Evaluation of models for the mechanism of action of 4-hydroxyphenylpyruvate dioxygenase

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Summary. 4-Hydroxyphenylpyruvic acid was oxygenated with various complexes of oxygen with Fe^{2+}, superoxide ion, hydroperoxide anion, triplet and singlet oxygen. Oxidation occurred giving varying amounts of the 4-hydroxy derivatives of benzaldehyde, benzoic acid, phenol, phenylacetic acid and 4-carboxymethylquinone, but no homogentisic acid. 4-Hydroxyphenylperacetic acid was prepared and shown not to undergo self-oxidation. Its ferrous salt decomposed to 4-hydroxybenzyl alcohol. It is concluded that the α-keto carboxylic function is the site of oxygenation in the enzyme process and that a trioxalanone rather than a peracid intermediate may be implicated.

The manner in which Fe^{2+}-requiring dioxygenases catalyze the combination of molecular oxygen with organic substrates continues to attract attention[2]. One such enzyme of long-standing interest[3], 4-hydroxyphenylpyruvate dioxygenase (E.C. 1.13.11.27), catalyzes the conversion of 4-hydroxyphenylpyruvic acid (**1**) to homogentisic acid (**5**). By analogy with other α-keto carboxylic acid-decarboxylating dioxygenases, this enzyme is expected to engender 2 catalytic phases, namely a) the combination of molecular oxygen with the α-ketocarboxylate group to generate an oxenoid function (**1** → **2**) and b) the intramolecular transfer of the oxene oxygen atom to the aromatic nucleus (**3**) with subsequent rearrangement of the side-chain[4] (**4** → **5**) (eq. 1). In this study we investigate firstly how an enzyme might catalyze the combination of molecular oxygen with the substrate and secondly examine an intermediate which could transfer an oxygen atom to its own aromatic nucleus. In general, the catalytic ability of a metallo-enzyme may depend on the nature of enzyme-bound dioxygen[5]. Accordingly, 4-hydroxyphenylpyruvic acid (**1**) was allowed to react with several dioxygen species; triplet and singlet oxygen, hydroperoxide anion, superoxide ion and various complexes of oxygen with ferrous ion[5]. Oxidation occurred to give 4-hydroxy derivatives of benzaldehyde (**6**), benzoic acid (**7**), phenol (**8**), phenylacetic acid (**9**) and 4-hydroxy-4-carboxymethyl quinone (**10**) (table) (scheme). While not wishing to infer that the enzyme generates singlet oxygen, we have included the photo-oxygenation of **1** as it may clarify our understanding of the nature of the oxenoid species arising in the enzyme-catalyzed process.

It is immediately clear that the slowness of reaction with ground-state molecular oxygen (entry 1) clearly demonstrates the need for catalysis. Although **1** exists in its keto-form under the experimental conditions[6], the reaction undoubtedly involves the enol **11** which is easily oxygenated[7] to give **6**.

The nature of the reactive species in oxygenated ferrous EDTA complexes depends on the concentration of ferrous ions[8]. The superoxo form predominates at lower concentrations (entry 2), whereas the more active oxidant, the peroxo species, is found at higher concentrations (entry 3). Judging by the formation of the benzaldehyde **6**, both complexes oxidize the enol tautomer **11**. Moreover, the appearance of benzoic acid **7** at higher ferrous ion concentrations is due to further oxidation of the aldehyde (**6**). The formation of some phenylacetic acid **9** reveals that oxidative decarboxylation of the keto tautomer **1** also occurs (entry 3)[9].

Notwithstanding the suggestion that ferrous-bound dioxygen radical is the reactive species in oxygenated ferrous ascorbate mixtures (entry 4)[8], the products are essentially the same as those found for the other oxygenated ferrous complexes. In all cases the 3 ferrous/oxygen systems (entries 2-4) apparently initiate autoxidation of the enol tautomer (**11**)[7] and do not generate 'activated' oxygen.

The reactions of the superoxide anion per se (entry 5) are difficult to interpret in view of its tendency to form other reactive species[10,11]. When protons are not available (en-

try 5), **9** will form by nucleophilic attack of a superoxide ion on **11**, while free radical oxidation of **11** accounts for benzaldehyde formation (**6**)[11]. When protons are present (entry 6), superoxide ion co-exists with its conjugate acid, the hydroperoxy radical, as well as the product of acid-catalyzed dismutation, the hydroperoxide anion[10]. These 2 species react by further oxidizing the aldehyde **6** to acid **7** and hydroquinone **8** respectively[11]. Hydroperoxide anion alone simply causes the rapid oxidative decarboxylation of the α-keto acid **1** to the 4-hydroxyphenylacetic acid **9** (entry 7).

Saito and co-workers[12] have previously found that the dye-sensitized photo-oxygenation of arylpyruvic acids is solvent-dependent. In methanol (entry 8) formation of the benzaldehyde **6** as the sole product is due to the predominance of the enol **11**. When the photo-oxygenation is carried out with rose bengal sensitization in water (entry 9), 4-hydroxyphenylacetic acid (**9**) and hydroquinone (**10**) are also formed. It is well to remember that, under aqueous conditions, photo-oxygenation of α-keto acids proceeds via 2 competing processes, namely dye-initiated autoxidation[7] of the enol **11** to the aldehyde **6** and reaction of the α-keto carboxylate function itself with singlet oxygen to give **9** (v.infra)[13]. We therefore conclude that the aforementioned

Reaction of 4-hydroxyphenylpyruvic acid (**1**) with various dioxygen species

Entry number	Proposed active dioxygen species	Time for complete reaction (h)	Product yields[a]				
			6	7	8	9	10
1	$^3O_2$[b]	300	100	–	–	–	–
2	$(Fe-O_2)^{+}$[c]	24	85	–	–	15	–
3	$(Fe-O_2-Fe)^{4+}$[d]	24	30	55	–	15	–
4	$Fe-O-O\cdot$[e]	24	92	–	–	8	–
5	O_2^-[f]	24	65	–	–	35	–
6	$O_2^-/HO_2\cdot/HO_2^-$[g]	24	–	10	80	10	–
7	HO_2^-[h]	1	–	–	–	100	–
8	$^1O_2/^3O_2$, dye[i]	6	100	–	–	–	–
9	$^1O_2/^3O_2$, dye[j]	6	30	–	–	20	50

[a] Products identified by isolation. Yields determined by NMR-spectroscopy of crude mixture. Average of 2 runs. [b] Oxygen-saturated aqueous solution of hydroxyphenylpyruvate, 1 mM; phosphate buffer pH 7.0, 100 mM. [c] Oxygen-saturated aqueous solution of hydroxyphenylpyruvate, 10 mM; $FeSO_4$, 25 mM; ethylenediaminetetraacetic acid (EDTA) and acetate buffer; pH 5.6, 10 mM. [d] As for c, but EDTA and $FeSO_4$ both 250 mM. [e] As for c, but ascorbic acid 150 mM replaces EDTA. [f] Trisodium salt of hydroxyphenylpyruvic acid, 1.0 mmol (prepared by lyophilization with 3.0 mmol 50% NaOH) in DMSO, 50 ml under nitrogen atmosphere treated with 8.0 mmole potassium superoxide and 0.4 mmol 18-crown-6-ether. [g] Free hydroxyphenylpyruvic acid, 1.0 mmol as substrate. [h] 10-fold excess of alkaline 30% hydrogen peroxide. [i] Conditions of Saito et al.[12]; methylene blue in methanol. [j] Rose bengal in water (Saito[12]).

Scheme

5

6

7

8
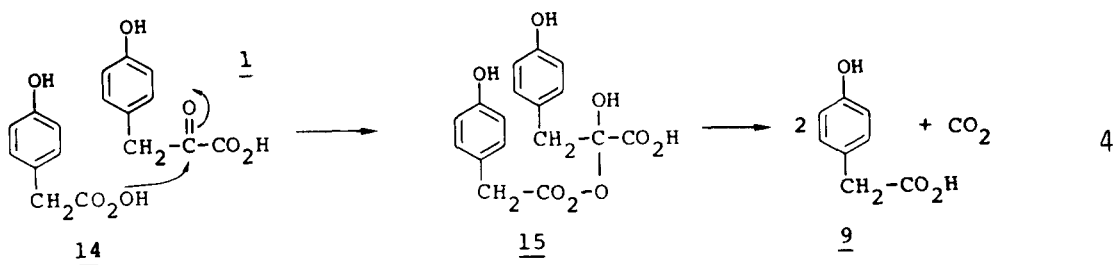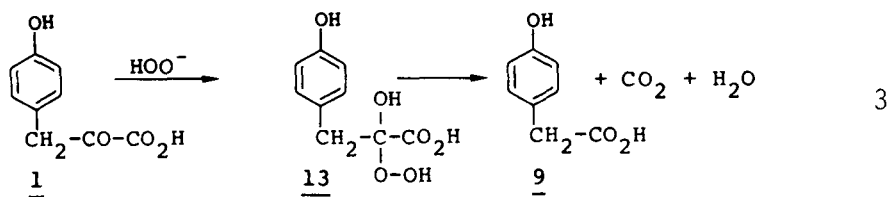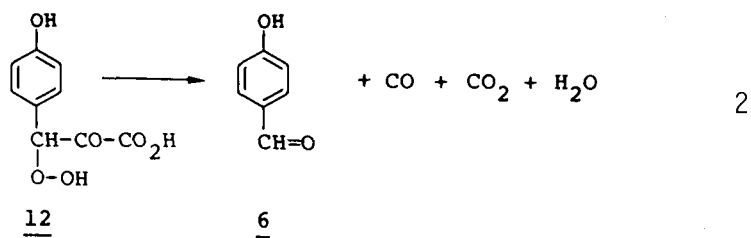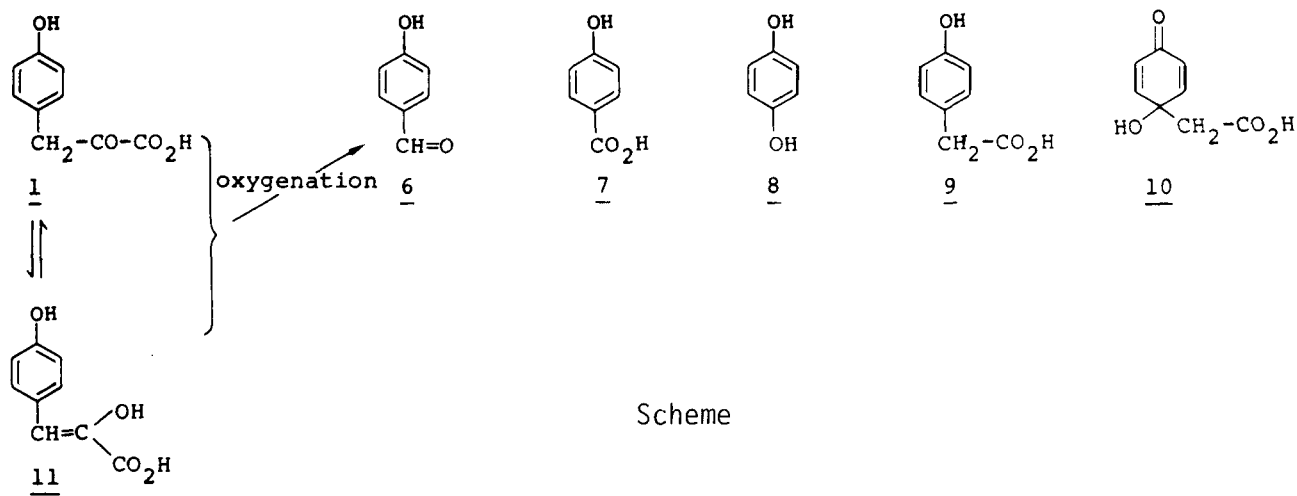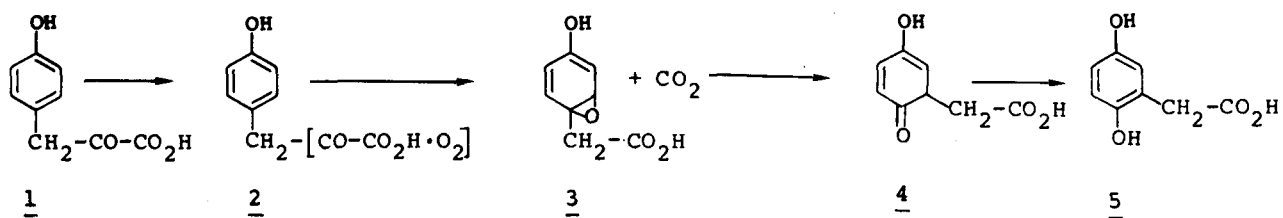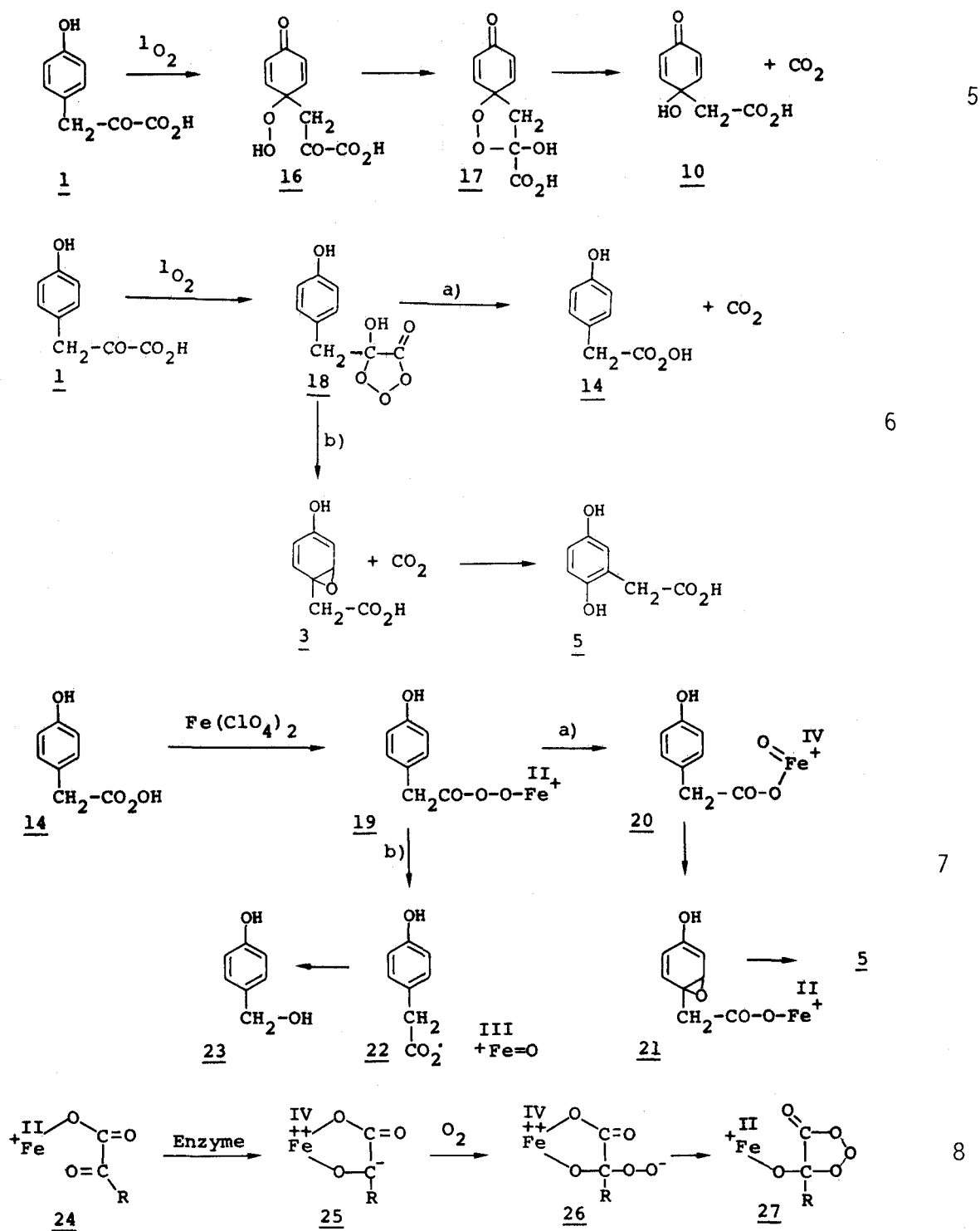
products **9** and **10** derive from the reaction of singlet oxygen with the a-keto acid tautomer **1** which indeed predominates in equilibrated aqueous solution[6].

The fact that none of the 9 different oxidizing systems produce homogentisic acid (**2**) clearly proves that none are models for the enzymatic process. Nevertheless, they show that molecular oxygen can react with the a-ketocarboxylic acid function and indicate the potential for catalysis. What needs to be shown next is that the putative oxene intermediate is capable of transferring an atom of oxygen to the aromatic nucleus.

Although we have no proof of any of the intermediates, the origin of the arylaldehyde **6** undoubtedly springs from the rapid and exothermic decomposition of the β-hydroperoxy compound **12**[7]. This intermediate would be expected to be unstable and fragment to a molecule of water and the oxides of carbon, thereby generating the aldehyde **6** (eq. 2). The provenance of the benzoic acid **7** and hydroquinone **8** is attributable to the further oxidation of the benzaldehyde[11]. 4-Hydroxyphenylacetic acid (**9**) is produced from the hydroperoxytetrahedral intermediate **13** on decarboxylation (eq. 3). This reaction (entries 2-7) identifies the

hydroperoxide ion as the oxidative decarboxylating agent. In any event, we have previously demonstrated that the oxidative decarboxylation of a-keto acids is easily accomplished by peracids[14]. Therefore, the 4-hydroxyphenylacetic acid 9 obtained in the singlet oxygen reaction comes from the interaction of peracid (14) with the a-keto acid 1 (v. infra) (eq. 4).

Of these 3 intermediates, namely the β-peroxycarboxylate (12), the a-peroxycarboxylate (13) and the peracid (14), the last is the best electrophile and the most likely to transfer an oxygen atom to an aromatic ring. This peracid has already been proposed as the intermediate in the enzyme-catalyzed process[4]. The results of photo-oxygenation (entry 9) add further strength to this proposal. We have already mentioned that the initially formed peracid rapidly combines with an a-keto acid to give a Baeyer-Villiger type intermediate (15) which generates 2 molecules of carboxylic acid liberating 2 molecules of carbon dioxide for each mole of singlet oxygen consumed (eq. 4)[13,14]. However, the a-keto acid 1 produces an additional product, the quinol 10 in which the aromatic ring has been oxidized. Saito and co-workers[12] originally suggested that this donor was singlet oxygen itself and that it had attacked the para position of the phenol moiety. Subsequent cyclization (16 → 17) followed by intramolecular oxidative decarboxylation then gave the quinol 10 (eq. 5). In fact, this reaction is well-documented and occurs with 4-hydroxyphenylacetic acid[12]. However, it is unlikely to account for the enzymic process as it only occurs under alkaline conditions and then too slowly, at about $\frac{1}{10}$ of the rate observed for the conversion of the a-keto acid 1 to the quinol 10. Indeed, Hecker et al.[15] had earlier shown that the incubation of labelled 1 and 10 with liver homogenate resulted in no incorporation of label in 10, thereby invalidating its intermediacy.

Returning to the peracid 14, can it give the epoxide 3? We prepared a sample of 4-hydroxyphenylperacetic acid (14) (80% pure by titration). It was found to be stable at room temperature. No rearrangement to 3, 5 or 10 was detected. In boiling water it regenerated the parent acid 9. We therefore conclude that although 14 is formed in the photo-oxygenation of 1, it merely causes the decarboxylation of 1 and does not give the quinol 10. Clearly, for intramolecular oxene transfer to occur, the acid side-chain must bear a more powerful oxidant than the peracid function.

The most plausible candidate is the trioxalanone 18, notwithstanding the failure or ambiguity of experiments to trap it so far. By analogy with primary ozonides, 18 should readily cleave to carbon dioxide and the peracid (eq. 6a). Moreover, the exothermicity of the liberation of carbon dioxide could be exploited by a suitable acceptor which undergoes mono-oxygenation. This hypothesis has been partially corroborated[16,17]. The aromatic nucleus in trioxalanone 18 could act as an acceptor as it is correctly placed to receive an intramolecular transfer of an oxygen atom, giving 3 and then 5 (eq. 6b). Such a process might account for the formation of quinol 10 from 1 on photo-oxygenation[18].

It is by no means certain whether an enzyme, without benefitting from the 23 kcal mol^{-1} of extra energy available to singlet oxygen, could generate high energy molecules such as trioxalanone. If the enzyme could find a less energy-demanding route to the peracid 14, it might still be able to increase its oxene-donating powers. For example, it has been reported that ferrous ions reduce percarboxylate to give Fe(IV)-oxo species which when appropriately coordinated can oxygenate inert carbon atoms by a two-step free radical mechanism[19]. The expectation is that the ferrous salt (19) of the peracid 14 would rearrange to its ferryl derivative 20 and then transfer an oxygen atom to give the epoxide 21, whence homogentisic acid 5 (eq. 7a).

In an exploratory experiment, we mixed the peracid 14 with an equal quantity of ferrous perchlorate in acetonitrile. 4-Hydroxybenzyl alcohol 23 was the main product. It can be thus inferred that 23 is the result of a one-electron process, giving the precursive carboxyl radical 22 (eq. 7b). It is not clear how an enzyme, even by appropriate coordination of Fe^{2+}, could bring about the two-electron reduction required for formation of homogentisic acid (eq. 7a).

Of the other possibilities, 1,4-oxygenation of the enol 11 has been discounted[20]. An intriguing alternative, which we will discuss elsewhere, is that ferrous a-keto carboxylate (24) in the enzyme complex generates a counterpolarized keto function (25) which is readily oxidized to the zwitterionic peroxide 26 or its trioxalanone tautomer 27 (eq. 8)[2].

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