfold the rate constant of the interaction of E_1 with *o*-dianisidine, which also evidences the direct participation of the peroxidase globule in oxidation of organic substrates.[19] Thus, peripheral mechanisms of electron transfer are realized in the course of peroxidase-cata-

lyzed oxidation of organic substrates, and in this case, the active site of the enzyme involves the functional groups of the protein.

The type of interaction of inorganic ions, which are peroxidase substrates (iodide, ferrocyanide, nitrite, thiocyanate), with the enzyme is still unclear. The completely different forms of the pH-dependences of the rate constants of individual steps, the absence of an effect of a nucleophile on the process of peroxidase-catalyzed oxidation, and the smaller size of the ions, all make it possible to propose the existence of a different mechanism of peroxidase-catalyzed oxidation. However, this does not mean that in the case of inorganic ions the enzyme—substrate interaction is of essentially different type. According to NMR spectroscopy data, peroxidase heme is not involved in the coordination of such ions as iodide and thiocyanate.[41] In addition, mutual inhibition is observed upon peroxidase oxidation of EDTA—iodide, EDTA—thiocyanate, and iodide—thiocyanate substrate pairs.[42—44] Binding of these substrates with the enzyme is controlled by a group of the protein with pK_a 5.4. Thus, the organic substrate (EDTA) interacts with the same peroxidase site as the inorganic substrates (iodide and thiocyanate). It is also known that the bisulfite ion inhibits simultaneously the peroxidase-catalyzed oxidation of both inorganic (iodide) and organic (pyrogallol) substrates.[45] Hence, direct contact with the heme is not obligatory in the peroxidase-catalyzed oxidation of inorganic substrates. In any case, the problem concerning the site for inorganic substrates in the peroxidase active center remains unsolved.

The reason for the broad substrate specificity of peroxidase can hardly be reduced to classification of substrates according to one or another principle. The currently available data on the mechanism of peroxidase catalysis in reactions with different substrates and on the structure of this enzyme are explained best of all within the framework of a concept regarding peroxidase as a protein conductor that provides several different channels for electron transport from substrates contacting with the protein surface to the heme iron. Some of these channels may also involve the porphyrin macrocycle.

Studies of the mechanisms of peroxidase-catalyzed oxidation of different phenols using peroxidase models, *i.e.*, different heme complexes, rather than peroxidase itself, as catalysts showed that the interaction and electron transport occur through heme periphery even in the case when the iron of the heme is accessible.[46] It is possible that peroxidase-catalyzed (one-electron) oxidation with the formation of free radicals occurs only in the case of peripheral mechanisms of electron transport. It is worth noting that the peroxidase-type electron transport is impossible for an other heme-containing enzyme, cytochrome P-450, in which the heme iron is more accessible for substrates than in peroxidase, but whose periphery is absolutely inaccessible.[47] Chloroperoxidase, which is extensively studied at present, possesses a peroxidase function and is capable of catalyzing epoxidation reactions typical of cytochrome P-450, and occupies an intermediate position in this respect.[48—51]

Substrate-substrate activation in reactions of peroxidase-catalyzed oxidation

In the peroxidase-catalyzed oxidation of two substrates, activation or inhibition of the reaction of one substrate by another is often observed. This fact is interesting by itself and is very important for practical purposes, since a slowly-oxidizable substrate, *e.g.*, luminol, often appears to be very convenient, because the product formed can be easily detected. Furthermore, there are compounds, in particular, 4-aminoantipyrine, that are not peroxidase substrates under normal conditions but can become peroxidase substrates only in the presence of rapidly oxidizable substrates. Co-oxidation of 4-aminoantipyrine and phenol affords a single product, antipyrylquinoneimine, which does not undergo further chemical transformations. This compound has a high extinction coefficient in the visible spectrum; therefore, it is extremely convenient for detection.[52] As for rapidly oxidizable substrates, phenols and amines, the products of their oxidation are unstable and difficult to identify.

Peroxidase-catalyzed co-oxidation of two substrates with close reactivities results in the formation of compounds with a mixed structure.[53] In this case, simultaneous oxidation of these substrates probably occurs, followed by cross interaction of intermediate free-radical products.

When two substrates, whose reactivities differ greatly, undergo peroxidase-catalyzed co-oxidation, mutual activation or inhibition is observed.[54—62] In this case, activation of a slowly oxidizable substrate and partial or total inhibition of the reaction of a rapidly oxidizable substrate (activator) take place. For example, peroxidase oxidation of 2,3-dichloro-1,4-naphthoquinol-1-dimethylphosphate does not begin until 50 % conversion of 2,3-dimethyl-1,4-naphthoquinol-1-dimethylphosphate is reached, provided that both substrates are present in the reaction mixture.[57] Ascorbic acid inhibits peroxidase-catalyzed oxidation of *p*-cresol and chloropromazine, and potassium ferrocyanide inhibits oxidation of *p*-cresol, 2,4-dichlorophenol, and hydroxamic acids.[55,60]

In the case of co-oxidation of luminol and substituted phenols or luciferin, only partial inhibition of peroxidase-catalyzed oxidation of substrates-activators and a manifold (by a factor of 10—50) activation of luminol oxidation are observed.[58,63] 4-Aminoantipyrine behaves similarly in the presence of substituted phenols.[59] Peroxidase-catalyzed co-oxidation of potassium ferrocyanide and *o*-dianisidine results in total inhibition of the oxidation of the latter, until practically 100 % of ferrocyanide is oxidized. In this case, a 10—20-fold activation of ferrocyanide oxidation is observed. Oxidation of *o*-dianisidine occurs only after total oxidation of ferrocyanide, and the rate of the former does not depend

Table 1. Substrate-substrate activation upon peroxidase-catalyzed co-oxidation of two substrates

Peroxidase substrates		Methods of detection[a]	Activation effect, k_a/k [b]	Reference
Slowly oxidizable	Rapidly oxidizable (activator)			
2,3-Dimethyl-1,4-naphthoquinol-1-dimethylphosphate	2,3-Dichloro-1,4-naphthoquinol-1-dimethylphosphate	EAS	5	57
Ascorbic acid	*p*-Cresol	ESR	16	60
Ascorbic acid	Chloropromazine	ESR	10	60
Ascorbic acid	Thyroxine	EAS	—	45
Adrenaline	Thyroxine	EAS	—	45
Uric acid	Thyroxine	EAS	—	45
Potassium ferrocyanide	*p*-Cresol	EAS	12	55
Potassium ferrocyanide	2,4-Dichlorophenol	EAS	15	55
Potassium ferrocyanide	Hydroxamic acids	EAS	5—10	60
Potassium ferrocyanide	*o*-Dianisidine	EAS	10	54
4-Aminoantipyrine	Phenol and its derivatives	EAS	—[c]	56
4-Dimethylaminoantipyrine	Phenol and its derivatives	EAS	—[c]	56
Luminol	Phenol and its derivatives	CL	10—15	58,63
Luminol	Luciferin	CL	20	58
NADH	Phenol	EAS	20—60	60
NADH	Hydroxamic acids	EAS	20—60	60

[a] EAS indicates electronic absorption spectra; CL is chemiluminescence. [b] Ratio of the rate constants for the oxidation of a "slow" substrate in the presence and in the absence of a substrate activator, respectively. [c] Oxidation does not occur in the peroxidase—H_2O_2 system in the absence of an activator.

on whether the ferrocyanide was initially present in the reaction mixture.[54,62] The degree of activation increases with an increase in the concentration of a substrate-activator for all of the substrate pairs studied. Table 1 presents the systems in which the substrate-substrate activation phenomenon has been observed.

Three different mechanisms have been proposed to explain the effects of substrate-substrate activation.

1. A difficultly oxidizable substrate is oxidized by radicals of an easily oxidizable substrate more effectively than by peroxidase intermediates E_1 and E_2, and radicals of an easily oxidizable substrate interact with those of a difficultly oxidizable substrate more effectively than with each other. This mechanism was proposed for the following systems: ascorbic acid—*p*-cresol,[55] 2,3-dimethyl-1,4-naphthoquinol-1-dimethylphosphate—2,3-dichloro-1,4-naphthoquinol-1-dimethylphosphate,[57] luminol—substituted phenols,[55] substituted antipyrin—phenol,[59] ferrocyanide—phenols, ferrocyanide—hydroxamates.[56] The kinetic scheme (Scheme 2) demonstrates the above mechanism.

Scheme 2

$$E + H_2O_2 \xrightarrow{k_1} E_1 , \quad (5)$$

$$E_1 + S_1 \xrightarrow{k_{21}} E_2 + S_1^{\cdot} , \quad (6)$$

$$E_1 + S_2 \xrightarrow{k_{22}} E_2 + S_2^{\cdot} , \quad (7)$$

$$E_2 + S_1 \xrightarrow{k_{31}} E + S_1^{\cdot} , \quad (8)$$

$$E_2 + S_2 \xrightarrow{k_{32}} E + S_2^{\cdot} , \quad (9)$$

$$2\,S_1^{\cdot} \xrightarrow{k_{41}} P_1 + S_1 , \quad (10)$$

$$S_1^{\cdot} + S_2^{\cdot} \xrightarrow{k_{41,2}} P_1 + S_2 , \quad (11)$$

$$2\,S_2^{\cdot} \xrightarrow{k_{42}} P_2 + S_2 , \quad (12)$$

$$S_1 + S_2^{\cdot} \xrightarrow{k_5} S_1^{\cdot} + S_2 , \quad (13)$$

where E, E_1, and E_2 are peroxidase and oxidized forms of peroxidase; S_1 and S_2 are slowly and rapidly oxidizable substrates, respectively; $S_1^{\cdot}$ and $S_2^{\cdot}$ are free radicals formed from these substrates.

Scheme 2 presumes that the activation phenomenon results from conjugation of oxidation of substrates that differ in reactivity at the steps of formation and further transformation of radicals. Ferrocyanide is probably oxidized by radicals of another substrate more effectively than by peroxidase intermediates E_1 and E_2. However, free radicals were not found during peroxidase-catalyzed oxidation of hydroxamates.

Applying the method of stationary concentrations to Scheme 2 and taking into account that for the majority of peroxidase substrates $k_1 \gg k_{21} \gg k_{31}$ and $k_1 \gg k_{22} \gg k_{32}$, one can obtain the following equation for the reaction rate

$$v_0 = 2[E]_0(k_{31}[S_1]_0 + k_{32}[S_2]_0). \quad (14)$$

Equation (14) satisfactorily describes the experimental data presented in a series of papers.[5,55,57,59,60]

The validity of the proposed mechanism is also confirmed by ESR spectra recorded during peroxidase-catalyzed co-oxidation of ascorbic acid and *p*-cresol.[55] It was shown that if ascorbic acid is added to the peroxidase—H_2O_2—*p*-cresol system, the ESR signal of monodehydro-*p*-cresol is completely replaced for a signal of monodehydroascorbate. Monodehydro-*p*-cresol also acts in a similar way in the case of nicotinamide adenine dinucleotide (NADH). Thus, *p*-cresol, which forms dimeric products on individual peroxidase-catalyzed oxidation, acts as an effective electron carrier during co-oxidation and activates peroxidase-catalyzed oxidation of "slow" substrates. A similar mechanism of activation has been also proposed for other substituted phenols.

2. To explain the activation of peroxidase-catalyzed oxidation of luminol in the presence of luciferin and substituted phenols, a kinetic scheme similar to Scheme 2 has been proposed. In this case, S_1 is a luminol anion, AH^-, and S_2 is a substrate-activator, luciferin or phenol. In the presence of an activator, luminol radicals ($AH^{\cdot}$) are formed both in the reaction with the enzyme (Eq. (15)) and upon interaction with the half-oxidized activator (Eq. (16)).

$$v_{AH} = (k_{21}[E_1] + k_{31}[E_2]) \cdot [AH^-] \quad (15)$$

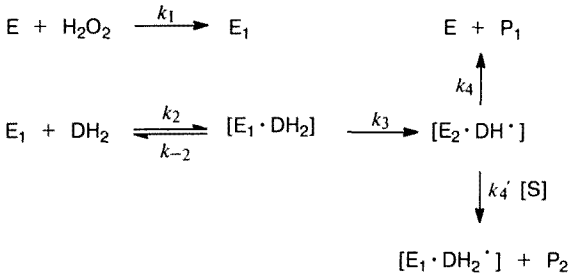
$$v'_{AH} = k_5[AH^-][S_2^{\cdot}] \quad (16)$$

In the presence of a substrate-activator, the concentrations of compounds E_1 and E_2 are higher than in the absence of this substrate, therefore, activation of peroxidase-catalyzed oxidation of luminol is explained both by the acceleration of the enzymatic generation of luminol radicals, $AH^{\cdot}$, and by the appearance of non-enzymatic pathways of their generation in the reaction of the $S_2^{\cdot}$ radical with luminol. In this case, the role of peroxidase is only the generation of intermediate free-radical particles, whereas half-oxidized substrates (free radicals) reside in solution and are not bound to the enzyme.

However, the kinetic regularities of the peroxidase-catalyzed oxidation of such substrates as pyrogallol, luminol, and *o*-dianisidine cay be explained only in the assumption that the peroxidase intermediates (E_1 and E_2) form complexes with these substrates.[40,64—66] Peroxidase complexes with aromatic substrates have been recorded by NMR spectroscopy.[51,67] It was found that the binding constants are not high (10^2—10^3 L mol^{-1}), which indicates the absence of strong interaction with the enzyme. In the case of other heme-containing peroxidases (myeloperoxidase, haloperoxidase), a hydrophobic site adjacent to heme was found by ESR and NMR spectroscopy, which serves as a binding site for aromatic substrates.[48,68] Higher values of binding constants, 10^3—10^4 L mol^{-1}, are typical of these peroxidases, which suggests stronger interaction of these enzymes with aromatic substrates. Aromatic compounds that are not peroxidase substrates are bound to another center. A complex of peroxidase with half-oxidized *o*-dianisidine was detected spectroscopically in the course of its peroxidase-catalyzed oxidation, but no free radicals were found in solution.[64]

3. To explain the kinetics of the co-oxidation of potassium ferrocyanide and *o*-dianisidine, Scheme 3 has been proposed, which takes into account the complex formation between the compounds E_1 and E_2 and substrates.

Scheme 3



where $[E_1 \cdot DH_2]$ is a complex of an intermediate E_1 with *o*-dianisidine (DH_2); $[E_2 \cdot DH^{\cdot}]$ is a complex of E_2 with half-oxidized *o*-dianisidine; S is ferrocyanide; P_1 and P_2 are products of individual oxidation of *o*-dianisidine and ferrocyanide, respectively.

Using the method of steady-state concentrations and taking into account that the initial rate (v_0) of the reaction characterizes only the appearance of the product of ferrocyanide oxidation

$$v_0 = \frac{d[P_2]}{dt} = k_4'[S][E_2 \cdot DH^{\cdot}],$$

a Michaelis-type equation was obtained. Assuming that $k_4 \ll k_4'[S]$ (*o*-dianisidine is not oxidized in the presence of potassium ferrocyanide) and $k_4'[S] > k_3$, the following expressions for k_{cat} and K_m were obtained:

$$k_{cat} = k_3, \; K_m = k_{-2}/k_2.$$

Hence, the experimentally determined k_{cat} characterizes the rate constant for the reduction of the $[E_1 \cdot DH_2]$ complex into $[E_2 \cdot DH^{\cdot}]$. According to the literature data,[40] this constant is approximately 10 times greater than the k_4 constant determined from individual oxidation of *o*-dianisidine. Thus, a mechanism of substrate-substrate activation, which presumes the direct participation of the enzyme in this process, is suggested for the

potassium ferrocyanide—*o*-dianisidine—peroxidase system. This mechanism is also confirmed by the fact that *o*-dianisidine does not accelerate the oxidation of ferrocyanide in the absence of peroxidase, although the nonenzymatic reaction also involves the participation of intermediate radical forms.

The activation of oxidase-type oxidation has been described for some peroxidase substrates (*e.g.*, NADH and indolyl-3-acetic acid) in the presence of phenol derivatives.[4,45,50,61] It was assumed that phenols decrease the concentration of the oxygenated low-active form of ferroperoxidase E_3. The activation of peroxidase-catalyzed oxidation of styrene into styrene oxide by *p*-cresol has been described (the oxidation does not occur in the absence of *p*-cresol).[69] At present, there are no literature data on the mechanism of substrate-substrate activation for the oxidase (oxidation with oxygen) and oxygenase (epoxidation) functions of peroxidase.

In the peroxidase-catalyzed co-oxidation of such reducing substrates as iodide—EDTA, thiocyanate—EDTA, bisulfite—iodide, and bisulfite—pyrogallol, activation is not observed, but competitive inhibition of oxidation of iodide and thiocyanate with EDTA[42,43] occurs in the reaction both with compounds E_1 and E_2. Ethylenediaminetetraacetic acid is a "rapid" substrate with respect to E_1 and a "slow" substrate with respect to E_2. Binding of these substrates with peroxidase is controlled by the same ionogenic group of the protein with pK_a 5.4, and competition for binding with the protein can exist.[44] In this case, a complex of the enzyme with one substrate is probably not an effective oxidant for the other substrate. Up to now, substrate-substrate activation has been observed only for substrates with noticeably different reactivities, whereas EDTA, iodide, and thiocyanate have similar reactivities.[43]

Analysis of the literature data on peroxidase action in mixtures of reducing substrates allows us to make the following conclusions.

1. When different peroxidase substrates are simultaneously present in solution, the effects of mutual activation and inhibition are observed. In this case, inhibition is either a competitive process or results from differentiated oxidation of substrates in the presence of peroxidase. In the latter case, the inhibition of oxidation of one substrate must be accompanied by the activation of the oxidation of the second substrate.

2. The substrate-substrate activation phenomenon, *i.e.*, acceleration of oxidation of a slowly oxidizable substrate by a rapidly oxidizable substrate, is a general property of peroxidase systems, typical of the peroxidase, oxidase, and oxygenase functions of this enzyme.

3. The mechanisms of substrate-substrate activation probably differ for different substrate pairs. Using ESR spectroscopy and kinetic methods, it has been shown that the activation in a number of substrate pairs (*p*-cresol—ascorbic acid, luminol—substituted phenols, 4-aminoantipyrine—substituted phenols, *etc.*) results from non-enzymatic processes, and the role of peroxidase is reduced to the generation of active intermediate free-radical species. The experiments in 4-aminoantipyrine—substituted phenols systems, in which the native peroxidase was replaced by microperoxidase, also confirm the absence of direct participation of the enzyme in the phenomenon under discussion.[56]

Conversely, no free radicals of half-oxidized *o*-dianisidine and hydroxamates were observed in the reaction mixtures during peroxidase-catalyzed oxidation of ferrocyanide—*o*-dianisidine and ferrocyanide—hydroxamic acid substrate pairs while an ESR signal, which most likely corresponds to enzyme-substrate radical complexes, was recorded.[60,64] The kinetics of co-oxidation of potassium ferrocyanide and *o*-dianisidine can also be satisfactorily described using the assumption that the species that effectively oxidizes ferrocyanide is a complex of peroxidase with half-oxidized substrate rather than the *o*-dianisidine free radical. In this case, the enzyme plays the key role in the activation.[54]

The mechanism of co-oxidation of compounds in the presence of peroxidase probably depends on the structure of substrates and on the mechanisms of their individual peroxidase-catalyzed oxidation, which can differ greatly, as we have mentioned above.

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