Hypothesis

On a revised mechanism of side product formation in the lignin peroxidase catalyzed oxidation of veratryl alcohol

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Abstract The O_2-dependent formation of side products during the oxidation of veratryl alcohol (VA) by lignin peroxidase has previously been proposed to start with the attack of H_2O on the VA radical cation ($VA^{\bullet+}$). This initial reaction is unlikely since it would also lead to side product formation in the absence of O_2, which is not the case. In the current mechanism $VA^{\bullet+}$ reacts first with O_2, whereafter H_2O attacks. Furthermore, this paper describes an alternative explanation for the inhibitory effect of Mn^{2+} on VA side product formation. It is proposed that Mn^{2+} reduces reactive intermediates back to VA. © 2001 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

Key words: Veratryl alcohol; Lignin; Manganese; Lignin peroxidase; Radical cation

1. Introduction

White-rot fungi make use of extracellular oxidative mechanisms to degrade lignin, a heterogeneous aromatic polymer which provides woody tissues with strength and rigidity. Underneath the lignin there are carbohydrates which are potential food stocks and this is the main reason for fungal lignin degradation. Among the enzymes involved in the lignin degrading mechanism are peroxidases, of which lignin peroxidase (LiP) and manganese peroxidase are the most common [1].

Once LiP is activated by H_2O_2 it can oxidize a large variety of both phenolic and non-phenolic compounds of which the fungal secondary metabolite 3,4-dimethoxybenzyl alcohol or veratryl alcohol (VA) is one. VA is a good reducing substrate for LiP and may therefore overcome H_2O_2-dependent inactivation of LiP [2]. VA functions also as a redox mediator between LiP and lignin, as concluded from studies in which lignin model compounds were used [3,4]. Redox mediation involves the oxidation of VA to the VA radical cation ($VA^{\bullet+}$), which is bound to the enzyme [5], and the subsequent removal of one electron from lignin by $VA^{\bullet+}$, resulting in oxidized lignin and VA [3,4].

In the absence of alternative reducing substrates, VA is predominantly oxidized to veratraldehyde (VAD). The mechanism involves two successive single electron oxidation reactions catalyzed by LiP [5,6]. Only in the presence of O_2, side products are formed [6,7] which in total account for 30% of the molar yield [8]. The reaction mechanism in which these side products are formed has been studied in detail [6,9,10].

Leisola et al. [11] reported for the first time on the formation of lactones (total molar yield 19% [8]) from VA, indicating that LiP can initiate an O_2-dependent cleavage of the aromatic ring in VA. This ring cleavage reaction was proposed to occur via a dioxetane intermediate that was formed by a reaction between superoxide and $VA^{\bullet+}$ [12]. However, this was discounted because only half, and not all, of the incorporated oxygen originated from O_2 [9] as would have been expected in the case of a dioxetane intermediate.

In 1987, Haemmerli et al. [6] proposed a reaction mechanism that accounts for all VA side oxidation products which is still generally accepted. In this mechanism the perhydroxyl radical ($HOO^{\bullet}$) acts as an essential reactant which was evidenced by the finding that Mn^{2+}, a well-known peroxyl radical reductant [13,14], decreased the formation of side products. This view has been questioned because the very fast spontaneous decay of the hydroperoxyl radical to O_2 and H_2O_2 would not allow such an involvement [9]. Nevertheless, the mechanism remained unchanged for 13 years.

In this paper it is pointed out that the reaction mechanism proposed by Haemmerli et al. [6] contains several inconsistencies. The most important one involves the first step, which is the addition of H_2O to $VA^{\bullet+}$. In our view this cannot be correct because such a reaction route would also yield side products in the absence of O_2 and those have not been observed [6]. By discounting the first step, it appeared that the entire reaction mechanism involved in VA side product formation needed revision.

2. Results and discussion

2.1. Inconsistencies in the previous mechanism

A schematic representation of the commonly accepted reaction mechanism that accounts for product formation from VA (**1**) is depicted in Scheme 1. LiP oxidizes VA to the corresponding radical cation ($VA^{\bullet+}$, **1a**) which may lose a proton to form a radical that is oxidized further by LiP to VAD (**2**).

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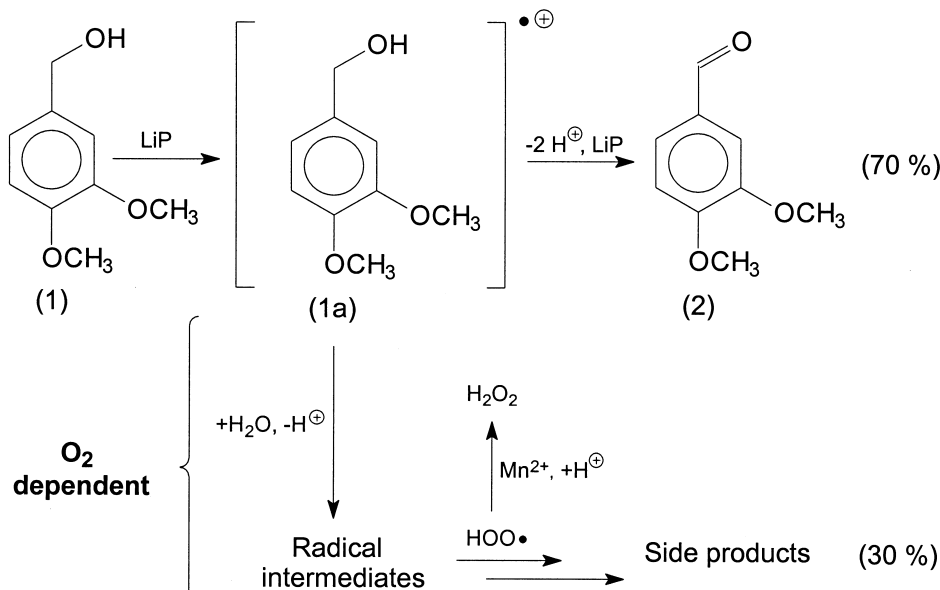
Scheme 1. A schematic representation of the reaction mechanism involved in the formation of VAD and side products by the LiP catalyzed oxidation of VA as proposed by Haemmerli et al. [6]. The indicated molar yields are from [8].

Under air the molar VAD yield is 70% [8], but in the absence of O_2 this yield is nearly 100% [6]. This indicates that under air products other than VAD are formed in an O_2-dependent manner. In relation to this, it is remarkable that the mechanism in Scheme 1 starts with the attack of H_2O on $VA^{\bullet+}$, because such a step occurs independently of the presence of O_2. Furthermore, such an initial attack of H_2O would in any case yield radical intermediates which do not lead to VAD. It is rather unfortunate that the same inconsistency occurs in the reaction mechanism that explains side product formation from VA methyl ether [8], but it is beyond the scope of this paper to revise that mechanism too.

Scheme 1 shows that the second step in VA side product formation is a coupling reaction between the hydroperoxyl radical ($HOO^{\bullet}$) and a VA derived radical ($VA^{\bullet}$) as proposed before [6]. This proposal, however, is questionable for several reasons.

The first reason is that there is much more O_2 available than $HOO^{\bullet}$ for a reaction with $VA^{\bullet}$. Moreover, $HOO^{\bullet}$ dismutates very fast under these acidic conditions [13,15] making it unlikely that it can significantly contribute to possible coupling reactions with $VA^{\bullet}$.

The second reason is another inconsistency in the mechanism itself. Haemmerli et al. [6] proposed that Mn^{2+} scavenges $HOO^{\bullet}$ and that Mn^{2+} therefore caused a decrease in the formation of most of the identified side products. If this were true then the intermediate radical products would still be available for subsequent reactions that do lead to other side products than those that were identified. However, no other products have been detected in the presence of Mn^{2+} [16]. Thus, the role of Mn^{2+} in VA side product formation needs reexamination too.

2.2. The alternative mechanism

In the proposed alternative mechanism that is described here, the first step in VA side product formation is the attack of O_2 on $VA^{\bullet+}$. The phenomenon that a radical cation readily reacts with O_2 has been shown before [17,18]. Furthermore, there is literature evidence that this reaction is also applicable to $VA^{\bullet+}$. In the case of $VA^{\bullet+}$ it has been shown that its decay is enhanced in the presence of O_2 [19,20] which cannot be attributed to the fast follow up reaction between $VA^{\bullet}$ and O_2 as explained in literature [19] because deprotonation of $VA^{\bullet+}$ is irreversible [20]. In our opinion, the effect of O_2 on the decay of $VA^{\bullet+}$ demonstrates that O_2 can react with $VA^{\bullet+}$ to the product $VAOO^{\bullet+}$ which has more routes to decay than $VA^{\bullet+}$.
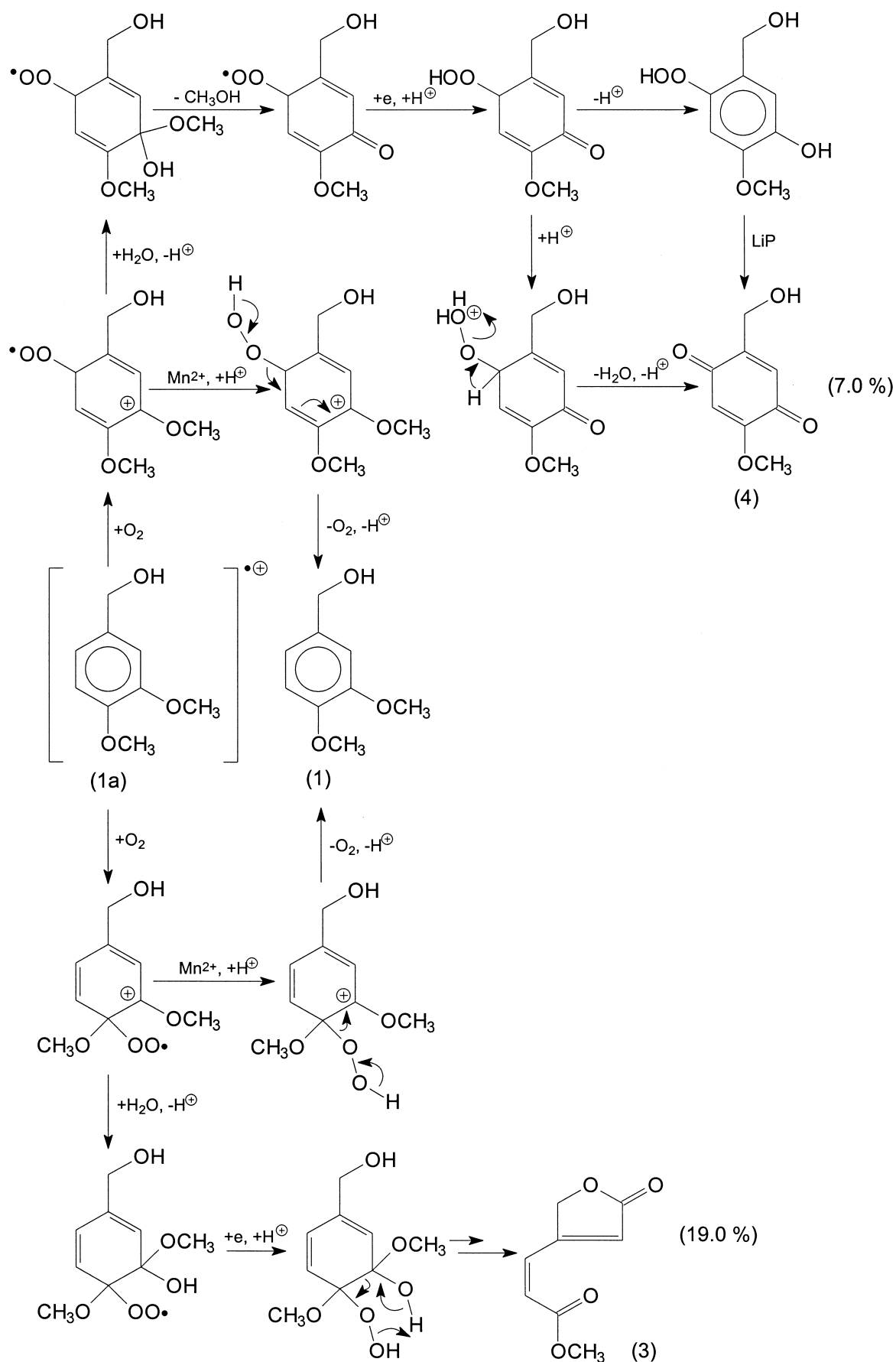
Scheme 2 shows the novel mechanism involved in the formation of two VA side products namely the Z-γ-lactone (3) and 2-hydroxymethyl-5-methoxy-2,5-cyclohexa-diene-1,4-dione (4). The first step in the mechanism is the attack of O_2 on the VA radical cation which yields intermediate peroxyl radical cations. The subsequent step is the attack of water on the carbocation.

In Scheme 3 the reaction mechanism towards 2-methoxy-1,4-benzoquinone (5), and 4,5-dimethoxy-3,5-cyclohexadiene-1,2-dione (6) is depicted which in essence follows the same route as shown in Scheme 2.

2.3. Alternative explanation for the effect of Mn^{2+} and pH on VA side products

Mn^{2+} reacts fast with peroxyl radicals in general [13,14]. Thus, in the presence of Mn^{2+}, the peroxyl radical cations are reduced back to VA. Without any Mn^{2+} present, H_2O attacks the peroxyl radical cation, which is an irreversible route towards the side products (see Schemes 2 and 3).

Once the VA radical is formed, its reaction with O_2 yields VAD almost instantly [20]. This fact allows us to propose an alternative explanation for the finding that VAD is produced in a higher molar yield upon increasing the pH value from 3.5 to 4.5 as reported before [6]. Evidently, deprotonation of $VA^{\bullet+}$ occurs faster at a higher pH value, making a reaction between $VA^{\bullet+}$ and O_2 towards side products a less likely event.

Scheme 2. A proposed alternative mechanism involved in the formation of the Z-γ-lactone (3) and 2-hydroxymethyl-5-methoxy-2,5-cyclohexa-diene-1,4-dione (4) from VA. The indicated molar yields are from [8].
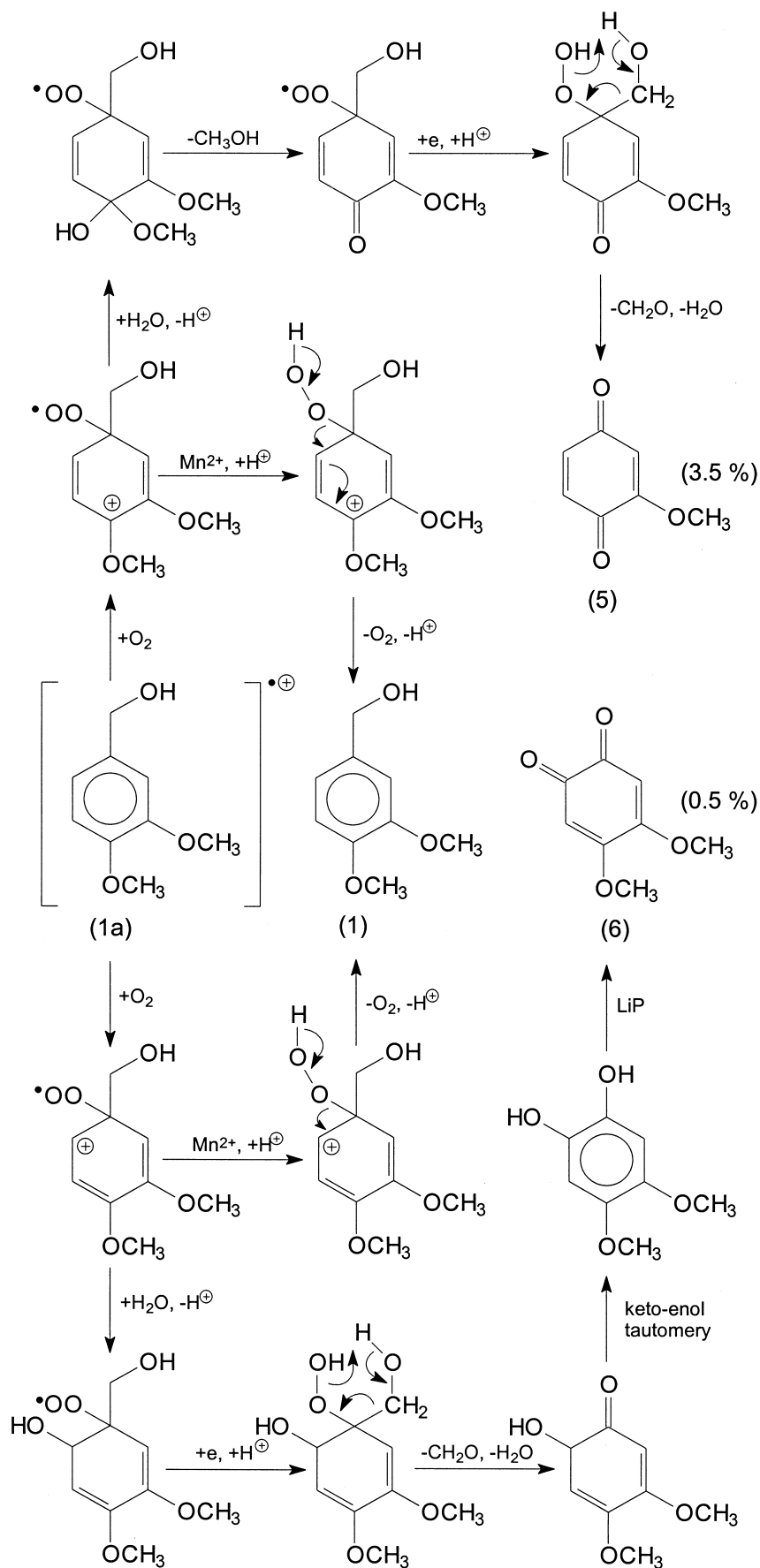
Scheme 3. A proposed alternative mechanism involved in the formation of 2-methoxy-1,4-benzoquinone (5) and 4,5-dimethoxy-3,5-cyclohexa-diene-1,2-dione (6) from VA. The indicated molar yields are from [8].

2.4. Critical evaluation towards the alternative mechanism

In this section some other first steps in the reaction mechanism are discussed. By starting with VA$^\bullet$, it is impossible to get side products because the addition of O_2 and subsequent elimination to VAD and superoxide occurs extremely fast [20] and therefore this possibility is eliminated.

In theory it is also possible that HOO$^\bullet$ reacts with VA$^{\bullet+}$ first. However, such a reaction would not be coupling as proposed before [12], but will be the reduction of VA$^{\bullet+}$ back to VA because the redox potential of VA$^{\bullet+}$ exceeds that of HOO$^\bullet$ [20,21] which indicates that a redox reaction is more favorable.

Also the direct attack of H_2O on VA$^{\bullet+}$ was ruled out as described before (this work), and therefore the only remaining option is that side product formation has to occur via an initial attack of O_2 on VA$^{\bullet+}$ as indicated in Schemes 2 and 3.

2.5. Consequences of the current mechanism

An intermediate VA derived peroxyl radical can abstract a benzylic hydrogen atom from, for example, fresh VA. This reaction is depicted in Schemes 2 and 3 as the uptake of an electron and a proton. The consequence of this reaction is that VA is consumed chemically and enzymatically if aerobic conditions are applied. Such a manner of substrate consumption has been described before in the case of the LiP substrate 1-(3′,4′-dimethoxyphenyl)propene [22].

If the origin of the hydrogen atom is indeed fresh VA, then it is expected that VA consumption is somewhat (maximally 30%) faster under aerobic than under anaerobic conditions. In such a case it is also expected that under air, Mn^{2+} inhibits the VA consumption to a similar level as expected under anaerobic conditions.

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