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Coordination Chemistry Reviews
185–186 (1999) 619–628

COORDINATION CHEMISTRY REVIEWS

Inhibitor binding studies on ascorbate oxidase

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Abstract

The characteristic features of the plant multicopper enzyme ascorbate oxidase are described, together with the current knowledge about its catalytic mechanism and substrate specificity. A variety of small anionic inhibitors have been used as spectroscopic probes for the enzyme metal sites, but recently interest has arisen for a new type of phenolic inhibitors which act competitively against ascorbate. These simple phenolic compounds can bind to the enzyme in the same pocket near type 1 copper as the substrate ascorbate binds, as shown by

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PII: S0010-8545(99)00014-4

docking and molecular mechanics computations.

Keywords: Ascorbate oxidase; Enzyme inhibition; Catalytic mechanism; Spectroscopic studies; Molecular mechanics

1. Introduction

Ascorbate oxidase (L-ascorbate: oxygen oxidoreductase, EC 1.10.3.3) is a member of the family of multicopper blue oxidases, which includes laccase, ceruloplasmin and other less well characterized enzymes [1]. The blue oxidases are among the few enzymes which are able to catalyze the four-electron reduction of dioxygen to water, a reaction releasing free energy without production of reactive, partially reduced species such as hydrogen peroxide or hydroxyl radical, which are potentially harmful to cells. Dioxygen reduction by these enzymes is accomplished at a functional unit composed by one type 1 copper ion and a trinuclear copper cluster (one type 2 and two type 3 coppers), and is coupled with the oxidation of substrate molecules, which generally occurs through one-electron steps [2]. Ceruloplasmin differs from the other multicopper oxidases in that it contains additional copper centers [3].

Ascorbate oxidase is a homodimeric enzyme with subunits of M_r 70 000 [4] which is found in plants [5,6] and bacteria [1]. It is commonly purified from green zucchini squash (*Cucurbita pepo medullosa*) [7] or cucumber peelings (*Cucumis sativus*) [8]. The physiological role of ascorbate oxidase is not known [9]. In vitro it is very active in the oxidation of L-ascorbate, which is involved in a variety of cell processes associated with plant growth, protection and development. Therefore, ascorbate may indeed be the natural substrate of the enzyme. The picture of the complex ascorbate system in plants [10,11] has been enriched recently by the finding that its biosynthesis depends on L-galactose [12], an intermediate ultimately formed from D-glucose. Ascorbate oxidase is the first of the multicopper oxidases to be crystallographically characterized [13,14]. Each subunit contains 552 amino acid residues, has a globular shape and is built up by three domains of similar β-barrel folding. The active site contains a type 1 copper separated by about 12.5 Å from the trinuclear cluster. The ligands of type 1 copper (His, Cys, His, Met), as well as its overall structural features, are the same as those found in type 1 copper proteins [15,16] and account for the strong optical absorption at 610 nm and the EPR signal with small hyperfine constant ($A_{\parallel} \sim 60 \times 10^{-4}$ cm^{-1}) of ascorbate oxidase [7]. The trinuclear cluster has eight histidine ligands and may be subdivided into a spectroscopic type 3 pair of copper atoms, each bound to three histidines and bridged by a hydroxide group, and a type 2 copper center, with two histidine ligands and a terminal hydroxide ligand. The bridging hydroxide group between the type 3 copper centers mediates strong antiferromagnetism and makes this pair EPR silent, but an optical absorption at 330 nm is commonly associated with this center. The type 2 copper has no optical feature and is responsible for the normal EPR

signal ($A_{\parallel} \sim 190 \times 10^{-4}$ cm^{-1}) of the enzyme. The average copper–copper distance in the trinuclear cluster is 3.74 Å, with individual distances within 0.16 Å from this mean value [13]. Another unusual feature in the ascorbate oxidase structure is the connection between Cys-507 ligand of type 1 copper and His-506 and His-508 which are both ligated to one of the type 3 copper atoms. A schematic view of the active site arrangement of ascorbate oxidase is shown in Fig. 1.

2. Catalytic mechanism

A catalytic mechanism for ascorbate oxidase, based on the available spectroscopic and mechanistic studies [5,17] and on the crystal structure of the enzyme in its resting (oxidized) form [13], and those of other derivatives [18], including the fully reduced form, has been proposed [14,18]. Starting from the resting form, the cycle proceeds by sequential one-electron reduction of the enzyme. Electrons are provided by the substrate and donated to the type 1 copper, the primary electron acceptor, in a fast, bimolecular second-order reaction [5,19–22]. Ascorbate is oxidized to semidehydroascorbate radical in this process, and the radical spontaneously dismutates in solution [23] (Fig. 2). The electrons are then transferred from type 1 copper to the trinuclear cluster. Anaerobic reduction studies carried out with a variety of electron donors show that this electron transfer appears to be monophasic or multiphasic depending on the actual electron donor used in this experiment [5,20,22]. The values of the kinetic constants found for this intramolecular electron transfer, though, do not exceed ~ 200 s^{-1} and are one to two orders of magnitude smaller than the turnover numbers observed for the enzymatic oxidation of various substrates [17]. It is therefore likely that the enzyme form used in the conditions of the anaerobic reduction experiments was not in its fully active form.

After four electrons have been transferred, the enzyme is fully reduced. In this state the hydroxyl bridge between the type 3 coppers has been released and the

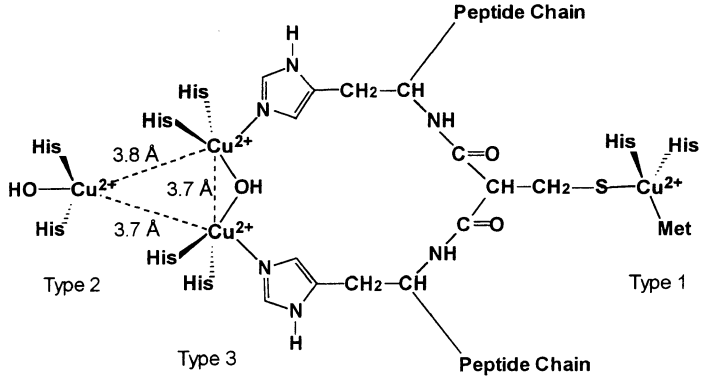


Fig. 1. Schematic view of the active site structure of ascorbate oxidase.

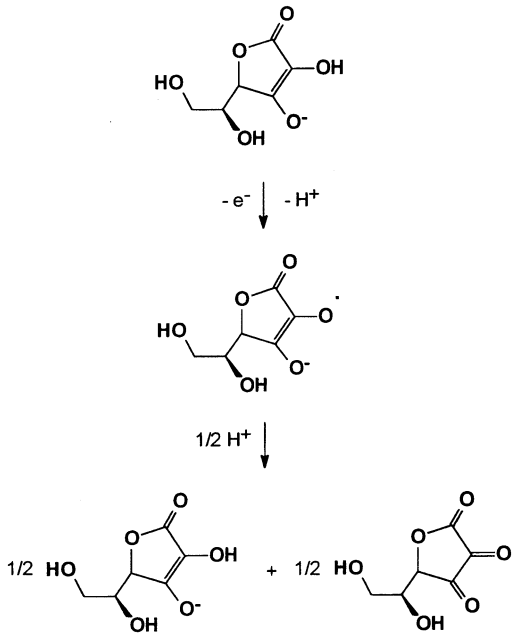


Fig. 2. Enzymatic one-electron oxidation of ascorbate and dismutation of the resulting semidehydroascorbate radical.

copper–copper distances in the trinuclear cluster increase from an average of 3.7 to 4.1–5.1 Å [18]. Dioxygen reacts with the reduced enzyme to form a putative peroxide-level intermediate, which is formulated as a hydroperoxide intermediate. A hydroperoxide derivative of ascorbate oxidase, containing a terminal $^{-}$O–OH group bound to one of the type 3 coppers (with no hydroxyl bridge) has been structurally characterized [18], but this adduct was obtained by reaction of hydrogen peroxide with the resting enzyme. An oxygen intermediate obtained by the reaction of fully reduced laccase with dioxygen has been proposed to contain an $^{-}$O–OH group bridging between type 2 copper and one of the type 3 coppers, while the hydroxide bridge is maintained [1,24]. In any case, the peroxide-level intermediate is further reduced by the third reduced copper in the cluster and by a fourth electron delivered from type 1 copper. The O–O bond may be broken at the third reduction level, with formation of an oxygen radical intermediate. An intermediate previously formulated as an oxyl or hydroxyl radical in the reaction of reduced laccase with dioxygen [25,26], has been recently reformulated as a fully reduced, dibridged dihydroxyl species [1,24] (Fig. 3). This assumes that the sequence of delivery of the third and fourth electron is very fast and in practice corresponds to a second two-electron transfer to the peroxide intermediate. The enzyme form which is produced upon reduction of dioxygen may relax to the resting state or be reduced by other substrate molecules for further turnover.

3. Inhibition by small anionic ligands

Since the catalytic mechanism of the blue oxidase is still little understood and also, until a few years ago, the structural information on these enzymes was scarce, small anions like fluoride or azide that are capable to bind to the copper centers have been extensively employed to gain insight into the complex structural and mechanistic features of the enzymes and to emphasize the differences in behavior within the blue oxidases [1]. Both azide, fluoride and thiocyanate inhibit ascorbate oxidase [27,28], but the inhibition pattern is complex, at least above pH 5.6. The anions give mixed-type inhibition, suggesting the existence of multiple binding sites in the enzyme. Spectral studies with a variety of techniques show that the site of interaction of these ligands is the trinuclear cluster [8,29–35], where more than one molecule can bind in terminal or bridging modes, and can even cause the cleavage of the endogenous bridge between the type 3 copper pair [36]. The latter feature is confirmed by the X-ray structural determination of the azide adduct of ascorbate oxidase [18]. Type 1 copper remains unaffected by the anions, but the observation that they act competitively with respect to ascorbate and, in addition, that the kinetically determined binding constants are much larger than those determined spectroscopically [28] suggest that the anions have an additional binding site close to the type 1 center (vide infra).

4. Substrate binding site

From the calculation of a Connolly surface for the X-ray structure model of ascorbate oxidase, Messerschmidt et al. identified a possible binding pocket for the substrate near the type 1 copper center [13]. The pocket is delimited by two tryptophan residues (Trp-163 and Trp-362) that may be important to stabilize the interacting substrate. Docking experiments showed that the lactone ring of ascorbate may give a stacking interaction with the aromatic ring of Trp-163, while the aromatic ring of Trp-362 is approximately parallel to His-512, one of the type 1 copper ligands [13]. We performed some computational calculations by coupling rigid body docking experiments of the substrate ascorbate in the protein with molecular mechanics (MM) [37]. According to the procedure we followed, the energy minima obtained by rigid body docking in the first step were subsequently

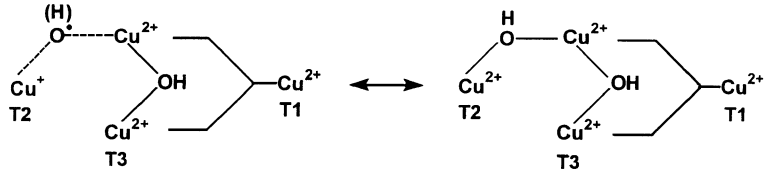
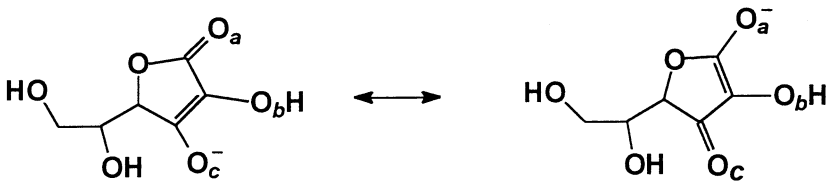


Fig. 3. Limiting formulations, as three- or four-electron reduced species, for the laccase 'oxygen intermediate' [1].

subjected to MM optimization by relaxation of the side chains of the amino acid residues in the active site. The monoanionic form of ascorbate was used in the calculations, because this is the form present in physiological conditions ($pK_a = 4.17$). While the substrate negative charge can be formally localized on oxygen atoms *a* and *c*, ab initio, calculations performed with the STO-3G basis set indicate an almost even distribution of this charge on all three oxygen atoms *a*, *b* and *c*, irrespective of the presence of the proton on atom O_b [37]:



Limit structures of ascorbate ion

This finding is important because the docking experiment invariably sets the ascorbate molecule in the protein cleft with the oxygen atom *b* hydrogen-bonded to the His-512 N_ε–H group. The two energy minima resulting from MM optimization contain an additional hydrogen bond between oxygen atom *a* of ascorbate and the Trp-362 N–H group, but differ in the disposition of the side chain, which can interact with the Glu-443 carboxylate group with either the primary or secondary hydroxyl group (Fig. 4). The feasibility of a direct hydrogen-bonding interaction between the substrate ascorbate and the histidine ligand of type 1 copper is extremely important from the mechanistic point of view because it can account for the extremely fast (completed within the dead time of the stopped flow instrument) electron transfer to type 1 copper [5].

5. Enzyme specificity

Ascorbate oxidase can oxidize other organic substrates besides ascorbate, provided some structural features are maintained in the molecule. In the most detailed account, the substrate specificity of a series of derivatives of L-ascorbic acid (6-amino-, 6-deoxy-, 6-deoxy-6-bromo-, 6-*O*-phenyl-, and 6-*S*-phenyl-L-ascorbic acid) was investigated [38]. The variations in enzyme turnover were marginal, while some significant variation in affinity was observed. In particular, a 4-fold decrease occurred for 6-amino-L-ascorbic acid, possibly due to the presence of a positive charge, and a 6-fold increase in affinity was found for the two 6-phenyl derivatives, probably because the binding of these substrates may be supported by additional hydrophobic interactions by their aromatic nucleus. With the structural model given in Section 4, this additional interaction can be attributed, on the enzyme side, to the aromatic nucleus of Trp-163, which is not involved in the binding of unsubstituted ascorbate. Structural modifications at other carbon positions of the

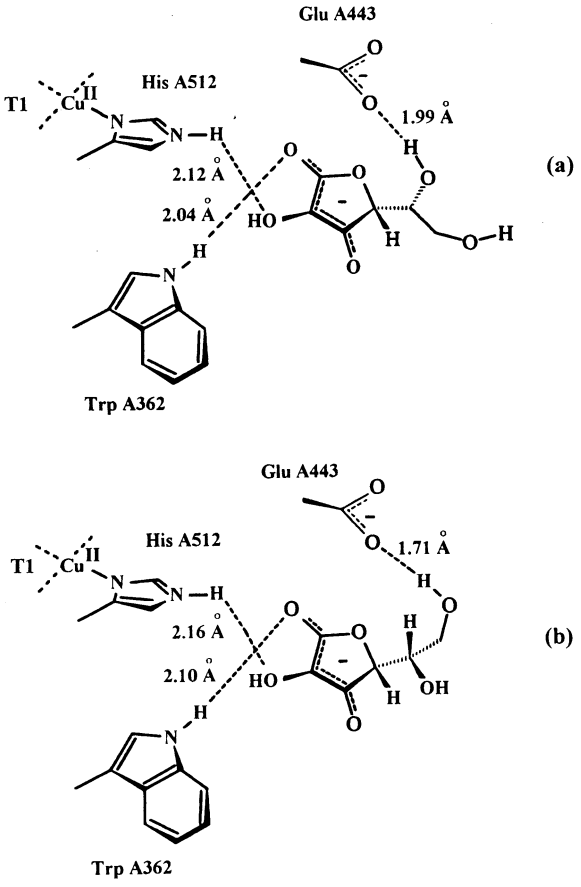


Fig. 4. Schematic view of the minimized interaction modes of ascorbate to the type 1 site pocket of ascorbate oxidase [37].

L-ascorbate molecule led to loss of activity [38]. Substrates more loosely related to ascorbate are oxidized with low rates by the enzyme. Among these are a small group of substituted hydroquinones (not hydroquinone itself) [39], some catechol compounds [40,41], and *N*,*N*-dimethyl-*p*-diaminobenzene [37]. Interestingly, the latter compound exhibits a K_m value which is almost as small as that of ascorbate, whereas in general, the enzyme affinity of the other aromatic substrates is much smaller than that of ascorbate.

6. Competitive inhibitors against ascorbate

With the picture emerging from the structural, spectroscopic and mechanistic studies performed on ascorbate oxidase, two main mechanisms of inhibition of the

enzyme can be envisaged. Small inorganic anions such as azide, fluoride and thiocyanate can act as competitive inhibitors as they bind to the trinuclear cluster of the enzyme. Other organic compounds structurally related to ascorbate can inhibit the enzyme by binding to a different site of the enzyme, i.e. near type 1 copper. Our group has followed the latter approach by investigating the behavior of a series of simple phenolic compounds through kinetic and spectroscopic studies [42], and more recently, also docking and MM calculations [37]. Phenols competitively inhibit the enzyme against ascorbate with inhibition constants in the millimolar range. The best inhibitors contain an uncharged, electron-withdrawing substituent on the phenol ring, preferably in *para* position, and their action is stronger when they are in anionic form. The inhibitory effect is not simply related to phenol acidity, since steric effects at the *para* substituent are important as well. When the size of this substituent is large, as in *p-tert*-butylphenol, the inhibitory effect is completely prevented. It is interesting to note that the presence of a phenol affects the binding of the azide ligand to the trinuclear cluster. In particular, azide binding to type 2 copper is completely prevented (from EPR evidence) and the affinity of the ligand for type 3 copper is reduced. These effects are not completely understood and further investigation is required to gain a clearer picture. However, since phenol itself does not appreciably change the spectroscopic properties of ascorbate oxidase, its effect in, e.g. preventing azide binding to type 2 copper, is not due simply to phenol competition for this site. Phenol may obstruct the access channel to type 2 copper [13], or induce some protein conformational change when bound at the type 1 copper site.

Docking and MM calculations performed on the three fluorophenols account for the competitive inhibition mechanism of action of this group of compounds [37]. Energy minima corresponding to two basic binding modes are found for the three compounds. In the first one the phenol binds to the access channel to type 1 copper, thus preventing substrate approach to this enzyme site. In the second binding mode the phenol interacts with protein residues responsible for substrate recognition and interaction in the active site. In particular, the phenol hydrogen-bonds to the type 1 copper ligand His-512 N_{ε}–H group in the same way as the substrate ascorbate does. A schematic view of the binding modes of *p*-fluorophenol to the enzyme is shown in Fig. 5. Unfortunately, it is impossible to give a preference to either binding mode, because absolute energy values are unreliable with the current computational procedure.

The possibility to arrange the molecules of substrate or inhibitor, preferably in their anionic form, in a protein pocket close to type 1 copper, but not directly binding to the metal center, can reconcile an apparent inconsistency existing in the literature between the binding constants of small anionic inorganic anions (fluoride, azide) to ascorbate oxidase determined spectroscopically, and those determined kinetically (which are larger [28]). It is conceivable that these small anions have easy access to the type 1 binding site even though this interaction cannot be characterized spectroscopically. As the spectroscopically characterized binding site of the anions to the enzyme is type 2 copper, their competitive inhibition affect against ascorbate led previously to the suggestion that type 2 copper was the ascorbate

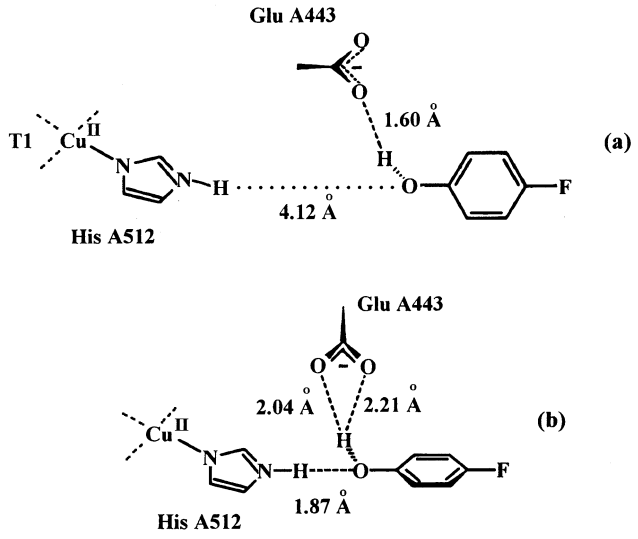


Fig. 5. Schematic view of the two binding modes of *p*-fluorophenol to the type 1 pocket of ascorbate oxidase resulting from docking and MM calculations [37].

binding site [27–29], a hypothesis which we now know is in contrast with a large body of evidence [19–22]. It seems likely that the small anions bind to the type 1 pocket with an affinity higher than to type 2 copper, and are therefore able to inhibit the enzyme at concentration smaller than those necessary to produce changes in the type 2 copper spectroscopic features.

Acknowledgements

This work was supported by the Italian M.U.R.S.T. The authors thank their colleagues involved in the project 'NMR of metallobiomolecules' for helpful discussions.

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