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Role of Ascorbate in Biological Hydroxylations: Origin of Life Considerations and the Nature of the Oxenoid Species in Oxygenase Reactions

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The proposition is made that the enzymes that catalyze ascorbate-dependent hydroxylation reactions in metabolism make use of a primordial dioxygen fixation system, in which a ferrous ascorbate complex is the central and permanent catalytic unit. It is suggested that dioxygen on bonding to the complex becomes a nucleophile that will react with an electrophilic and easily oxidizable compound, such as an α-ketoacid. This gives rise to a presumably fairly stable intermediate ferryl ascorbate complex that acts as an electrophilic oxenoid both in ascorbate-dependent oxygenase reactions and in chemical models thereof, such as Udenfriend's system (ascorbate and $Fe^{2\oplus}$, with or without EDTA in water). A similar ferryl species with easily oxidizable ligands is suggested to be the oxenoid in other oxygenase reactions such as the tetrahydrobiopterin and the cytochrome *P*-450-dependent monooxygenases.

Considered in the context of the origin of life, present-day enzymes can be divided conceptually into two types with regard to the principles they use for catalysis. The first type in its most extreme form requires no nucleotide cofactors for activation and is highly specific, often acepting only one conformation of one enantiomer of a particular substance. The mechanism of action of this type of enzyme is most easily understood with the concept of complementarity of the enzyme surface to the transition state of the reaction to be catalyzed (*1*). Examples of this type can be found in many hydrolytic enzymes and biotin-dependent enzymes (*2*). These can be rationalized as being post-genetic-code biocatalysts.

The second type, ideally, bears a prosthetic group or makes use of coenzymes (often nucleotides, or nuclotide-like substances) that play an important role in activation of substrates. Such enzymes often show a much broader substrate specificity. Good examples are the adenosylcobalamin-dependent enzymes, which in addition to their usual substrates can also often use analogs thereof with methyl or other substituents bonded to the carbon atoms that are directly involved in the reaction (*3*). Some will even catalyze the (irreversible) isomerization of the unnatural enantiomer *with equal rate* (*4*). In this type of activation bonding of substrates to the chiral peptide part of the enzyme system must be of lesser importance. These enzymes can be rationalized as being derived from a primor-

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dial catalyst upon which, after the development of the genetic code, the polypeptide apoenzyme evolved. In such primordial catalysts, e.g., transition metals and complexes thereof, sugars and especially (poly)nucleotides (sugar derivatives also) must have played an important role along with solid minerals and noncoded polypeptides.

It is suggested that the ascorbate-dependent oxygenases also belong to this second type in view of the nature of the cofactor, the universal requirement of a transition metal ion, and the relatively low substrate specificity (*5*). A departing point for this article is therefore the assumption that ferrous ascorbate (a complex of two reduced ions) in chemical evolution was one of the systems that reduced molecular oxygen coming from photolysis of water in the overall reducing environment. Present-day oxygenases still make use of this catalytic unit. Since ascorbate is not an especially strong ligand, the combination must have a particular fitness for such a task.

Ascorbic acid. Vitamin C (AH_2) is a reductant and an acid of $pK_a = 4.1$, and it is thus normally present as ascorbate ($AH^{\ominus}$), which *in vivo* therefore can reduce $Fe^{3\oplus}$ to $Fe^{2\oplus}$ (in blood for instance) and $O_2^{\ominus}$ to H_2O_2 (*6*). This means that whenever iron is present in catalytic amounts relative to ascorbate it is like to be present in the ferrous state. These one-electron redox reactions of ascorbic acid are reminiscent of those of (*ortho*) hydroquinones, but there is also an important difference. On losing one electron ascorbate becomes a semiquinonoid radical ($AH\cdot$) that is a strong acid of $pK_a = -0.45$ (*7*); in consequence it immediately loses a proton under physiological conditions. The so-formed radical anion ($A\cdot^{\ominus}$) is strongly stabilized by resonance, does not measurably react with dioxygen (*8, 9*), and behaves normally as an oxidant (*9*). In fact the only detectable reaction is the fast disproportionation to ascorbate ($AH^{\ominus}$) and dehydroascorbic "acid" (A). In solution there is a general tendency to form quinhydrone-like complexes between the different species, for instance, AH_2–AH_2, AH_2–$AH^{\ominus}$ (*10*), and $A\cdot^{\ominus}$–AH_2 (*11*), the latter isolable as yellow crystals; the fast disproportionation of $A\cdot^{\ominus}$ can probably also be explained by the formation of a complex $A\cdot^{\ominus}$–$AH\cdot$ which rapidly collapses to A–$AH^{\ominus}$ (deprotonated quinhydronoid complex). Dehydroascorbic acid forms a covalent dimer (*12*).

Ascorbate as a ligand. The structure of ascorbate metal complexes is not known. A priori three possibilities seem feasible.

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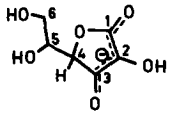


Structures in which oxygen atoms of ascorbate bridge between two metal ions as in thallous ascorbate crystals (*13*) are not considered. First, there is the possibility of a tridentate chelate (O-3, O-5, and O-6); this structure is found for hard metal ions such as $Ca^{2\oplus}$ (*14*), but seems less likely for softer ions such as $Fe^{2\oplus}$ and $Cu^{\oplus}$. Since owing to resonance an ester carbonyl oxygen is a slightly better Lewis base than a ketone oxygen (*15*), one can expect O-1 to be a better ligand than O-3. In the X-ray structure of ascorbic acid the unusually short C-1, O-4 bond length is

explained by that resonance (*16*). The effect remains on deprotonation (*17*). Inspection of the three known X-ray structures, sodium ascorbate (*17*), calcium ascorbate dihydrate (*18*), and barium 2-O-sulfonato-ascorbate dihydrate (*19*), confirms this. The total number of O-1 ligating atoms is equal to that for O-3. Moreover, in all three structures the metal to O-1 distance is the shortest of all metal oxygen distances by a marked amount. Until more direct evidence to the contrary is available it is therefore assumed that $Fe^{2\oplus}$ and $Cu^{\oplus}$ bridge O-1 and O-2 in ascorbate metal complexes.

If transition metal ascorbates were of major importance in dioxygen "detoxification" on the primordial earth the above short characterization of ascorbate chemistry should be enough to clarify their role. In the remaining part of this article the components of the various enzymatic reactions are therefore simply put together and analyzed for anticipated chemistry in the hope that a definite mechanistic scheme emerges.

α-KETOACID-DEPENDENT HYDROXYLATIONS

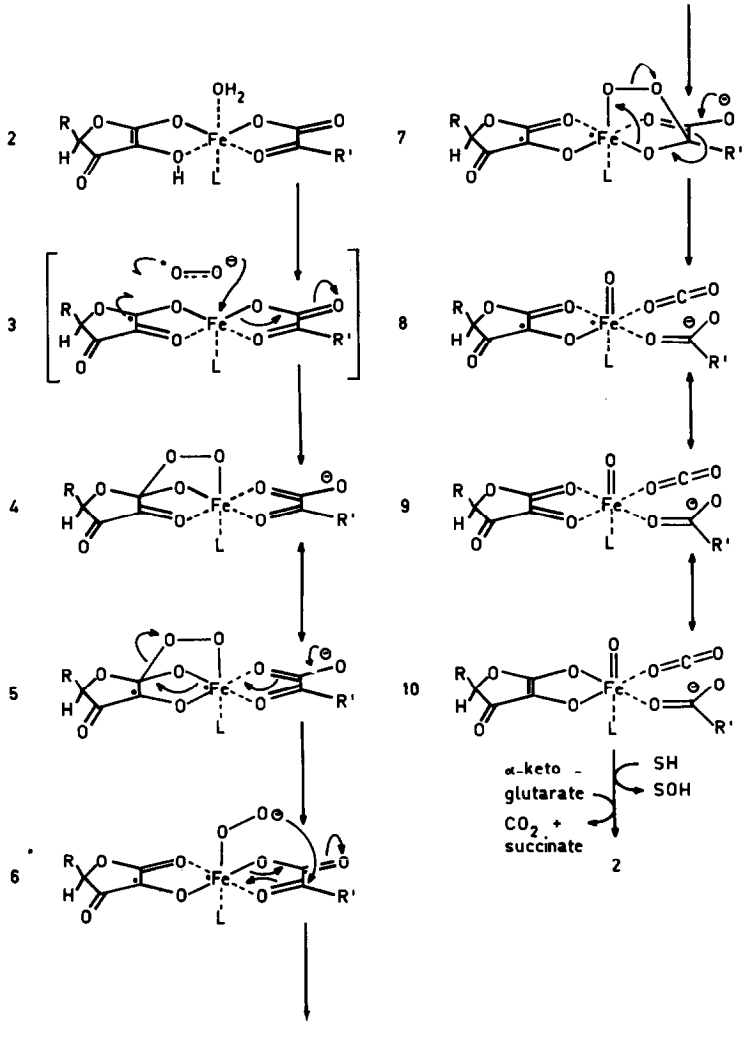
The α-ketoglutarate-dependent dioxygenases (*20*) catalyze hydroxylations as shown in the following general equation:

$$\mathrm{HOOC{-}CH_2{-}CH_2{-}\overset{\overset{O}{\|}}{C}{-}COOH + O_2 + RH \xrightarrow[Fe^{2\oplus}]{\text{ascorbate}} HOOC{-}CH_2{-}CH_2{-}COOH + CO_2 + ROH.}$$

The hydroxylation can best be viewed as an O-insertion (*21*) (Hamilton's oxenoid mechanism) in various types of C–H bonds. Prolyl- and lysylhydroxylase hydroxylate the growing procollagen polypeptide chain on essentially nonactivated carbon atoms with retention of configuration. All of the prolyl and lysyl groups in procollagen that are hydroxylated can in fact be considered as different substrates, which means that the enzymes have a very broad substrate specificity. Prolylhydroxylase from plant sources can catalyze hydroxylations of underhydroxylated procollagen from chick embryos (*22*). γ-Butyrobetaine hydroxylase catalyzes a similar hydroxylation on a nonactivated carbon atom leading to carnitine (*20*). Thymine 7-hydroxylase catalyzes demethylation to uracil via three successive hydroxylations on the same carbon atom (*20*) and uses therefore three different types of substrates (methyl, hydroxymethyl, and formyl carbon). Pyrimidine deoxyribonucleoside 2′-hydroxylase transforms thymidine and deoxyuridine into thymine and uridine, respectively.

All of these enzymes have a specific requirement for $Fe^{2\oplus}$ and α-ketoglutarate, but less so for ascorbate as a cofactor. Although the enzymes are relatively nonspecific for the substrate, they catalyze hydroxylations with complete retention, at least for the examples investigated. In examined cases, the oxygen source of both added oxygen atoms is molecular oxygen (*20*).

When the reaction components for these reactions are put together, Scheme 1 emerges.[1]

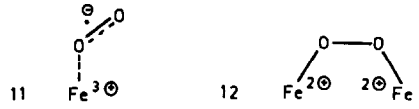


SCHEME 1. Proposed reaction sequence for α-ketoglutarate-dependent enzymes. R = –CHOH–CH_2OH; R' = –CH_2–CH_2–$COO^{\ominus}$; L = not identified ligand from the enzyme.

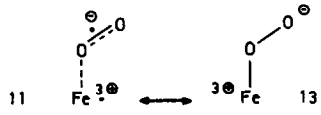
When it is assumed that the cosubstrate α-ketoglutarate is complexed to ferrous ascorbate, then there remain two coordination places on high-spin Fe(II), one for the enzyme and the other for dioxygen activation, structure 2. The complexation of dioxygen to transition metals such as high-spin $Fe^{2\oplus}$ can—in present-day terms—most easily be described by covalent bonding of a superoxide radical

[1] In Scheme 1 and other reaction mechanisms to follow one or more of the ligands are oxidizable. That means that the signed bonds (full and dotted lines) have only formal sense, just as the valency of the transition metal. In order not to "lose" electrons, formal charge and uneven number of electrons are consequently shown. Usually only one or two resonance structures of a great number are given.

anion with Fe(II) (superoxo Fe(III)), **11**, when one metal ion is involved and as a bridged peroxo Fe(III) complex, **12**, when two metal atoms are available (*23*).



When dioxygen approaches Fe(II) the bonding occurs by a formal one-electron transfer from high-spin Fe(II) to dioxygen. Whenever a good (one-electron) reducing ligand such as ascorbate is available, this lost electron is replenished and the now-oxidized ascorbate ligand loses concomitantly a proton (pK_a free AH· = −0.45), leading to **3**. Although the system has only one transition metal, a peroxo bridge as in **4** is stereochemically possible. In a formal sense the one-electron redox ligand ascorbate can be considered as a second "transition metal." Structure **4** is given preference above bridging to C_2 of ascorbate, because bridging to C_1 leaves conjugation in the ascorbate ligand intact, whereas on bridging to C_2 it is broken. Because in **4** ascorbate is highly oxidized, a better resonance structure for it is **5**. In the next step the oxygen bridge is broken to give an Fe(III) superoxo complex, **6**. A more direct route to **6** is of course also possible. Because of the fact that ascorbate is a good reducing agent, **6** is not expected to be a normal Fe(III) superoxo complex. The tendency to lose electrons will make resonance structure **13** (ferryl peroxide)—unimportant in complexes such as, for instance, oxyhemoglobin—more important in **6**. In other words **6** must be a good oxygen nucleophile and because a good electrophilic center is available in α-ketoglutarate, ring closure to form structure **7** is expected.

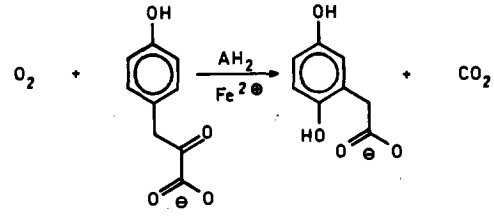


Structure **7** can be looked upon as a ferric complex of a carbonic peracid and should be a good oxenoid complex. The oxygen atom attached to Fe would thus be transferred. A second possibility is that first CO_2 and succinate are split off as in structure **8**[2] (**9** and **10** are two other resonance structures—out of a large number—for this species). This will be called ferryl ascorbate (see structure **10**). The chemistry of this species should be variable. The great number of resonance structures suggests it to be fairly stable. Among these structures all conceivable oxygen intermediates are found. Perhaps it is best described as an electrophilic (triplet?) oxenoid complexed to the good leaving group ferrous ascorbate. Such a species in the evolution of life on earth would have been well suited to become incorporated into an enzyme system for oxygen atom transfer, because the oxidizable ligand not only tends to stabilize the ferryl ion, but also is a good possible target for regulation of the reactivity of the system by the evolving apoenzyme. The direct environment of ascorbate (enzyme surface) can in this

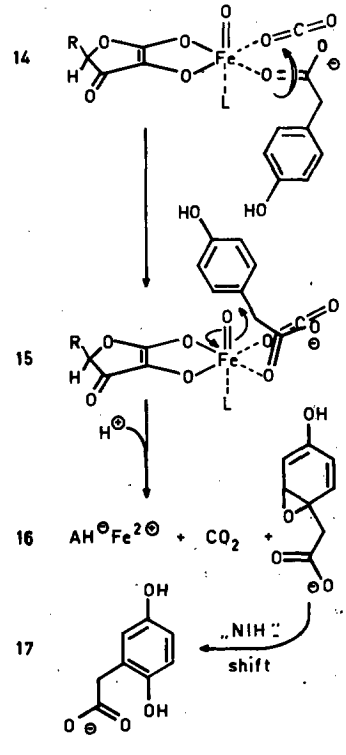
[2] A similar proposal for the role of α-ketoglutarate has recently been made by Moriarty (*24*) by way of entirely different reasoning (analogy with singlet oxygen chemistry).

manner make the system react like an oxygen radical or a singlet or triplet oxenoid and influence the electrophilicity of the oxygen atom being transferred.

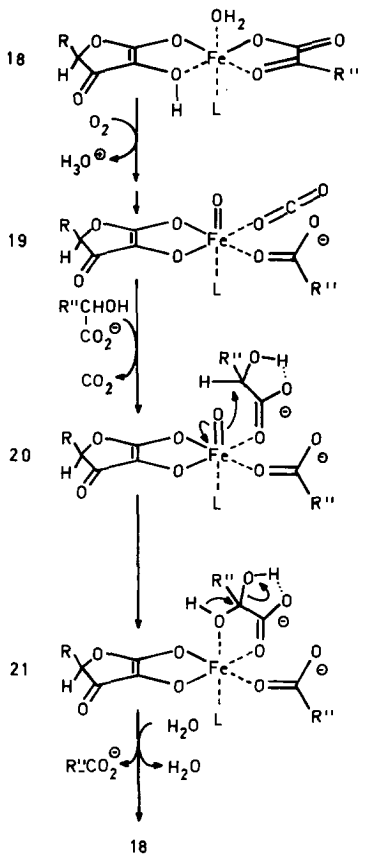
In addition to the α-ketoglutarate-dependent enzymes mentioned above, there is an enzyme that oxidizes a substrate which bears its own α-ketoacid, namely, hydroxyphenylpyruvate hydroxylase, which catalyzes (again among others) the following reaction:



The product, homogentisate, is presumed to originate from an "NIH shift" of an arene oxide (*25*). Thus, a scheme analogous to Scheme 1 can be proposed for this enzyme, leading to **14** (=**10** with R′ = *p*-hydroxybenzyl), from which the arene oxide can originate by simply turning the decarboxylated ligand as in **15**. For this enzyme a concerted decarboxylation–hydroxylation via an analog of structure **7** is stereochemically impossible, which suggests a stepwise process as depicted in Scheme 2.



SCHEME 2. Proposed reaction sequence for hydroxyphenylpyruvate hydroxylase. R = –CHOH–CH_2OH; L = not identified enzymic ligand.



SCHEME 3. Proposed reaction sequence for α-oxidation of cerebronic acid. R = –CHOH–CH_2OH; R″ = –$(CH_2)_{21}$–CH_3; L = not identified enzymic ligand.

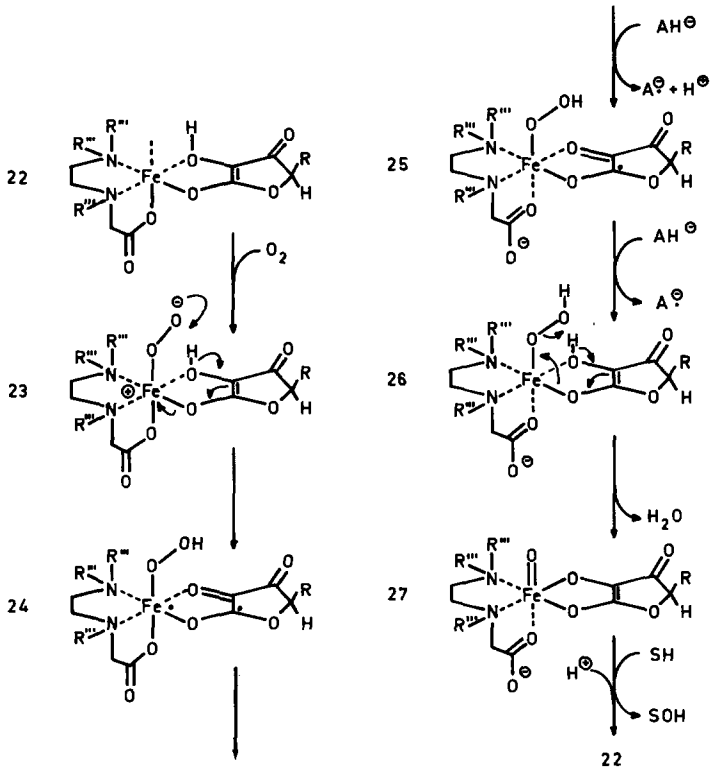
Finally, a satisfying reaction mechanism closely related to that shown in Scheme 1 can be proposed for the α-oxidation of cerebronic acid in the brain, which is also dependent on vitamin C and $Fe^{2\oplus}$ (*26*). The requirement for an α-ketoacid is fulfilled by the intermediary α-ketotetracosanoic acid (Scheme 3).

UDENFRIEND SYSTEM

If present-day ascorbate-dependent oxygenases make use of a primordial nonpeptide oxygen fixation system, it should be relatively easy to design model systems for the oxygenases. In this respect the discovery in 1954 by Udenfriend and co-workers that aromatic compounds are hydroxylated under physiological conditions by a mixture of ascorbate, Fe(II), EDTA, and O_2 is of special importance (*27*). There has been much research since then into the mechanism of action of this system. (For a recent review see Ref. (*21*).) Product analysis allowed demonstration only of what was *not* an intermediate in this system (H_2O_2,

$OH\cdot$, $OH^{\oplus}$, $\cdot OOH$, singlet oxenoid) (*21*). Hamilton concluded that a somewhat electrophilic triplet oxygen atom, arising from a complex of ascorbate, Fe(II), and dioxygen, could fit the accumulated evidence best (*21*), and this suggests strongly a reaction sequence like that in Scheme 1. However, there are two major differences with the situation there. First, in the absence of an electrophilic substance such as α-ketoglutarate, the only electrophile present is the proton. Second, ascorbate is used as a substrate in the Udenfriend system.

With this in mind a reaction sequence as in Scheme 4 is consistent with the known facts:



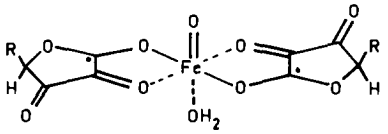
SCHEME 4. Proposed reaction sequence for Udenfriend's system. R = $-CHOH-CH_2OH$ R‴ = $-CH_2-COO^{\ominus}$.

EDTA is known not to be a hexadentate ligand for Fe(II), which makes a tridentate complex such as **22** possible (*28*, see also the discussion). A possible function of the EDTA ligand is to protect the nucleophilic oxygen in **23** from intermolecular reactions (*29*). In the oxycomplex **23**, the ferryl peroxide resonance structure again is favoured to some extent by the reducing ligand (ascorbate). Reaction with the only electrophile present, the proton on O-2, is stereochemically feasible. Of course, the alternative route in Scheme 1 is also possible. This leads to an ascorbate–iron hydroperoxide such as **24**. With the known tendency of ascorbate and related ions to form dimeric complexes (see

above), this complex can be readily reduced by one-electron transfer from free ascorbate to **25** and subsequently to **26**. Concomitantly with the latter reduction O-2 will be reprotonated and the system can lose water in an again stereochemically feasible fashion, leading to the ferryl ascorbate complex **27**. This can react as an oxenoid in hydroxylation reactions analogously to Scheme 1 and thereby regenerate the starting complex **22**. Ascorbate in this system is therefore at the same time substrate and part of a catalytic center (which presumably was a normal case in primordial times). A good indication for the involvement of electron transfers via ascorbate dimers is found in the strong dependence of the system on ascorbate concentration (*27*). Again, in this scheme ascorbate plays in a formal sense the role of a transition metal, namely, to pass electrons from substrate ions to the reaction center.

Of course EDTA is not a likely primordial substance, but the system works also without it (be it more slowly (*30*)). For this system a scheme analogous to Scheme 4 can be depicted, leading to **28** as the hydroxylating species.

28



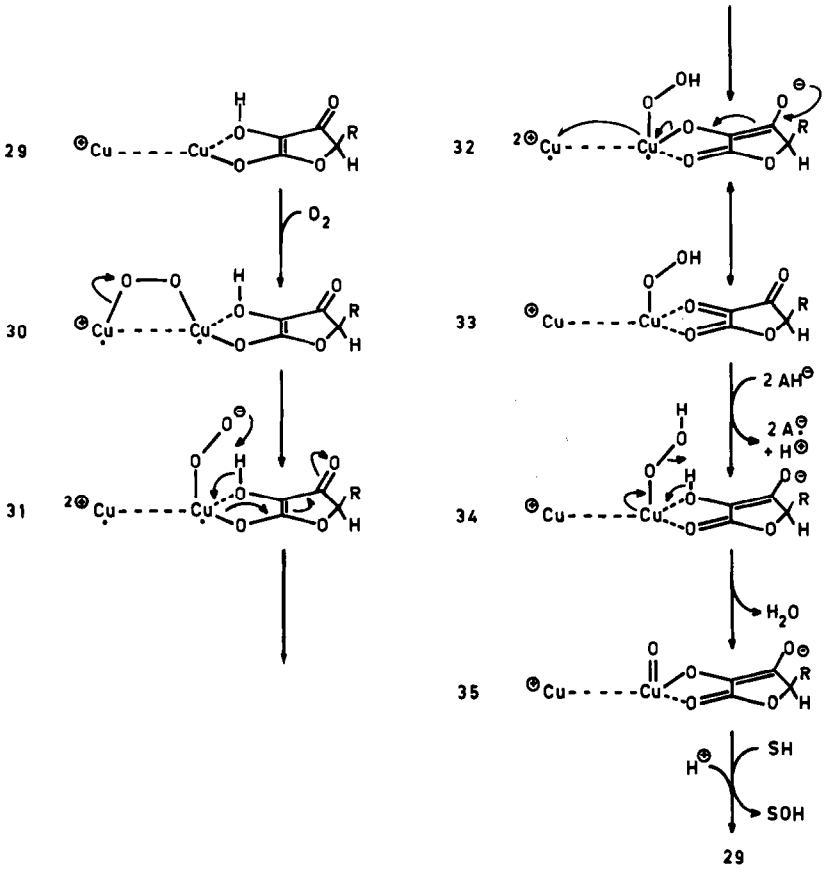
In the past this system has even been suggested to be one of the detoxification systems working *in vivo*. A better interpretation, however, is to consider it as one of the primordial (oxygen) "detoxificators," nowadays built into polypeptide apoenzymes.

Other Hydroxylases

Besides the Fe(II)–ascorbate-dependent hydroxylases, there is a copper-containing monooxygenase, namely, dopamine β-hydroxylase (*31, 32*), that normally uses ascorbate as a cosubstrate. It is a nonspecific enzyme that can make use of various substrates and cosubstrates. It is presumed that it contains two electronically coupled copper ions at its active site. The reaction is of the same oxenoid type as the Fe(II)–ascorbate-dependent ones and a related reaction sequence for it can be written (Scheme 5).

Scheme 5 is essentially a hybrid of Scheme 1, in which ascorbate and Fe(II) are replaced each by a Cu(I) ion, and Scheme 4. The scheme results in a cupryl species ($Cu^{\oplus}$=O) stabilized by ascorbate, as the oxenoid species. A similar scheme can be used for the also nonspecific copper enzyme tyrosinase, in which the intermediary *ortho*-diphenol takes the place of ascorbate. The latter can be used to initiate the reaction (*31*).

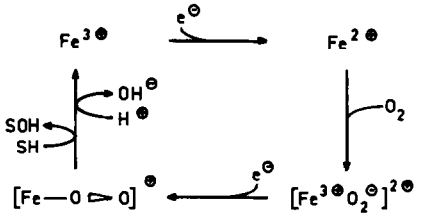
Still another group of related enzymes are the pterin-linked monooxygenases. These enzymes depend on Fe(II) as cofactor and use tetrahydrobiopterin as a cosubstrate (*33*). In almost all ascorbate-dependent hydroxylases tetrahydrobiopterin can fulfill the requirement partially, and inversely, ascorbate can often function as a cosubstrate instead of the pterin. For one enzyme it recently has



SCHEME 5. Proposed reaction sequence for dopamine β-hydroxylase. R = $-CHOH-CH_2OH$.

been suggested that ascorbate *in vivo* can also play the cosubstrate role. Consequently, a related mechanism seems highly probable for these enzymes.

Finally, it may be wise to reconsider the possibility of a ferryl ion as an intermediate in the case of the *P*-450 hydroxylases (*34*). The current working hypothesis for these oxenoid enzymes goes along the following lines (*31, 35, 36*):



In this scheme it is claimed that the good oxygen nucleophile $Fe^{3\oplus}\,O_2^{\ominus}\cdot$ *by adding an electron* is turned into an electrophilic oxenoid. This is against all chemical logic. Perhaps a ferryl ion stabilized by the protoheme ligand is a better intermediate.

ACKNOWLEDGMENTS

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