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A Survey of Oxidative Paracatalytic Reactions Catalyzed by Enzymes That Generate Carbanionic Intermediates: Implications for ROS Production, Cancer Etiology, and Neurodegenerative Diseases

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A SURVEY OF OXIDATIVE PARACATALYTIC REACTIONS CATALYZED BY ENZYMES THAT GENERATE CARBANIONIC INTERMEDIATES: IMPLICATIONS FOR ROS PRODUCTION, CANCER ETIOLOGY, AND NEURODEGENERATIVE DISEASES

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I. Overview

Enzymes that generate carbanionic intermediates often catalyze paracatalytic reactions with O₂ and other electrophiles not considered “normal” reactants. For example, pyridoxal 5′-phosphate (PLP)—containing pig kidney dopa decarboxylase oxidizes dopamine with molecular O₂ to 3,4-dihydroxyphenylacetaldehyde at about 1% of the rate at which it catalyzes nonoxidative dopa decarboxylation. The mutant Y332F enzyme, however, catalyzes stoichiometric conversion of dopa to 3,4-dihydroxyphenylacetaldehyde, suggesting that even minor structural changes may alter or initiate paracatalytic reactions catalyzed by certain enzymes. Carbanions generated by several thiamine diphosphate (ThDP)—dependent enzymes react with different electrophiles, transforming some xenobiotics and endogenous compounds into potentially biologically hazardous products. The detrimental effects of paracatalytic reactions may be greatly increased by cellular compartmentation of enzymes and intermediates. For example, in two of the the three multienzyme complexes involved in oxidative α-keto acid decarboxylation, paracatalytic reactions of the third component inactivate the first carbanion-generating component.

In this review we provide an outline of carbanion-generating enzymes known to catalyze paracatalytic reactions. We also discuss the potential of some of these reactions to contribute to irreversible damage in cancer and neurodegeneration through disease-induced alterations in the metabolic state and/or protein structure.

II. Paracatalytic Reactions: Definition

In a series of seminal articles published more than 25 years ago, Philipp Christen and co-workers showed that the carbanionic intermediates of several enzymes [e.g., class I and class II fructose 1,6-bisphosphate aldolase, 6-phosphogluconate dehydrogenase, cytosolic aspartate aminotransferase, and pyruvate decarboxylase (PDC)] were accessible to various oxidants (electrophiles). Depending on the enzyme, these oxidants included, for example, 2,6-dichloroindophenol (DCIP), hexacyanoferrate(III), porphyrindin, tetranitromethane, and H_2O_2 (1–8). Christen coined the word paracatalytic to describe enzyme-catalyzed interactions between substrate and a reagent (especially an oxidant) not generally considered to be a physiological reactant (4, 5, 7). Reactions of enzyme-generated carbanions with O_2 were not included in Christen's original definition of paracatalytic reactions. However, several enzymes have been shown to catalyze oxidative side reactions with O_2 as the oxidant.¹ Therefore, in the present chapter we include enzyme-catalyzed oxidation side reactions with O_2 as a type of paracatalytic reaction. In many cases, the side products, whether generated from an artificial electron acceptor or from O_2 , are reactive and can lead to inactivation of the enzyme catalyzing the reaction or result in other types of oxidative damage. These reactive species might also be released from the active site of one enzyme to affect neighboring enzymes and macromolecules within the cellular milieu. As discussed below, these para-catalytic side reactions may contribute to disease processes.

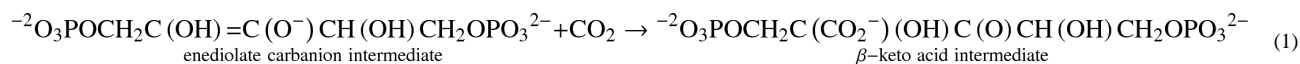
III. Discovery of Enzyme-Catalyzed Oxygenase Side Reactions: Rubisco

Originally it was not suspected that enzymes catalyzing reactions with carbanionic intermediates might also catalyze reactions with O_2 . Therefore, it came as a surprise to enzymologists when it was shown that ribulose 1,5-bisphosphate carboxylase is capable of catalyzing an oxygenase reaction [see the article by Bowes and Ogren (9) for the original discovery]. Since ribulose 1,5-bisphosphate carboxylase was the first enzyme shown to catalyze an unexpected oxygenase side reaction, we include a discussion of this enzyme here for historical reasons and to use this enzyme as a prototype in our discussion of enzyme-catalyzed oxidase/oxygenase side reactions.

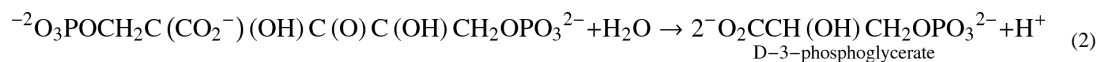
Ribulose 1,5-bisphosphate carboxylase, which is probably the most abundant enzyme on earth, catalyzes the first and controlling step of carbon fixation in photosynthesis (capture of atmospheric CO_2 to form 3-phosphoglycerate). However, ribulose 1,5-bisphosphate carboxylase also catalyzes carbon photorespiration (consumption of O_2 with concomitant production of 2-phosphoglycolate) (9–12). Because of this finding, the enzyme is now generally referred to as ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco). Photosynthesis results in a net gain of carbon biomass, whereas photorespiration does not. Photorespiration is energetically inefficient because it does not capture released energy as ATP or as a reduced nicotinamide adenine dinucleotide (phosphate). Thus, the oxygenase activity of Rubisco impedes the carbon biomass productivity of plants (9).

¹In this chapter we use the Enzyme Commission guidelines in defining oxygenases and oxidases. Oxygenases catalyze reactions in which at least one O atom derived from molecular oxygen (O_2) is incorporated into the main substrate. A monooxygenase catalyzes the incorporation of one O atom into the main substrate (AH), generating AOH; the other O atom is reduced to water, with concomitant oxidation of the co-substrate (BH_2) to B. The reaction may be written formally as $\text{AH} + \text{BH}_2 + \text{O}_2 \rightarrow \text{AOH} + \text{B} + \text{H}_2\text{O}$. Dioxygenases catalyze the incorporation of two O atoms into a substrate. In some cases a single oxidized product retains both O atoms derived from O_2 . In other cases, however, oxidation may generate two identical products, each containing an O atom derived from O_2 . In yet other cases, oxidation may yield two dissimilar products from a single substrate, each containing an O atom derived from O_2 . Oxidases catalyze oxidation reactions in which electrons are removed from the substrate and are used to reduce an electron acceptor, usually, but not always, O_2 . A one-electron reduction of O_2 will generate superoxide anion radical ($\text{O}_2^{\bullet-}$). More common is a two-electron reduction of O_2 to hydrogen peroxide (H_2O_2). In other cases, a four-electron reduction of O_2 yields water. In none of these three cases is O derived from O_2 incorporated into the oxidized product.

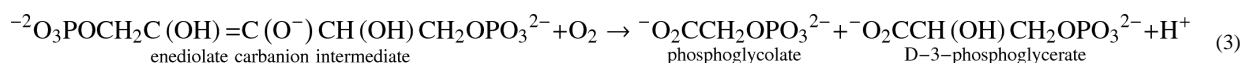
During CO₂ fixation catalyzed by Rubisco, a carbanionic intermediate (enediolate) derived from ribulose 1,5-bisphosphate reacts with CO₂ to form a β-keto acid:



which then undergoes hydrolysis at the active site to yield two equivalents of D-3-phosphoglycerate:



The net carbon gain is 1 (i.e., 5C + 1C → 3C + 3C). In the oxygenase reaction catalyzed by Rubisco, the electrophilic O₂ attacks the enediolate intermediate, generating a peroxide intermediate, which then fragments into phosphoglycolate and D-3-phosphoglycerate. The net reaction is



There is no carbon gain (i.e., 5C → 2C + 3C). These and other reactions catalyzed by wild-type and mutant forms of Rubisco are discussed in more detail by Schloss and Hixon (13).

Although the oxygenase reaction of Rubisco may be regarded as a side reaction, the maximal oxygenase rate at atmospheric levels of O₂ and CO₂ may reach about 50% of the CO₂ fixation rate (13). Because the ratio of CO₂/O₂ utilization varies markedly among CO₂-fixing higher plants, algae, and cyanobacteria, much effort has been devoted to understanding the topology of the active site that controls the CO₂/O₂ utilization ratio in the various Rubisco enzymes (14). It has been hoped by some that by suitable molecular engineering, the CO₂/O₂ utilization ratio in Rubisco of commercially important crops may be increased. If successful, the increased biomass yields would be of enormous benefit to the burgeoning human population (12). At the present time, however, it appears to be extraordinary difficult, if not impossible, to divorce the O₂-consuming reaction of Rubisco from its CO₂-consuming reaction.

Possibly, the oxygenase reaction catalyzed by Rubisco has an important physiological role in its own right and was therefore not eliminated during evolution. Remarkably, the intermediate peroxide in this reaction does not leave the active site, there is no release of reactive oxygen species (ROS), and O₂ is consumed. Therefore, the oxygenase reaction catalyzed by Rubisco may be a mechanism to lower the concentration of potentially harmful O₂. However, many other enzyme-catalyzed oxidative side reactions may on occasion be at best neutral, or at worst detrimental. Indeed, we suggest later that such side reactions may play roles in cancer and neurodegenerative diseases. As we discuss below, it is likely that even relatively slow oxidative side reactions may be physiologically important in some cases. Thus, although we use the term *side reaction* liberally here, it should be borne in mind that such reactions are not necessarily merely enzymological curiosities and may have biological and clinical relevance.

IV. Reaction of Carbanions with Oxygen Catalyzed by Selected Enzymes (Excluding Pyridoxal 5'-Phosphate-Containing Enzymes)

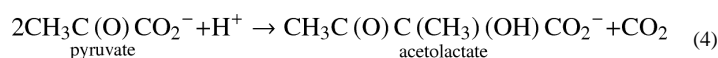
A. BACKGROUND

By the early 1990s it was well known that Rubisco catalyzes an oxygenase side reaction and that several enzymes catalyze the transfer of electrons from substrate to artificial oxidants. However, the full extent to which carbanion-forming enzymes catalyze side reactions directing the transfer of electrons from substrate to ambient O₂ was not appreciated at that time. In 1991, Abell and Schloss pointed out that any enzyme that generates a carbanionic intermediate is *theoretically* capable of reacting not only with electrophiles (oxidants) other than O₂ but also with O₂ itself (15). Accordingly, these authors investigated several enzymes that catalyze reactions in which carbanionic species are intermediates for their ability to catalyze oxygenase/oxidase side reactions. Carbanion-generating enzymes reported to catalyze oxygenase/oxidase side reactions included *Salmonella typhimurium* acetolactate synthase isozyme II (ALS II), brewer's yeast PDC, *Escherichia coli* glutamate decarboxylase (GAD), *Staphylococcus aureus* fructose 1,6-bisphosphate aldolase, and *E. coli* L-rhamnulose 1-phosphate aldolase (15–17). On the other hand, no oxygenase/oxidase side reactions could be detected for several other carbanion-forming enzymes, including rabbit muscle fructose 1,6-bisphosphate aldolase, *E. coli* [(phosphoribosyl)amino]imidazole carboxylase, *Torula* yeast 6-phosphogluconate dehydrogenase, pig heart isocitrate dehydrogenase (NADP⁺), baker's yeast triose phosphate isomerase, and *E. coli* L-fuculose 1-phosphate aldolase (15–17).

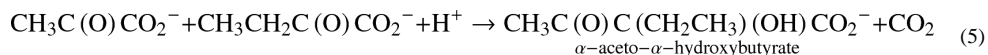
Four well-studied examples of enzymes that catalyze a paracatalytic side reaction with molecular O₂ are discussed in detail below. Two of these enzymes (ALS II and PDC) are thiamine diphosphate (ThDP)-dependent. The third and fourth examples are fructose-1,6-bisphosphate aldolase and L-rhamnulose-1-phosphate aldolase. These aldolases do not contain an organic cofactor. Several pyridoxal 5'-phosphate (PLP)-containing enzymes that catalyze paracatalytic reactions with O₂ are discussed in Section V. The α-ketoglutarate dehydrogenase complex (KGDHC), which contains ThDP in one of its component enzymes, catalyzes paracatalytic reactions with electron acceptors. Under certain circumstances, KGDHC also catalyzes the formation of O₂^{•-} (dismutating to H₂O₂). Because of the likely biological importance of these reactions catalyzed by KGDHC, they are discussed separately (Sections VII and VIII C).

B. ACETOLACTATE SYNTHASE

ALSs are enzymes that catalyze important steps in the biosynthetic pathway to the branched-chain amino acids (18). When two equivalents of pyruvate are used as a substrate pair, the product is acetolactate: a precursor of valine and leucine:

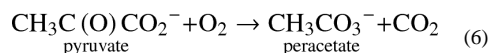


On the other hand, when pyruvate and α-ketobutyrate are used as a substrate pair, the product is α-aceto-α-hydroxybutyrate:



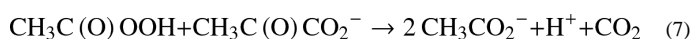
Hence, the alternative name for ALS is acetohydroxy acid synthase (AHAS). α-Aceto-α-hydroxybutyrate is a precursor of isoleucine.

As noted above, ALS II catalyzes a paracatalytic side reaction with O₂. The ultimate product of the reaction is acetate. The mechanism of the reaction catalyzed by *S. typhimurium* ALS II has been studied extensively. The enzyme contains both flavin adenine dinucleotide (FAD) and ThDP within the active site. 4,5-Deaza-FAD (a substitute for FAD that is not competent for one-electron chemistry) and added superoxide dismutase had no effect on O₂ consumption, indicating that the FAD in the active site and adventitious metal ions do not play a role in the oxygenase reaction [(15); see also below]. These data, together with the labeling patterns obtained with ¹⁸O₂, [2-¹³C]pyruvate, and H₂ ¹⁸O were consistent with the oxygenase reaction:



in which FAD plays mostly a structural role (15).

Taken together, the data suggested that a (hydroxyethyl)thiamine diphosphate hydroperoxide intermediate is converted to peracetate [CH₃C(O)O₂⁻] and ThDP [reaction (6)]; the peracetate then reacts with one equivalent of pyruvate, forming two equivalents of acetate (19):



The 2 : 1 stoichiometry between acetate produced and O₂ consumed was confirmed in careful labeling studies with [¹⁸O]O₂ (15, 19). In the normal condensation reaction, the second pyruvate (or α-ketobutyrate) competes with O₂ for reaction with a common intermediate (20). It was noted that the enzyme was eventually inactivated by the products of the reaction (15). This inactivation is presumably paracatalytic, as noted previously, for example, with rabbit muscle fructose 1,6-bisphosphate aldolase during trapping experiments with the external oxidant hexacyanoferrate(III) (21).

Note that the product of the oxygenase reaction catalyzed by *S. typhimurium* ALS II has one less carbon than in the substrate (pyruvate). There is no H₂O₂ produced from the peroxide intermediate in the oxygenase side reaction catalyzed by ALS II [reaction (6)], although a peracid is produced as an intermediate. This situation is similar to that observed for the oxygenase reaction catalyzed by Rubisco, where no H₂O₂ is produced [reaction (3)].

S. typhimurium ALS II was previously reported to either reduce the enzyme-bound FAD or to support attack of the carbanionic intermediate on the isoalloxazine ring of the flavin (22, 23). Recently, it was proposed that *E. coli* ALS II (AHAS) transfers electrons to the enzyme-bound FAD (i.e., reduces the flavin) and produces H₂O₂ as one of the products of the O₂-consuming reaction (24). However, no evidence was provided for H₂O₂ production. Further, as noted above, *S. typhimurium* ALS II does not produce H₂O₂ under aerobic conditions (15, 25), but does produce per-acetate (25). ALS II (*ilvG*) is thought to have evolved from pyruvate oxidase (*poxB*) or from a common ancestor that used the tightly bound FAD cofactor to convert pyruvate and O₂ to acetate, CO₂, and H₂O₂ (26). Rather than reducing O₂ to H₂O₂, pyruvate oxidase transfers electrons in vivo to a lipophilic quinone (Q₁₀).

A number of commercial herbicides, exemplified by sulfonylureas and imidazolinones, are thought to bind to the evolutionary vestige of the quinone-binding site in ALS (27). Consistent with this notion, water-soluble quinones (Q₀ and Q₁) compete with these herbicides for binding to ALS II and can reversibly inhibit the enzyme in a noncompetitive fashion with respect to pyruvate (27). Similar to the binding of quinones to pyruvate oxidase, the herbicides bind most tightly to ALS II after addition of pyruvate (27). In

contrast to pyruvate oxidase, however, *S. typhimurium* ALS II does not reduce Q_0 in the presence of pyruvate (23). On the other hand, if FAD at the active site of ALS II is photoreduced, the enzyme is able to reduce Q_0 . However, the enzyme does not catalyze this reaction. Half of the enzyme-bound photoreduced flavin is oxidized by Q_0 at a rate similar to the rate at which free FAD is oxidized by Q_0 ($>10^8 \text{ M}^{-1} \text{ s}^{-1}$). The other half of the enzyme-bound photoreduced flavin is oxidized more slowly ($28,200 \text{ M}^{-1} \text{ s}^{-1}$) (23).

These data suggest that the flavin quenching observed for *E. coli* ALS II (24) and *S. typhimurium* ALS II (22, 23) is not due to a reduction of the flavin to FADH_2 , but rather to a reversible attack of the carbanion on the flavin isoalloxazine ring. Furthermore, ALS II, which has been stripped of its tightly bound flavin and reconstituted with FAD, no longer exhibits quenching of flavin absorbance upon addition of pyruvate (28). Substitution of FAD with flavins that are incompetent for one electron transfer (e.g., 8-demethyl-8-chloro-FAD, 5-deaza-5-carba-FAD, or 8-demethyl-8-hydroxy-5-deaza-5-carba-FAD) produces a catalytically competent ALS II, exhibiting 84, 100, or 57% specific activity, respectively, relative to that of enzyme containing FAD (23). Similarly, substitution of the FAD in the active sites of ALS I or ALS III (two additional isozymes of ALS) with 8-demethyl-8-hydroxy-5-deaza-5-carba-FAD has only modest effects on the specific activity of their synthase reactions [$2 \text{ pyruvates} \rightarrow \text{acetolactate} + \text{CO}_2$; reaction (4)], exhibiting 98 and 39% specific activity, respectively, relative to that of enzyme containing FAD (23). By contrast, substitution of the FAD in pyruvate oxidase with 8-demethyl-8-hydroxy-5-deaza-5-carba-FAD results in a catalytically incompetent enzyme ($<2\%$ activity) (23).

The accumulated evidence strongly suggests that O_2 reacts directly with the carbanionic intermediate (radical form) generated by the ALS II-catalyzed decarboxylation of pyruvate. The commercial herbicides that inhibit ALS II (e.g., sulfometuron methyl, a sulfonylurea) trap an O_2 -sensitive reaction intermediate on the enzyme, thereby inducing paracatalytic inactivation of the enzyme by O_2 (29). The structures of several ALS-sulfonylurea complexes have been reported (18, 30–32). The crystal structures and structures deduced by homology modeling are consistent with the herbicides closing the active site. Paracatalytic inactivation of ALS, induced by interaction with commercial herbicides, is an important aspect that dramatically enhances herbicidal activity at the level of the whole plant. The peracetate produced in the oxygenase reaction of ALS II is a highly reactive oxidant which will react readily with thiols ($6000 \text{ M}^{-1} \text{ s}^{-1}$), pyruvate ($20 \text{ M}^{-1} \text{ s}^{-1}$), and various assay components (e.g., buffers, cofactors, and other enzymes) (25, 33). Peracetate also reacts with various metals, such as Co^{2+} ($2000 \text{ M}^{-1} \text{ s}^{-1}$), Mn^{2+} , Pb^{2+} , and Fe^{3+} , to produce molecular O_2 , light, and other products (e.g., CO_2 and methane) (13, 19, 25, 33). The light produced by peracetate is likely to be a consequence of radiation produced by an activated metal, as it does not have the proper spectral characteristics to be produced by decay of singlet oxygen to its triplet state (13, 19, 25).

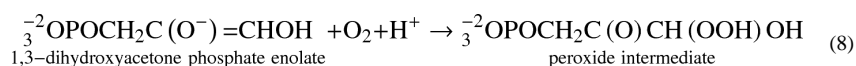
C. PYRUVATE DECARBOXYLASE

Yeast PDC, which catalyzes the decarboxylation of pyruvate to acetaldehyde, utilizes ThDP as a cofactor. The enzyme catalyzes formation of the same (hydroxyethyl)thiamine diphosphate intermediate as ALS II, but lacks the flavin in the active site (15, 25). This enzyme was shown to catalyze an oxygenase side reaction, the end product of which is acetate (15, 25). The reaction proceeds through the oxidation of pyruvate with peracetate as shown for ALS II [reactions (6) and (7)]. The corresponding PDC from *Zymomonas mobilis* supports a somewhat higher level of oxygenase activity than the yeast enzyme (25). The *Z. mobilis* PDC oxygenase activity is about twice that of yeast PDC in the presence of 50 mM 2-(*N*-morpholino)ethanesulfonic acid (MES)-NaOH (pH 6.0), 10 mM MgCl_2 , 0.1 mM ThDP, and 25 mM sodium pyruvate (33 versus $16 \text{ nmol min}^{-1} \text{ mg}^{-1}$, respectively) (25).

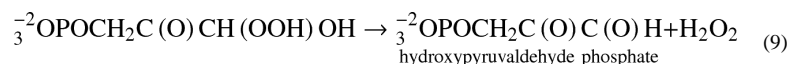
Yeast PDC, *Z. mobilis* PDC, and ALS II were confirmed to produce a peracid as their initial oxygenase product by relying on the more rapid interaction of peracetate with 5-thionitrobenzoate (TNB) than with pyruvate (25). By using TNB, a continuous assay for the formation of peracetate was devised for both ALS II and the *Z. mobilis* PDC. The activity of this assay was dependent on the respective substrate and O₂. This assay could also distinguish between the production of H₂O₂ and peracetate, due to the much greater reactivity of peracetate toward TNB (25). Under the conditions used for assay of ALS II, for example, TNB reacted about 3000 times faster with peracetate (6000 M⁻¹ s⁻¹) than with H₂O₂ (2 M⁻¹ s⁻¹).

D. ALDOLASES

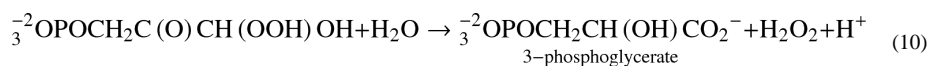
S. aureus fructose 1,6-bisphosphate aldolase is a class I aldolase. Class I aldolases do not require a cofactor but make use of Schiff-base chemistry involving a lysine residue during the catalytic cycle. *S. aureus* fructose 1,6-bisphosphate aldolase catalyzes the slow uptake of O₂ in the presence of substrate (15). For every equivalent of O₂ taken up, 0.25 equivalent of hydroxypyruvaldehyde phosphate and 0.25 equivalent of 3-phosphoglycerate are generated. Evidently, the hydroperoxide formed from the carbanionic intermediate (1,3-dihydroxyacetone phosphate, enolate form) plus O₂:



can break down to form either hydroxypyruvaldehyde phosphate and H₂O₂:



or 3-phosphoglycerate and H₂O₂:



Interestingly, the enzyme undergoes inactivation during the course of the reaction (15). It should be pointed out that hydroxypyruvaldehyde phosphate in the form of its hydrate (gem-diol) can isomerize to 3-phosphoglycerate under alkaline conditions (19).

In later work, the ability of two metal-containing class II aldolases to catalyze oxygenase-type side reactions was investigated (16, 17). It was found that *E. coli* l-rhamnulose 1-phosphate aldolase catalyzes an O₂-consuming side reaction. In a fashion similar to Rubisco, this enzyme catalyzes the formation of an ene-diolate intermediate. However, the Rubisco oxygenase reaction is unlike that of l-rhamnulose 1-phosphate aldolase, in that the former reaction results in scission of the intermediate with no production of H₂O₂ [reaction (3)], whereas in the side reaction catalyzed by l-rhamnulose 1-phosphate aldolase, H₂O₂ is generated in amounts roughly stoichiometric with O₂ consumption (16).

Unlike *E. coli* l-rhamnulose 1-phosphate aldolase, *E. coli* l-fuculose 1-phosphate aldolase is unable to support an oxygenase/oxidase side reaction (16). Despite limited sequence homology, l-rhamnulose 1-phosphate aldolase and l-fuculose 1-phosphate aldolase are structurally and mechanistically similar (34–36). Thus, some carbanion-forming enzymes have evolved the ability to either fully protect intermediates from attack by O₂ whereas others have not (15) or have substantially more radical-forming character than others have (13).

E. MECHANISMS

The question arises as to why some carbanion-forming enzymes catalyze oxygenase/oxidase side reactions, whereas others do not. In the absence of stabilizing factors, any peroxide intermediate formed between carbanion and O₂ could decompose back to carbanion and O₂. Stabilizing factors could include protonation of the peroxide anion and/or metal coordination (15). These factors may differ even within conserved active sites in closely related enzymes, due to variations in the conformational mobility of the protein. For example, the difference observed in the oxygenase activity between yeast PDC and *Z. mobilis* PDC may correlate with differences in the role of conformational changes in regulation of the enzymes. Whereas the yeast enzyme is subject to allosteric activation by pyruvate, the *Z. mobilis* enzyme is not (37).

It is interesting to note that a metal cofactor is not an absolute requirement for an enzyme to catalyze a side reaction with molecular O₂. For example, *S. aureus* fructose 1,6-bisphosphate aldolase, but not the rabbit muscle fructose 1,6-bisphosphate aldolase, catalyzes an oxygenase side reaction (15). Although the former enzyme was initially misidentified as a class II (metal-dependent) aldolase (15–17), neither of these enzymes possesses a metal cofactor. Thus, with *S. aureus* fructose 1,6-bisphosphate aldolase and rabbit muscle fructose 1,6-bisphosphate aldolase, the difference in reactivity toward molecular O₂ is not related to the presence of metal cofactor.

Nevertheless, in other cases a metal cofactor may be essential for an enzyme to carry out side reactions with molecular O₂. For example, both L-rhamnulose 1-phosphate aldolase and L-fuculose 1-phosphate aldolase contain Zn²⁺ as a cofactor. The substrates for these two enzymes are epimeric at a single position, and both enzymes catalyze similar reactions (35, 36). However, as noted above, only the former enzyme catalyzes an oxidase reaction. Interestingly, L-rhamnulose 1-phosphate aldolase has substantially greater oxidase activity with Co²⁺ or Mn²⁺ as the activating metal than with the natural cofactor Zn²⁺. Similar to the oxygenase activity of the *Rhodospirillum rubrum* Rubisco, where replacement of active site Mg²⁺ with Co²⁺ results in an enzyme with exclusive oxygenase activity (38), various metals support different relative levels of oxidase activities with the rhamnulose 1-phosphate aldolase. Although different metal ions cause different relative amounts of “normal” versus oxidase/oxygenase activities for both rhamnulose 1-phosphate aldolase and Rubisco, the rank order in which metals support these two activities varies. Moreover, substitution of various metals for Mg²⁺ in the oxygenase reaction of ALS II has only a modest effect on the relative levels of synthase and oxygenase activity (20). Clearly, the relative oxygenase/oxidase activities are not solely a feature of the activating metal ion, but also depend on the structure of the active site.

A summary of the carbanion-generating enzymes (excluding PLP-containing enzymes) currently known to catalyze oxygenase/oxidase side reactions is given in Table 1.

V. Oxidation Reactions Catalyzed by PLP-Containing Amino Acid Decarboxylases

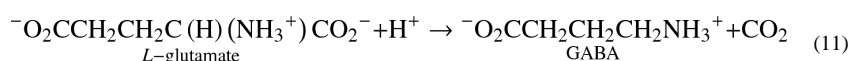
A. BACKGROUND

Healy and Christen in 1973 described the first paracatalytic reaction catalyzed by a PLP-dependent enzyme (2). These authors showed that in the presence of the amino acid substrate analog D,L-erythro-β-hydroxy aspartate, cytosolic pig heart aspartate aminotransferase catalyzes reduction of the artificial electron acceptor (oxidant) hexacyanoferrate(III). No enzyme-catalyzed reduction was observed in the absence of amino acid (2). At that time, however, no PLP-containing enzyme was known to interact

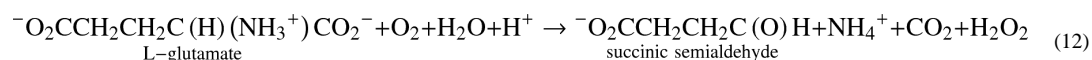
paracatalytically with O₂. Subsequently, several PLP-dependent decarboxylases were shown to react directly with O₂, and these enzymes are described individually below. In three cases [glutamate decarboxylase (GAD), dopa decarboxylase (DDC), ornithine decarboxylase (ODC)], the enzyme-catalyzed oxidative reaction with O₂ is slower than the enzyme-catalyzed “natural” nonoxidative decarboxylation reaction. However, in one case [phenylacetaldehyde synthase (PAAS)], evolutionary pressure has converted an ancestral decarboxylase that presumably catalyzed a nonoxidative amino acid decarboxylation to an enzyme that catalyzes the stoichiometric oxidative decarboxylation of its amino acid substrate. The considerations above raise the intriguing question of why PLP-dependent decarboxylases, among the many disparate classes of PLP-containing enzymes, are apparently unique in their ability to catalyze paracatalytic reactions with O₂.

B. GLUTAMATE DECARBOXYLASE

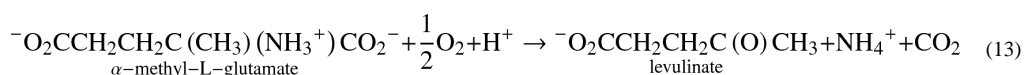
E. coli GAD catalyzes O₂ uptake in the presence of glutamate (15). The nonoxidative and oxidative decarboxylase reactions are



and



respectively. Bertoldi et al. showed that *E. coli* GAD oxidizes α-methyl-L-glutamate to ammonium and levulinate. Only half an equivalent of O₂ was shown to be consumed for every equivalent of ammonium and levulinate produced; no H₂O₂ was detected (39):



Bertoldi et al. stated that GABA is also an oxygenase substrate of *E. coli* GAD (39). Since other authors reported that this enzyme could not support an oxidation reaction with GABA (15, 40), the level of oxidase activity with GABA must be quite low.

More recent work has established that the *E. coli* GAD oxidase reaction has a rapid equilibrium ordered kinetic mechanism in which O₂ traps glutamate in a ternary complex with enzyme that turns over to products very slowly (40). Partitioning between the “normal” GABA-forming reaction and the oxidase reaction is shifted dramatically in ²H₂O relative to that in ¹H₂O. The addition rate of O₂ (*k*_{cat}/*K*_m oxygen) and the fraction of glutamate converted to oxidase-specific products (H₂O₂, ammonium, and succinic semialdehyde) are approximately 10-fold greater in ²H₂O than in ¹H₂O. This appears to be a consequence, under steady-state conditions, of a larger fraction of the enzyme intermediate being present as a radical form in ²H₂O than in ¹H₂O. It is the radical intermediate that reacts with O₂.

The recombinant isozymes of human GAD that are either GABA-vesicle associated (GAD65) or localized in the cytosol (GAD67) are sensitive to reversible inhibition by molecular O₂ or by nitric oxide (NO) under anaerobic conditions (41). Two other PLP-dependent decarboxylases, that is, two isozymes of porcine cysteine sulfinic acid decarboxylase (CSAD I and CSAD II), are also sensitive to reversible inhibition by O₂ and NO (41). Unlike molecular O₂, which is a biradical (i.e., contains two unpaired electrons), NO has only a single unpaired electron. It is important to specify that experiments with NO were conducted in the absence of O₂, since NO will react rapidly with O₂ to form other

products. NO is known to bind to various paramagnetic substances and is a commonly used paramagnetic probe.

E. coli GAD, recombinant human GAD65, recombinant human GAD67, recombinant porcine CSAD I, and recombinant porcine CSAD II are remarkably similar with respect to their sensitivity to reversible inhibition by O₂ ($K_I=0.2$ to 0.5 mM) or NO ($K_I=0.2$ to 0.5 mM) (41). However, the inhibition does not reflect the ability of the enzymes to use O₂ as a substrate. (The ability of porcine CSAD I and CSAD II to catalyze an oxidative decarboxylation reaction has not been studied.) Human GAD65 does not consume O₂ (as assessed with an O₂ electrode) or produce H₂O₂ (as assessed by the glutathione–glutathione peroxidase coupled reaction) at levels comparable to those observed for the *E. coli* enzyme (42). Also, the rate at which the *E. coli* enzyme utilizes O₂ in the H₂O₂/NH₄⁺/succinic semialdehyde-forming reaction [reaction (12)] is disproportionate to the effect that O₂ has on the GABA-forming reaction (41). Under normal atmospheric conditions, the oxidase reaction catalyzed by *E. coli* GAD proceeds at approximately 0.2% of the rate of the GABA-forming reaction. However, under the same conditions, O₂ inhibits the GABA-forming reaction by 30% relative to the reaction rate observed under anaerobic conditions. In the presence of 1 atm of pure O₂, the GABA-forming reaction is inhibited by 70%. However, under these conditions, the rate of the oxidase reaction is 1.2% of the rate of the GABA-forming reaction.

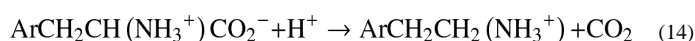
Clearly, there is more O₂-induced inhibition of the GABA-forming reaction than can be accounted for by diversion of glutamate into the oxidase reaction. Inhibition of the GABA-forming reaction correlates with saturation of the H₂O₂/succinic semialdehyde/NH₄⁺-forming reaction. Oxygen ($K_m=0.56$ mM) traps glutamate ($K_{IA}=13$ mM) into a complex that turns over to products (H₂O₂, succinic semialdehyde, and NH₄⁺) very slowly (0.9 min⁻¹). Although the complex formed between O₂ and human GAD65 does not turn over at a rate comparable to that of *E. coli* GAD, the rate of O₂ release is likely to be similar, since the amount of inhibition (uncompetitive, $K_{II}=0.2$ mM) of the GABA-forming reaction by O₂ is comparable. In the presence of O₂, GAD should exhibit slow binding kinetics, due to the slow rate of O₂ release ($t_{1/2}=0.8$ min). [See the article by Schloss (43) for a discussion of slow-binding enzyme inhibition and its relationship to reaction-intermediate analogs.] Further, assuming that O₂ actually binds rapidly to the radical enzyme form that is O₂ sensitive, the abundance of the radical form can be estimated. In ¹H₂O, k_{cat}/K_m for O₂ is 27 M⁻¹ s⁻¹ and increases to 175 M⁻¹ s⁻¹ in ²H₂O (40). If O₂ actually binds to the radical at a diffusion-limited rate [about 2.5×10^9 M⁻¹ s⁻¹ (13)], only 0.000001% of the enzyme exists in a radical form in steady state. The fraction of GAD existing as a radical in steady state would increase to 0.000007% in ²H₂O.

Unlike the *E. coli* GAD, human GAD65 is inactivated under aerobic assay conditions in the absence of various thiols. This has precluded the same type of detailed kinetic analysis of human GAD65 that has recently been completed for *E. coli* GAD. Attempts to determine whether human GAD65 can support an oxidase reaction under any conditions, similar to *E. coli* GAD, have met with mixed results (42). However, it has been reported that pig brain GAD produces succinic semialdehyde in the presence of glutamate, with concomitant inactivation of the enzyme by transamination of the PLP cofactor to PMP (44). A similar inactivation of pig brain GAD by aspartate has also been reported (45). It is unclear whether the inactivation of pig brain GAD by transamination is in addition to and independent of interaction of the enzyme with O₂ or whether this may also be an O₂-dependent process. However, inhibition of human GAD65 by O₂, which would interfere with loading of GABA vesicles and GABAergic synaptic function in the brain, is likely to be responsible for O₂-induced seizures (41) (see also Section XIII). Under conditions of hyperbaric oxygen, an unusual cyclic change in the concentration of GAD67 (cytoplasmic GAD), but not in

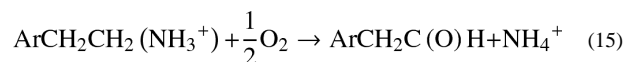
GAD65 (GABA vesicle-bound GAD), has been observed (46). Presumably, this cyclic change in the intracellular level of GAD67 is related to the reversible inhibition of GAD by oxygen, since there was no irreversible loss of either GAD65 or GAD67 during the period of observation.

C. DOPA DECARBOXYLASE

Bertoldi et al. showed that native pig kidney DDC catalyzes oxidation of aromatic amines (39, 47–53). The pig kidney DDC-catalyzed conversion of L-dopa to dopamine and L-5-hydroxytryptophan to serotonin, however, is about 100 times faster than the enzyme-catalyzed conversion of dopamine to 3,4-dihydroxyphenylacetaldehyde and serotonin to 5-hydroxyindoleacetaldehyde, respectively (51). Ammonium production from the amine is stoichiometric with aldehyde formation and with consumption of half an equivalent of O₂. In initial reports, no H₂O₂ formation was detected (51). However, more recently the production of both H₂O₂ and superoxide was inferred, based on the slower rate of oxygen consumption observed in the presence of catalase or superoxide dismutase (53). The nonoxidative decarboxylation of aromatic amino acids is shown as:

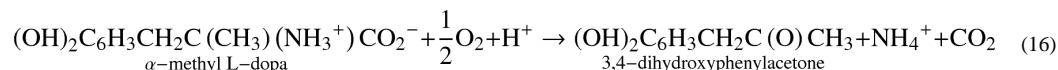


Oxidation of the corresponding amines apparently proceeds with the stoichiometry:



Presumably, the oxygen or superoxide produced in the first round of catalysis is consumed as a substrate in a second round of catalysis (53).

Earlier reports that pig kidney DDC catalyzes an abortive half-transamination with L-dopa under aerobic conditions (54) may require reinterpretation, since it appears that abortive transamination competes with the oxygen-consuming reaction, depending on the availability of O₂ or other oxidants (e.g., H₂O₂ or superoxide) (51, 53). Nevertheless, pig kidney DDC can catalyze half-transamination reactions with (1) B-aromatic amino acids under aerobic conditions, (2) with L-aromatic amino acids, B-aromatic amino acids, and aromatic amines under anaerobic conditions, and (3) α-methyl L-dopa under aerobic conditions (48, 51, 53). With α-methyl L-dopa, the product of oxidative decarboxylation is 3,4-dihydroxyphenylacetone (39, 48, 51, 53):



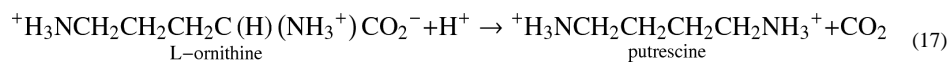
The partitioning ratio between oxidative decarboxylation and abortive transamination with this substrate is about 100 (51). The oxidized products generated from serotonin (47) and dopamine (51) eventually cause inactivation of the enzyme by covalent modifications of the enzyme/cofactor. Thus, molecular O₂ seems to play a role as substrate and as an enzyme regulator.

Bertoldi et al. showed that mutation of tyrosine 332 to phenylalanine converts pig kidney DDC to a decarboxylation-dependent deaminase, in which decarboxylation of amino acid is *stoichiometric* with oxidation (50, 53). Unlike the reaction catalyzed by the native enzyme, the amine product cannot be detected in the reaction catalyzed by the mutated enzyme. However, under aerobic conditions, both native and mutated enzymes catalyze oxidation of dopamine at about the same rate. Under anaerobic conditions, the mutated enzyme exclusively catalyzes a decarboxylation-dependent half-transamination (50, 53).

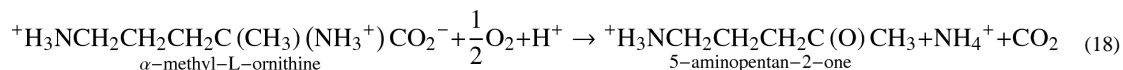
Several cases of dopa decarboxylase deficiency have been reported [reviewed by Allen et al. (55)]. The defect is inherited in an autosomal recessive fashion. Although rare, it is suggested that the incidence might be underreported. Activity may be low to undetectable. Some patients, although not all, are helped by treatment with a monoamine oxidase inhibitor, dopamine antagonist, and pyridoxine. In those patients helped by pyridoxine it is possible that the mutation results in less effective binding of PLP in the active site. Since, as noted above, a relatively conservative change in a tyrosine residue to a phenylalanine residue at the active site converts DDC to an oxidative decarboxylase, it would be interesting to determine whether mutations of DDC that have arisen naturally in the human population may enhance oxidation reactions at the expense of nonoxidative decarboxylation.

D. ORNITHINE DECARBOXYLASE

Bertoldi et al. showed that ODC prepared from *Lactobacillus* 30a catalyzes the oxidative deamination/decarboxylation of L- α -methylornithine (39). The product of the reaction was identified as 2-methyl- Δ^1 -pyrroline, which arises through cyclization/dehydration of the corresponding aldehyde (5-aminopentan-2-one). One equivalent of ammonium was generated at the expense of half an equivalent of O₂ consumed (39). The decarboxylation reaction with L-ornithine is:



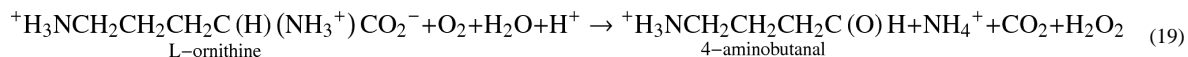
and the oxidative decarboxylation of α -methyl-L-ornithine is:



The authors also found that putrescine is oxidatively deaminated by *Lactobacillus* ODC at a relatively slow rate. Half an equivalent of O₂ was consumed per equivalent of putrescine oxidized (39).

The *Lactobacillus* ODC was shown to catalyze a half-transamination reaction with α -methyl-L-ornithine (39). The partition ratio between oxidative deamination and half-transamination with this substrate was 12 : 1 (39). The *Lactobacillus* ODC is therefore remarkably similar to the *E. coli* GAD in its ability to oxidatively deaminate/decarboxylate α -methyl amino acid substrates and to catalyze a low-efficiency half-transamination of PLP with α -methyl amino acid substrate (39).

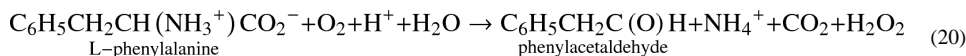
In earlier work (56), Sakai et al. showed that an ODC purified from *Hafnia alvei* catalyzes the oxidative decarboxylation of L-ornithine to 4-aminobutanal:



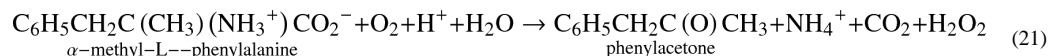
which reversibly cyclizes and dehydrates nonenzymatically to Δ^1 -pyrroline (not shown). The authors named this activity L-ornithine oxidase (decarboxylating) (OOD). Most turnover events at the active site lead to the release of CO₂ and to the formation of putrescine [reaction (17)]. Occasionally (about one turnover in 160), however, the enzyme converts L-ornithine and O₂ to 4-aminobutanal [reaction (19)]. Unlike DDC, which can oxidize dopamine (the end product of the nonoxidative decarboxylation of substrate L-dopa), ODC/OOD could not be shown to oxidize putrescine (the end product of the nonoxidative decarboxylation of substrate L-ornithine) (56).

E. PHENYLACETALDEHYDE SYNTHASE

Recent work has shown that petunia petal PAAS oxidatively converts L-phenylalanine stoichiometrically to phenylacetaldehyde, ammonium, H₂O₂, and CO₂ (57):



Phenylethylamine is neither a product nor a substrate (57). PAAS also converts α-methyl-L-phenylalanine to phenylacetone:



In the presence of catalase, the enzyme can catalyze turnover of phenylalanine to phenylacetaldehyde for several hours without loss of activity. Interestingly, however, in the absence of catalase to trap H₂O₂, PAAS is slowly inactivated (57).

As noted above, native pig kidney DDC catalyzes the *nonoxidative* decarboxylation of dopa to dopamine. However, mutation of an active-site tyrosine residue to a phenylalanine converts pig kidney DDC to an enzyme that stoichiometrically catalyzes an *oxidative* decarboxylation of dopa to an aromatic aldehyde (50, 53). It is interesting to note that during evolution the corresponding residue in the ancestral dopa-decarboxylase-like enzyme was converted from a tyrosine to a phenylalanine in the case of rose PAAS and to a valine in the case of petunia PAAS (57).

An interesting analogous reaction to that catalyzed by PAAS has recently been described in which *N*-hydroxy-L-phenylalanine is oxidatively decarboxylated to *z*-phenylacetaldoxime. The enzyme that catalyzes this reaction, tentatively named *N*-hydroxy-L-phenylalanine decarboxylase/oxidase, was isolated from a *Bacillus* strain and shown to contain only PLP as a cofactor (58). Although not measured, it is probable that H₂O₂ is generated in the reaction.

VI. Mechanisms Contributing to the Oxidation Reactions Catalyzed by PLP-Containing Amino Acid Decarboxylases

The oxidation reactions catalyzed by various PLP-containing decarboxylases are shown in Table 2. *E. coli* GAD, *H. alvei* ODC, and petunia PAAS catalyze the oxidative decarboxylation of their respective amino acid substrates to aldehydes, ammonium, and CO₂ with the stoichiometric production of H₂O₂. Oxygen in the aldehyde moiety of the product is derived from water. These enzymes, therefore, clearly meet the definition of oxidases. In the case of the petunia PAAS reaction, net oxidation is stoichiometric with decarboxylation. However, in the case of the *E. coli* GAD or *H. alvei* ODC, oxidative decarboxylation occurs only about once in every 500 or 160 turnovers, respectively, under normal atmospheric conditions (0.24 mM dissolved O₂; under 21% oxygen at 1 atm).

The putative mechanism by which petunia PAAS catalyzes the oxidative decarboxylation of phenylalanine is shown in Figure 1. We suggest that PAAS catalyzes a biradical mechanism similar to that proposed for *E. coli* GAD (41). There are many possible radical mechanisms that could be written for this reaction. Normally, even for reactions that are commonly accepted to involve radical intermediates, such as those involving the isoalloxazine ring of flavin cofactors, the radical intermediates are not shown. This is because, in most cases, radical forms are short-lived, high-energy intermediates, which are usually difficult to observe directly, due to their low abundance in the steady state. However, in the hypothetical reaction sequence illustrated in Figure 1, radical intermediates are included for

purposes of illustration only. These are not the only possible radical forms, nor are they likely to build up to detectable levels during steady state. The following mechanistic steps include those commonly accepted for PLP-dependent enzymes and additional steps unique to the oxygen-utilizing reactions. In the absence of substrate, the PLP coenzyme forms a Schiff base with the ϵ -amino group of a lysine residue at the enzyme active site (E-PLP aldimine). Binding of phenylalanine at the active site leads to formation of a new Schiff base (Phe-PLP aldimine) in which the α -amino group of phenylalanine (Phe) displaces the ϵ -amino group of the active site lysine. The double bond of the imine is converted from a singlet state (paired electrons) to a triplet state (unpaired electrons) (biradical intermediate 1). Subsequent decarboxylation generates biradical intermediate 2. Since O_2 is a biradical, it should react extremely rapidly with biradical intermediate 2. Addition of O_2 could theoretically occur at either of the two unpaired electrons of the biradical 2 intermediate (41). Here we show the addition of O_2 at the aldehyde carbon of the intermediate, generating biradical intermediate 3. Transfer of an electron from the nitrogen base to the peroxy radical of biradical intermediate 3 will generate a zwitterionic structure containing a peroxy anion and a radical cation (biradical intermediate 4). This zwitterionic structure is expected to be short-lived, producing a hydroperoxy intermediate. Reaction of the ϵ -amino group of a lysine residue in the active site will generate the E-PLP aldimine with the liberation of 1-peroxy-2-phenylethylamine, which undergoes nonenzymatic elimination of H_2O_2 with the formation of 2-phenylethylimine. Hydrolysis of the imine will yield phenylacetaldehyde and ammonium. Generation of a biradical form of ribulose 1,5-bisphosphate from an ene-diol intermediate has been proposed as part of the oxygenase reaction of Rubisco (13). Quantum mechanical calculations have indicated that the energy level of the radical intermediate can be reduced substantially by appropriate adjustment of the molecular geometry (59–61). Since the PLP-imine and other PLP intermediates, especially the quinonoid intermediate, more closely resemble the structure of the flavin isoalloxazine ring, it is likely that these radical intermediates will be even lower in energy than those in the oxygenase reaction catalyzed by Rubisco.

The oxidative decarboxylation reactions catalyzed by *E. coli* GAD and *H. alvei* ODC presumably occur via a radical mechanism similar to that depicted in Figure 1 for petunia PAAS. However, there are differences among the three enzymes. Petunia PAAS has evolved to ensure maximal oxidation of substrate intermediate, so that the oxidation is stoichiometric with decarboxylation. On the other hand, the other two enzymes have either evolved to exclude O_2 from the active site or to reduce the steady-state levels of radical intermediates available for reaction with O_2 . Whichever method is employed, the oxidase reaction is not totally eliminated, since the oxidative decarboxylation reaction still occurs at 0.2 and 0.6% of the nonoxidative reaction rates, respectively. It will be interesting to compare the active sites of these enzymes to determine those steric factors that may exclude O_2 from the active site or that affect the steady-state levels of radical intermediates.

PAAS, *E. coli* GAD, and *H. alvei* ODC clearly catalyze oxidase reactions as O_2 consumption in the presence of L-amino acid substrate is stoichiometrically coupled to ammonium and H_2O_2 production. However, the situation with the pig kidney DDC is less clear. As noted above, in the oxidation reactions catalyzed by DDC, H_2O_2 cannot be detected directly and only half an equivalent of O_2 is consumed per equivalent of substrate oxidized. There are several possible explanations for this observation. If DDC were to utilize H_2O_2 produced in one round of catalysis as an oxidant in a second round of oxidation, two equivalents of aldehyde or ketone would be produced for each equivalent of oxygen consumed. In this explanation, H_2O_2 would be expected to serve as a substrate for the oxidation reaction. Such an explanation could be consistent with the recent observation that the rate of oxygen consumption by DDC is reduced in the presence of catalase or superoxide dismutase (53). To the best of our knowledge, H_2O_2 has not been tested as an alternative

substrate for any of the pig kidney DDC oxygen-consuming reactions. A second possible explanation for the inability to detect the H_2O_2 intermediate directly would be that the pig kidney DDC was prepared by including 0.1 mM dithiothreitol to preserve the enzyme activity (47). Any thiol present in the assay would effectively prevent detection of H_2O_2 produced by DDC (25). However, the interference by thiols in assaying for H_2O_2 would not explain the 1 : 2 stoichiometry between O_2 consumed and aldehyde or ketone produced, which was observed for DDC (47, 48, 51), *Lactobacillus 30a* ODC (39), and *E. coli* GAD (39). Since a 1 : 1 stoichiometry between O_2 consumed and H_2O_2 /aldehyde produced has been observed by other investigators for one type of bacterial ODC (56), *E. coli* GAD (15, 40, 41), and PAAS (57), there are clearly problems in the literature that need to be resolved.

Another point of interest is that all the native decarboxylases listed in Table 3, except pig kidney DDC, can oxidatively decarboxylate their L-amino acid substrates with varying efficiencies, but cannot effectively oxidize the amine product when this is added directly to the enzyme. On the other hand, pig kidney DDC can oxidize aromatic amines, but not the L-amino acid substrate. It would seem, however, that not much perturbation (either of the active site itself or with a modified substrate) is required to enable pig kidney DDC to catalyze an oxidative decarboxylation. Thus, as noted above, a Tyr \rightarrow Phe mutation at position 322 results in an enzyme that can effectively catalyze oxidative decarboxylation of dopa. Additionally, the native enzyme can catalyze the oxidative decarboxylation of L- α -methyldopa (51, 53).

Vitamin B₆ in its active form (usually, PLP, but occasionally, PMP) is a remarkably versatile biological catalyst. Enzyme-catalyzed reactions in which PLP is a cofactor include, but are not limited to, racemization, transamination, α -decarboxylation, β -decarboxylation, β -decarboxylation–addition, reverse aldol condensation, α,β -elimination, α,β -replacement, β,γ -elimination, and β,γ -replacement (62, 63). Most reactions catalyzed by vitamin B₆-containing enzymes are not generally considered to involve radical intermediates. The mechanism of almost all such enzymes is thought to be based on their ability to stabilize high-energy anionic intermediates in their reaction pathways by the pyridinium moiety of PLP/PMP (64). However, a few exceptions are known. One example is *Clostridium subterminale* lysine 2,3-aminomutase (65). This enzyme catalyzes the PLP-dependent interconversion of L-lysine and L- β -lysine. The reaction mechanism includes one-electron chemistry and uses a [4Fe–4S] cluster and S-adenosylmethionine (65). Another example of a PLP enzyme that catalyzes a radical mechanism is *Clostridium stricklandii* lysine 5,6-aminomutase. This enzyme catalyzes an adenosylcobalamin (vitamin B₁₂)–dependent interconversion of D-lysine with 2,5-diaminohexanoate and of L- β -lysine with 3,5-diaminohexanoate (66). A third vitamin B₆-dependent enzyme that participates in a radical mechanism is CDP-6-deoxy-L-threo-D-glycero-4-hexulose-3-dehydrase. This enzyme catalyzes the C-3 deoxygenation in the biosynthesis of 3,6-dideoxyhexoses in *Yersinia pseudotuberculosis* and is a PMP-dependent enzyme that also contains a [2Fe–2S] center (67).

Based on the findings that several PLP-containing decarboxylases can catalyze oxidations with O_2 as side reactions (and in at least one case an oxidative decarboxylation that is the predominant pathway), it would seem that the potential for PLP to participate in radical mechanisms is more widespread than is generally appreciated.

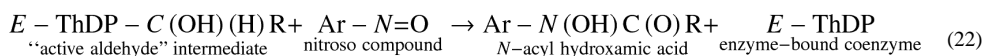
VII. Paracatalytic Oxidation of the α -Ketoglutarate Dehydrogenase–Generated Carbanion

α -Ketoglutarate dehydrogenase ($\text{E}_{1\text{k}}$) is a member of a family of α -keto acid dehydrogenases that also includes pyruvate dehydrogenase ($\text{E}_{1\text{p}}$) and the branched-chain α -

keto acid dehydrogenase (E₁b). The enzymes catalyze the first and rate-controlling steps of the overall reactions within the α -ketoglutarate dehydrogenase complex (KGDHC), pyruvate dehydrogenase complex (PDHC), and branched-chain α -keto acid dehydrogenase complex (BCDHC), respectively. These enzyme complexes are similar in their architecture and catalytic mechanism. Both PDHC and KGDHC have been shown to catalyze reactions with external oxidants, which can lead to paracatalytic inactivation. For example, pig heart PDHC was shown to be paracatalytically inactivated in the presence of [¹⁴C]pyruvate, ThDP, and the oxidant DCIP (68). Inactivation was accompanied by incorporation of radioactivity into the PDHC complex, mostly into the E₂p component (68). However, most of the work on the paracatalytic reactions of α -keto acid dehydrogenases has been carried out with KGDHC. Therefore, most of the discussion in this section is devoted to this enzyme complex.

The α -ketoglutarate dehydrogenase complex (KGDHC) self-assembles from multiple copies of α -ketoglutarate dehydrogenase (E₁k), dihydrolipoamide succinyl transferase (E₂k), and dihydrolipoamide dehydrogenase (E₃). KGDHC catalyzes the oxidative decarboxylation of α -ketoglutarate (KG) with the concomitant formation of succinyl-CoA and reduction of NAD⁺ to NADH (Figure 2). E₁k is a ThDP-dependent enzyme that generates a carbanionic intermediate after the ThDP-dependent decarboxylation of the substrate (Figure 2, reaction (1)). In the course of the overall reaction this intermediate undergoes oxidation by the lipoyl moiety covalently bound to the second enzyme of the complex, E₂k (Figure 2, reaction (2)). Free lipoate and lipoamide are relatively poor substrates of the α -keto acid dehydrogenases compared to the protein-bound lipoyl group (69, 70). Nevertheless, free lipoate and lipoamide may be reductively acylated in the E₁k-catalyzed reaction. Also, a number of small-molecular-weight electron acceptors may participate in the E₁k-catalyzed reaction and can therefore be considered as paracatalytic substrates of E₁k. Several aspects of these paracatalytic reactions deserve attention regarding their potential pathophysiological significance.

First, the E₁k-catalyzed oxidation of α -keto acids is supported by structurally and chemically different oxidants (electrophiles) (Table 4). Reaction rates at saturating concentrations of different oxidants may vary by more than an order of magnitude, but similar saturation with KG is observed with these oxidants (Table 4). Thus, the K_m^{KG} values determined for the paracatalytic reactions are close to the K_d^{KG} values measured in independent experiments under equilibrium conditions (74). This finding shows that the E₁k-generated carbanion is formed rapidly compared to the subsequent oxidation step. That is, the oxidoreduction step, which is the slowest step of the overall reaction (reaction (2) in Figure 2), is also slow compared to carbanion formation during paracatalytic side reactions. Most of the paracatalytic substrates [hexacyanoferrate(III), DCIP, 4-chloro-7-nitro-2,1,3-benzoxadiazole (NBD-Cl), tetranitromethane] of E₁k act as oxidants. After their reduction by the carbanionic intermediate at the active site, the resulting succinyl residue is transferred from succinyl-ThDP to water. However, the reaction with 4-chloronitrosobenzene includes both an oxidoreduction step and an acyl transfer step, producing an *N*-succinyl hydroxamic acid (72, 75–77). Analogous reactions are known for other ThDP-dependent enzymes transforming the “active aldehyde” intermediate of the “natural” substrate in the presence of 4-chloronitrosobenzene to an *N*-acyl hydroxamic acid (75). The overall reaction catalyzed by such an enzyme (E) is depicted by:



where R = $-\text{CH}_3$, $-\text{CH}_2\text{OH}$, and $-\text{CH}_2\text{CH}_2\text{CO}_2\text{H}$ for reactions catalyzed by pyruvate dehydrogenase ($\text{E}_{1\text{p}}$), transketolase, and $\text{E}_{1\text{k}}$, respectively. This finding implicates ThDP-containing enzymes in the metabolic transformation of aromatic nitroso compounds.

A unique feature of $\text{E}_{1\text{k}}$ in this paracatalytic reaction is that in addition to the *N*-acyl hydroxamic acid, $\text{E}_{1\text{k}}$ also produces significant amounts of other products. These products include some highly polar unidentified material and *N*-succinyl 1-hydroxy-4-chloroaniline (75–77). The *N*-succinyl 1-hydroxy-4-chloroaniline presumably arises through a Bamberger rearrangement, where the $-\text{OH}$ group migrates from the nitrogen to the aromatic ring (76). The distribution of the products among the different pathways depends on the reaction conditions (e.g., salts, pH) (77). The distribution also depends on the enzyme source, presumably due to subtle differences in active-site structures (72). Table 4 shows that compared to *E. coli* $\text{E}_{1\text{k}}$, both bovine and porcine $\text{E}_{1\text{k}}$ exhibit high affinities and high rates for the paracatalytic reaction with 4-chloronitrosobenzene. This is accompanied by a significant decrease in *N*-succinyl hydroxamic acid production in favor of the other products originating from an electrophilic aromatic intermediate stabilized at the $\text{E}_{1\text{k}}$ active site.

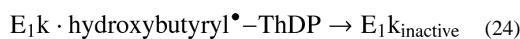
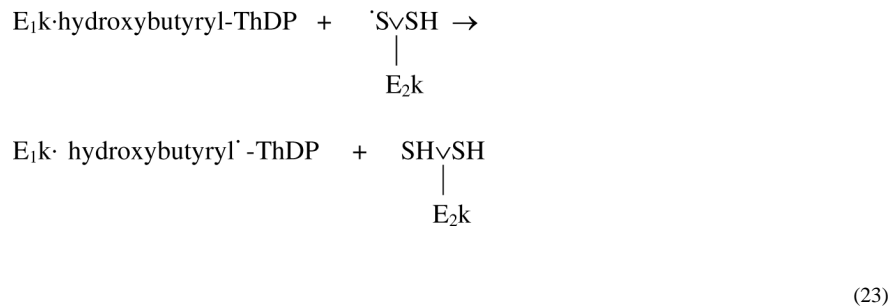
Second, the $\text{E}_{1\text{k}}$ -catalyzed paracatalytic reactions occur not only with isolated $\text{E}_{1\text{k}}$, which is *not* the *in vivo* form of the enzyme, but also with the $\text{E}_{1\text{k}}$ in its natural complex-bound state. Moreover, an increase in the maximal reaction rates with artificial electron acceptors in intact KGDHC compared to those with isolated $\text{E}_{1\text{k}}$ is observed, probably due to the overall stabilization of $\text{E}_{1\text{k}}$ when bound to the complex (Table 5). Thus, incorporation of $\text{E}_{1\text{k}}$ into the native structure of the multienzyme complex does not prevent the paracatalytic reactions. These findings further support the potential significance of such reactions *in vivo*.

Third, an irreversible inactivation of $\text{E}_{1\text{k}}$ occurs in the course of both paracatalytic and native reactions (78). This process is different from the inactivation resulting from the nonspecific action of oxidants *per se*. The catalysis-associated inactivation involves a reactive catalytic intermediate formed during the oxidoreduction step in the presence of both the α -keto acid and oxidant substrates of $\text{E}_{1\text{k}}$. The fate of this intermediate may differ depending on the surrounding medium, and in particular, on the substrates that are present. Table 5 shows that the hexacyanoferrate(III)-dependent oxidation of KG is characterized by an apparent rate constant for irreversible inactivation (k_{in}) $\sim 0.1 \text{ min}^{-1}$. With another α -keto dicarboxylate substrate of $\text{E}_{1\text{k}}$, α -keto adipate, the k_{in} value is increased up to fivefold, while dicarboxylates such as glutarate, succinate, and malonate protect $\text{E}_{1\text{k}}$ from the inactivation (78).

What are the intermediates and/or side reactions leading to $\text{E}_{1\text{k}}$ inactivation? As shown in Fig. 2, the $\text{E}_{1\text{k}}$ carbanionic intermediate is normally oxidized by a two-electron acceptor, the $\text{E}_{2\text{k}}$ -bound lipoyl residue (reaction (2)). However, the intermediate can also be efficiently oxidized by a one-electron acceptor such as hexacyanoferrate(III) (Table 4). A radical of the carbanionic intermediate of isolated $\text{E}_{1\text{k}}$ from *E. coli* was observed by EPR, presumably resulting from the one-electron oxidation of the intermediate by molecular oxygen (79). Similar one-electron oxidation reactions take place in pyruvate:ferridoxin oxidoreductases and in chemical models of key intermediates of thiamine catalysis [reviewed by Bunik and Sievers (80)]. A reactive intermediate containing an uncoupled electron generated in the course of a one-electron oxidation could induce a modification of the $\text{E}_{1\text{k}}$ active site leading to KGDHC inactivation. This would explain the first-order loss of activity of $\text{E}_{1\text{k}}$ operating through a one-electron oxidation in the presence of hexacyanoferrate(III) (Table 4). A strong oxidant of the $\text{E}_{1\text{k}}$ -generated carbanion is the stabilized thiyl radical of the complex-bound lipoyl residue. Experiments on the substrate-dependent reduction of the α -keto acid dehydrogenase complexes under anaerobic conditions showed that there is transient formation of complex-bound thiyl radicals of lipoyl residues, which arises from the

semiquinone species of the E₃-bound FAD (80). In the course of the anaerobic reduction of KGDHC, a carbon-centered radical and a complex-bound thiyl radical were trapped with 5,5'-dimethyl-1-pyrroline-*N*-oxide (DMPO) and α -phenyl-*tert*-butylnitron, respectively. Production of these radicals coincided with E₁k inactivation. These findings support the mechanism of E₁k inactivation during catalysis.

When terminal two-electron oxidation is perturbed or totally blocked (e.g., at limiting or no NAD⁺), thiyl radicals of the complex-bound lipoyl moieties may be formed. As shown by Bunik and Sievers (80), these radicals react with the E₁k-generated carbanion through one-electron oxidation (reaction (23), with the resulting carbon-centered radical species inactivating E₁k according to the following reactions:



Further evidence for this mechanism of the E₁k inactivation is provided by protection from the inactivation by a known thiyl radical scavenger thioredoxin (81–83). Thioredoxin prevents the substrate-induced inactivation by catalyzing dismutation of the complex-bound thiyl radicals, which arise upon the KGDHC reduction with KG and CoA (Figure 3). Remarkably, the formation of the thiyl radicals is transient under anaerobic conditions, but becomes permanent in the presence of O₂ because O₂ stimulates formation of the semiquinone species of the complex-bound FADH₂ through its one-electron oxidation (80, 82). That is, at saturating concentrations of KG and CoA, the E₂k-bound dihydrolipoyl residues and E₃-bound FAD of KGDHC are reduced (reactions (1) to (4) in Figure 2). At low or zero concentrations of NAD⁺, O₂ competitively oxidizes E₃, with the concomitant formation of O₂^{•-} and semiquinone form of the E₃-bound FAD. The semiquinone is in redox equilibrium with the catalytic thiol groups of KGDHC. This redox equilibrium gives rise to the permanent O₂-dependent generation of the complex-bound thiyl radicals of the lipoyl residues. As a result, the E₁k component is inactivated according to reactions (23) and (24) under both aerobic and anaerobic conditions. However, generation of the inactivating thiyl radicals under anaerobic conditions occurs by different mechanisms (incomplete reduction versus an oxidase reaction) and kinetics (transient versus permanent). The oxidative decarboxylation of KG may thus proceed under low concentrations of NAD⁺ only when thioredoxin protects E₁k by dismutating the complex-bound thiyl radicals (Figure 3). Under these conditions, however, the electron flow to O₂ is not blocked by E₁k inactivation, leading to accumulation of the superoxide anion radical and the product of its dismutation, H₂O₂ (80). Studies with isolated mitochondria (84, 85) and cultivated neurons (86) indicated the significance of KGDHC for the production of ROS in vivo. Thus, accumulation of ROS produced by KGDHC and interaction of these ROS with cellular metabolites may complicate the transformation pathways of the E₁k reactive intermediates in vivo.

It is possible that there are several mechanisms for the paracatalytic inactivation of E₁k, arising under conditions that stabilize different reactive intermediates. Some of these inactivation mechanisms may be due to the oxygenase side reaction of a catalysis-generated intermediate, which results in peracid formation. Such an oxygenase reaction at the E₁k active site is supported by the observation that under aerobic conditions, TNB formed upon thiol modification of E₁k by 5,5'-dithiobis(2-nitrobenzoic acid) is consumed in the presence of KG (87). As pointed out above, TNB consumption may be indicative of peracid formation resulting from the oxidase/oxygenase reaction of carbanions generated by ThDP-dependent enzymes (25). A peracid intermediate analogous to that observed in the PDC- and ALS II-catalyzed oxygenase reactions may well be involved, therefore, in the E₁k-catalyzed process, explaining why paracatalytic inactivation occurs not only in the reaction with the one-electron acceptor hexacyanoferrate(III), but also with the two-electron acceptor DCIP, and during the course of the native reaction with the lipoyl-bound moiety at saturating NAD⁺. Moreover, an O₂-dependent reaction on E₁k would explain the rather strong α -keto acid-dependent inactivation observed with some preparations of E₁k (88). This inactivation varies with the α -keto acid and E₁k preparation. These variations may be due to differences in the stability of reactive intermediates and to structural differences among the isolated E₁k preparations, resulting, for example, from binding of regulatory metal ions to the enzyme and/or posttranslational modifications. As shown above with other enzymes, such factors greatly affect the paracatalytic oxidase/oxygenase reactions.

Recently, the isolated E₁k component of the KGDHC from *E. coli* was shown to catalyze an O₂-dependent reaction with KG, the product of which could be detected by the Amplex Red-peroxidase assay (79). Because formation of resorufin in the Amplex Red assay is known to be catalyzed by peroxidase in the presence of H₂O₂, the authors concluded that H₂O₂ is the product of the E₁k-catalyzed reaction. However, although the Amplex Red-peroxidase kit is used to assay H₂O₂-generating enzymes, it cannot be employed as an analytical tool to prove H₂O₂ production. In particular, N-C bond scission, which in Amplex Red leads to resorufin, is known to be catalyzed by peroxidase in the presence not only of H₂O₂ but also of peracids (89, 90). Moreover, peracids may generate hydrogen peroxide hydrolytically (90). Taken together, formation of resorufin, interpreted by Frank et al. (79) as H₂O₂ generation by E₁k, is also consistent with an E₁k-dependent oxygenase reaction, resulting in peracid formation. EPR detection of the carbanion/enamine-ThDP radical intermediate in the presence of KG and oxygen (79) led Frank et al. to assume that the peroxide production occurs through two successive 1e⁻ oxidations of the carbanion/enamine-ThDP intermediate by two molecules of O₂, with the resulting superoxide anion radical dismutated to the peroxide. However, the radical intermediate detected could arise from secondary reactions in the presence of peracid or its decomposition product H₂O₂, or in a side reaction concomitant with the peracid formation. In addition, the suggested ping-pong mechanism, in which the ThDP radical intermediate is stable enough to let one superoxide anion radical leave the E₁k active site, and the other oxygen molecule arrive to accept the second electron, seems much less probable than the reaction sequence proposed for other ThDP-dependent enzymes in which the carbanionic intermediate reacts with O₂, resulting in peracid formation (25). Remarkably, the resorufin accumulation curves at 1 mM KG do not show any significant inactivation of the enzyme in the course of this reaction (79). This is in accord with our study of pig heart KGDHC, in which inactivation in the presence of KG alone occurred at a rate one order of magnitude lower ($k=0.01 \text{ min}^{-1}$) than under conditions in which the thiol radical of the complex-bound lipoate was efficiently generated ($k=0.1 \text{ min}^{-1}$) (80). Thus, both the isolated E₁k and complex-bound E₁k generate reactive species by oxidizing α -ketoglutarate with O₂ (79, 80) without significant inactivation. The isolated E₁p component also generates reactive species by oxidizing pyruvate with O₂ (79). However, under normal physiological conditions (i.e., when the components are integrated within the multienzyme complexes and the substrates of other

component enzymes are present as well), side reactions may be blocked rapidly by self-inactivation (80–82), which is subject to metabolic and redox regulation by substrate structural analogs (78) and by thioredoxin and/or NADH/NAD⁺ (81, 82). These data indicate that natural systems may use paracatalytic enzyme inactivation for limiting side reactions in vivo. Moreover, for enzymes that produce highly reactive compounds, self-inactivation in the course of catalysis was shown to be a feasible means of preventing cellular accumulation of such reactive compounds (91, 92). KGDHC may be classified as one such enzyme, as it produces ROS, and in vivo ROSs are known to be signal transducers that become toxic at excessively high levels.

VIII. Potential Role of Paracatalytic Side Reactions in Diseases

A. BACKGROUND

During the course of evolution, nature has made numerous efforts to prevent O₂ and other electrophiles from reacting at the active sites of enzymes that generate carbanionic and/or radical intermediates. For some enzyme reactions that do not benefit from radical chemistry, carbanionic intermediates may not require sequestration from molecular O₂, but may still need to be protected from other possible reactants. Those enzymes that do support reactions with substantial radical character must somehow prevent reaction with molecular O₂ and other radical species that may be present. With the luxury of hindsight, we now know that enzymes are only as “perfect” as required by natural selection (13). In other words, excluding O₂ may or may not be desirable, but will be decided upon by selection pressure.

Tipping the balance from an inefficient to an efficient oxygenase/oxidase in an enzyme that catalyzes the formation of a carbanionic intermediate may only require surprisingly minor alteration in an enzyme's architecture. There are a number of examples when post-translational modification of amino acid residues of proteins, which typically arise from the oxygenation of aromatic residues, covalent cross-linking of amino acid residues, or cyclization or cleavage of internal amino acid residues, may give rise to altered or even new catalytic functions (93). Thus, as noted above, native pig kidney DDC does not oxidatively decarboxylate a significant fraction of L-dopa, although it can oxidize dopamine at a relatively low rate (51, 53). A Y332F mutation results in an enzyme that retains the ability to catalyze the oxidation of dopamine at a relatively low rate. However, this mutated enzyme catalyzes a very active oxidative decarboxylation of L-dopa. The V_{\max} of this reaction is at least as great as, and possibly greater than, that of the nonoxidative decarboxylation of L-dopa catalyzed by the native enzyme (50, 53). The affinity of the mutated enzyme for L-dopa is, however, somewhat lower than that exhibited by the native enzyme (50). These findings may have important ramifications. It is conceivable, for example, that mutation or chemical modification could give rise to a modified form of DDC with substantial oxidase/oxygenase activity. Such modifications of DDC could convert an enzyme that catalyzes a purely nonoxidative dopa decarboxylation reaction to an enzyme that catalyzes an exclusive or partial oxidative decarboxylation of L-dopa to an aromatic aldehyde (i.e., 3,4-dihydroxyphenylacetaldehyde; dopal). Aromatic aldehydes are generated from the action of DDC on dopamine and serotonin (47), which can then inactivate the enzyme. A damaged or mutant DDC may generate a greater amount of reactive aromatic aldehyde than native DDC, resulting in pathology. It is interesting to note that dopal is far more toxic to the substantia nigra than is dopamine (94). It has been suggested that dopal is a major neurotoxin contributing to the death of neurons in the substantia nigra (94). Although dopal is generally regarded as arising via the action of monoamine oxidases on dopamine, it is conceivable that this reactive aldehyde may also arise as a new activity of a damaged DDC to catalyze oxidative decarboxylation of L-dopa or to increased ability of the enzyme to oxidize dopamine.

In this regard, as noted above, recent findings suggest that defects in DCC, although rare, occur in human populations (55). As summarized by Allen et al. (55), children with DCC deficiency present early in life with axial hypotonia, hypokinesia, choreoathetosis, developmental delay and episodes of dystonia, limb hypertonia, and oculogyric crises. Autonomic symptoms are common, including nasal congestion, temperature instability, and excessive sweating. Presumably, the symptoms are related to low levels of dopamine and serotonin (and products derived therefrom). However, the possibility should also be considered that a mutated enzyme can convert dopa to neurotoxic dopal.

As noted above, petunia PAAS has strong sequence homology to aromatic amino acid decarboxylases (57). Based on this finding and the finding that only a relatively simple mutation can convert DDC from a nonoxidative decarboxylase to an oxidative decarboxylase (50), it is reasonable to assume that nature readily exploited mutations in the basic structure of L-aromatic amino acid decarboxylases to maximize interaction of carbanionic intermediates with O₂ in the petunia PAAS. On the other hand, other PLP-dependent decarboxylases, where oxidative decarboxylation of the L-amino acid to an aldehyde is not quantitatively an important metabolic process, have evolved to minimize the interaction of carbanionic intermediates with molecular O₂. The fact that the reaction with O₂ has not been eliminated altogether suggests that due to the topology of the active site, some oxidation with O₂ is unavoidable if nonoxidative decarboxylation is to proceed at a reasonable rate. However, it is also possible that nature may use these side reactions with O₂ to regulate decarboxylase activity or to balance normal physiological function at various oxygen tensions. For example, inhibition of the human vesicle-associated GAD (GAD65) and porcine CSAD I/II (enzymes that catalyze an important step in the taurine biosynthesis pathway) by molecular O₂ may be involved in maintaining the balance between excitatory and inhibitory signaling during changes in atmospheric pressure. Failure to maintain balance due to excessive inhibition of GAD65 is thought to contribute to the genesis of oxygen-induced seizures (e.g., seizures that result from extended underwater dives on compressed air) (41, 46).

Certain enzymes can catalyze new or increased paracatalytic reactions if the natural metal ion cofactor is replaced by an inappropriate metal ion. For example, as noted above, replacement of active site Mg²⁺ with Co²⁺ in *Rhodospirillum rubrum* Rubisco converts the enzyme from a partial oxygenase into an exclusive oxygenase (38). We suggest that enhancement of oxygenase/oxidase activity in some carbanion-forming enzymes by alterations of the topology of the active site and/or exposure to an inappropriate metal may also occur in mammalian enzymes and contribute to metabolic stress in various diseases. For example, zinc homeostasis is thought to be perturbed in Alzheimer disease (AD) brain (95). Even sub- μ M amounts of Zn²⁺ can promote, by as much as fivefold, the NADH oxidation by O₂ catalyzed by the E₃ component of KGDHC (96).

B. POSSIBLE ROLE OF ThDP IN THE PARACATALYTIC BIOACTIVATION OF N-NITROSO COMPOUNDS AND RELEVANCE TO CANCER INDUCTION

As discussed above, some nitroso compounds are converted to N-acyl hydroxamic acids in the presence of "active aldehyde" [reaction (22)] at the active sites of E_{1p}, transketolase, and E_{1k} (97). Due to its ability to accommodate bulky substrates at the active site, the BCDHC can participate in the biotransformation of N-nitroso aromatic compounds producing N-hydroxyarylamines (98). In a similar fashion, a transketolase-mediated reaction of nitroso aromatic compounds with fructose-6-phosphate can produce N-hydroxy-N-arylglycolamides (98). The formation of N-hydroxy-N-acetamide and N-hydroxyarylamines is facilitated by PDHC and BCDHC, respectively (98, 99). The formation of these derivatives is enhanced by cofactors (Mg²⁺ and ThDP) and inhibited by thiamine thiazolone pyrophosphate, a

specific inhibitor of ThDP-dependent enzymes (98). These reactions may be very important in the bioactivation (toxification) of *N*-nitroso compounds.

Humans are exposed to *N*-nitroso compounds by exogenous routes (from processed meats in particular) and by endogenous routes. A variety of *N*-nitroso compounds are derived primarily from reaction of amides, substituted ureas, or aromatic amines within the stomach and intestines as a result of oral and gastric bacterial degradation of nitrates to nitrites, both common food additives used in the processing of ham, sausages, and other meat products to prevent the colonization of *Clostridia* bacteria. Red meat may contribute to the formation of colorectal cancers by exposure to *N*-nitroso compounds, and endogenous exposure to *N*-nitroso compounds is dose-dependently related to the amount of red meat in the diet (100). The polycyclic aromatic hydrocarbons (PAHs) contained in smoked and charcoal-broiled foods can be converted to *N*-substituted aromatic compounds and direct-acting mutagens by treatment with nitrite in acidic media. Thus, simultaneous consumption of charcoal-broiled and smoked foods with nitrite-containing food may contribute to esophageal, stomach, and colorectal tumors (101). Similarly, intragastric nitrosation of 4-chloroindole and of 4-chloro-6-methoxyindole in fava beans (*Vicia faba*) with nitrite yields a direct-acting mutagen, 4-chloro-2-hydroxy-*N*¹-nitroso-indolin-3-one oxime, a stable α -hydroxy *N*-nitrosylated compound (102). Other naturally occurring *N*-substituted aromatic compounds have been identified in a widely consumed tea from India (Kashmir), which are responsible for a high incidence of esophageal and stomach cancer in that area. Mononitrosocaffeidine and dinitrosocaffeidine are obtained from in vitro nitrosation of caffeidine, a hydrolysis product of caffeine present in the Kashmir tea (103). In addition, after nitrosation, naturally occurring aromatic amines, especially 2-carboline derivatives such as harman, norharman, harmaline, harmalol, harmine, and harmol, become more mutagenic to *Salmonella typhimurium* (104). Under gastric conditions, piperidine, detected in popular white and black pepper powders, reacts readily with nitrite to form carcinogenic *N*-nitroso-piperidine (105).

N-Substituted aromatic compounds have been implicated in induction of cancers and DNA mutations due to metabolic transformation into *N*-hydroxyarylamines and *N*-acyl hydroxamic acids, which can react with DNA after bioactivation into reactive electrophiles. As noted above, both of these classes of compounds can arise through paracatalytic reactions of aromatic *N*-nitroso compounds with aldehydes at the active sites of a number of ThDP-containing enzymes. The mechanism by which hydroxylamines and *N*-acyl hydroxamic acids are bioactivated is beyond the scope of this review, but it is worth mentioning that the bioactivation of the latter group of compounds appears to involve *N*-acyltransferase reactions (106). These reactions show certain degrees of similarity to *S*-acyltransferase reactions catalyzed by the ThDP-dependent E₁p and E₁k (99).

C. CRITICAL INVOLVEMENT OF KGDHC IN NEURODEGENERATIVE DISEASES

Considerable evidence has accumulated during the past two decades that reduction of brain KGDHC activity is inherent in the pathophysiology of thiamine deficiency and neurodegenerative diseases, such as AD, Parkinson disease, Huntington disease, Wernicke–Korsakoff disease, and progressive supranuclear palsy [for a review, see the article by Gibson et al. (107)]. Whether the neurodegeneration-associated inactivation of KGDHC is primary or secondary, the loss of activity would strongly aggravate the disease state, due to the key position of KGDHC at a branch point of the central metabolism of mitochondria, organelles known to play an important role in cell death. In particular, AD is associated with loss of neurons with a high KGDHC content and with a specific decrease of KGDHC activity in cortex and hippocampus (108). Cortex and hippocampus provide trophic support for cholinergic neurons of the forebrain basal nucleus and produce nerve growth factor

(NGF), a deficit of which occurs in AD brain (109). Impairment of energy metabolism due to diminished KGDHC activity will disturb the levels of trophic factors.

Although the mechanisms by which KGDHC is inactivated in diseased brain are not clear, it is thought that metabolic imbalance and oxidative stress under disease conditions may contribute to the reduced KGDHC activity. The findings summarized in the present review may suggest potential mechanisms. For example, KGDHC activity is diminished in Wernicke–Korsakoff syndrome, a neuropsychiatric disorder that is induced in humans by thiamine deficiency as a result of chronic alcoholism (110) and in some cases by hyperemesis gravidarum. Curiously, however, the content of the thiamine-dependent E₁k is increased by as much as threefold in rats exposed to ethanol (111). Taken together, these data suggest that increased E₁k expression may be a compensatory response to E₁k-dependent KGDHC inactivation resulting from alcohol-induced stress. It is well established that excessive alcohol consumption increases the NADH/NAD⁺ ratio in liver. This increased NADH/NAD⁺ ratio should promote thyl radical formation by KGDHC, thereby increasing E₁k inactivation according to reactions (23) and (24). The possibility of paracatalytic inactivation of E₁k under pathological conditions is supported by our finding that the glutamate excitotoxicity toward neurons is significantly alleviated by synthetic analogs of KG, which protect KGDHC from paracatalytic inactivation (112). Because glutamate excitotoxicity is known to contribute greatly to hypoxic–ischemic damage to both adult and developing brain, we tested analogs of KG in vivo as neuroprotective agents, using a model of prenatal hypoxia. Exposure of pregnant rats to KG analogs was shown to alleviate hypoxic damage to their offspring (113). It is worth noting that the glutamate neurotoxicity observed in neurodegenerative diseases is associated with increased ROS production in overstimulated neurons (114). Using specific inhibitors of cellular E₁k (115), we showed that neuronal KGDHC contributes significantly to the ROS production in response to glutamate overstimulation (86). The biological significance of the KGDHC-dependent paracatalytic reactions involving E₁k-E₃ interaction is supported further by the fact that KGDHC from brain possesses a specific isoform of E₁k and a lower E₃ content than that of the heart complex (116). Structural differences between the two E₁k isoforms (117) and the role of E₁k in the E₃ binding to the complex (118) suggest that the lower E₃ content of brain KGDHC is determined by the structure of the brain-specific isoform of E₁k. The decreased content of E₃ in the brain KGDHC may thus represent an additional means by which the brain is protected from excessive ROS generation.

On the basis of the observed diversity of the oxidants accepted at the E₁k active site (Table 4) and diverse chemistry of the interaction of these oxidants with the E₁k active site as discussed in the present review, we wish to emphasize the possibility that some xenobiotics and some metabolites not on the main catalytic sequence shown in Figure 2 may be paracatalytic substrates of E₁k in vivo, oxidizing the carbanionic intermediate formed in reaction (1). These paracatalytic reactions may result in products detrimental to KGDHC itself and/or to the mitochondrial milieu. For example, E₁k paracatalytic substrates might very well include cellular quinones, which are well known to be highly electrophilic. This possibility is supported by study of the cellular toxicity of coenzyme Q₀. KGDHC interacts with this quinone in vivo as documented by adduction of coenzyme Q₀ to E₂k and protection from this modification by coenzymes Q₁ and Q₂ upon incubation of cells with the various quinones (119).

An important factor in the KG plus CoA-dependent generation of ROS by KGDHC is that the ROS are produced concomitantly with, and in the vicinity of, CO₂/HCO₃⁻, which are known to greatly increase the damaging potential of ROS (120, 121) and peroxynitrite (122). For example, bicarbonate enhances the radical-dependent oligomerization and nitration of the neuronal presynaptic protein α -synuclein, which is known to be a component of

potentially toxic Lewy bodies in neurodegenerative disorders (122). Furthermore, $\text{CO}_2/\text{HCO}_3^-$ mediates the Mn^{2+} -catalyzed decomposition of H_2O_2 , initiating a chain of biologically hazardous processes (121, 123). The structural and functional interaction of E_1k and E_3 within KGDHC is especially important in this regard because (1) the E_3 -produced ROS may be coupled to KG oxidative decarboxylation, resulting in CO_2 formation, and (2) some mammalian E_1k subunits are known to bind Mn^{2+} and other metal ions in addition to the catalytically essential Mg^{2+} (124, 125). We conclude that changes in the cellular proteome and metabolome that occur as a result of neurodegenerative diseases may affect paracatalytic reactions of E_1k . Stimulation of such reactions may not only decrease the energy production by KGDHC but also damage the cellular milieu through generation of reactive metabolites by KGDHC. Further study of the mechanism of the E_1k catalysis and biologically occurring paracatalytic substrates will lead to a greater understanding of the complex processes which take place in diseased brain. Such an understanding may suggest new avenues for safe and effective treatment modalities against devastating neurodegenerative diseases.

IX. Conclusions

Many enzymes possess the ability to catalyze paracatalytic (including oxygenase/oxidase) side reactions with carbanionic intermediates. In some cases, this may be the result of an evolutionary balance between generating a biologically acceptable level of the “natural” product and a biologically acceptable level of a toxic or “nonproductive” side product. These side reactions may have additional consequences. For example, they may be used to regulate O_2 levels or enzyme activities. However, under pathological conditions, these side reactions may contribute to cancer induction and to the dysfunctions of cerebral metabolism during aging or in neurodegenerative diseases. The possibility exists that products resulting from enzyme-catalyzed paracatalytic reactions, including oxygenase/oxidase side reactions, may be elevated in aging and neurodegenerative diseases by (a) provision of an inappropriate metal, (b) increased availability of reactive electrophile/oxidant (e.g., quinones), and/or (c) oxidative stress-induced modification of an enzyme. Inhibition of PLP-dependent decarboxylases, such as human GAD65, by molecular O_2 could be responsible for acute manifestations of oxygen toxicity, such as O_2 -induced seizures during diving. Disruption of glutamate and dopamine levels as a result of dysregulation of GAD and DDC may also have strong implications for neurodegenerative diseases. Finally, decreased NADH production by KGDHC, which is a robust finding for several neurodegenerative diseases, no doubt contributes to the decline in cerebral energy metabolism in many neurodegenerative diseases. However, paracatalytic activities of KGDHC may be activated under these conditions and aggravate the disease state due to other factors as well. The disease-induced modifications of enzymes and cellular milieu (in particular, perturbed thiol redox status, an increased NADH/NAD⁺ ratio, and a perturbed metal ion balance) may increase oxidative stress in AD brain by stimulating production of $\text{O}_2^{\bullet-}$ and H_2O_2 . Pharmacological treatments may have side effects through the paracatalytic generation of hazardous compounds. Together, these factors could lead to a vicious cycle in which cellular metabolism is compromised.

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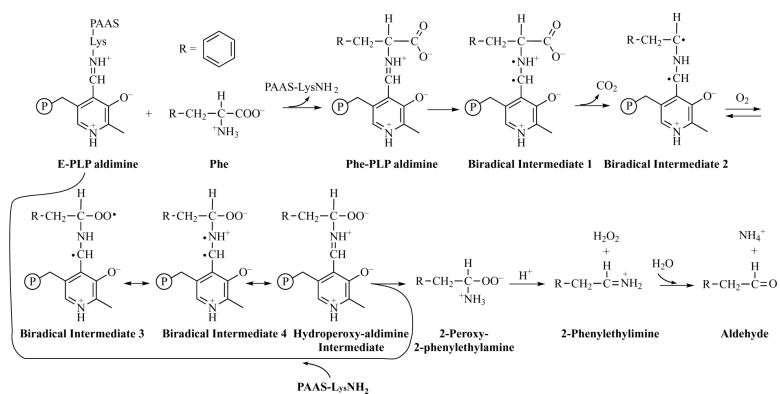
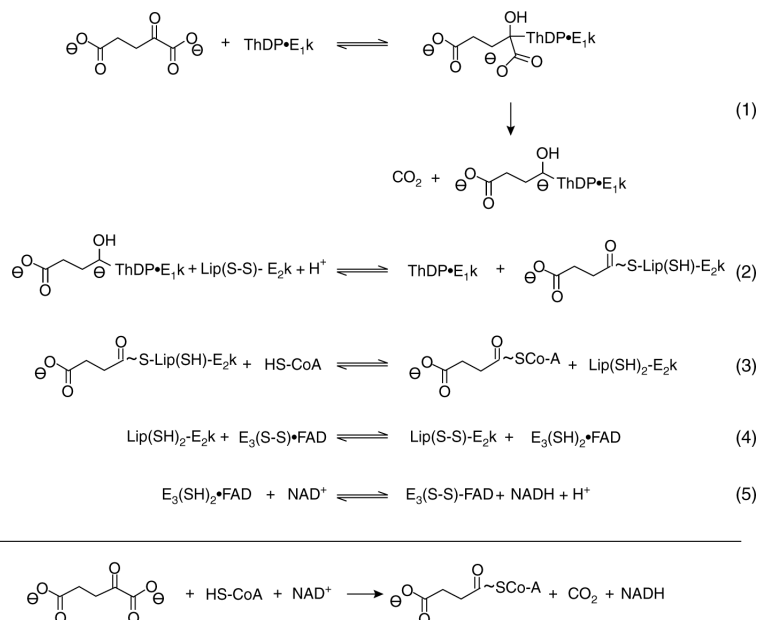


Figure 1. Proposed mechanism for the phenylacetaldehyde synthase (PAAS)-catalyzed reaction leading to biosynthesis of phenylacetaldehyde from L-phenylalanine. For details see the text. [Modified from (57).]

**Figure 2.**

α -Ketoglutarate dehydrogenase complex (KGDHC)-catalyzed reaction. In the first step, KG reacts with the ThDP cofactor and is decarboxylated to generate CO_2 and active aldehyde (carbanion) at the active site of E_1k (step 1). In the second step, ThDP is regenerated in the E_1k -catalyzed reductive transacylation reaction with the lipoyl residue covalently bound to E_2k (step 2). This thio ester contains a high-energy bond as depicted by \sim . In the next step, at the active site of E_2k , coenzyme A reacts with the succinyl dihydrolipoyl thio ester to generate the high-energy compound succinyl CoA and a dihydrolipoyl residue (step 3). Subsequently, a disulfide linkage in the dihydrolipoyl residue is regenerated concomitant with the $2e^-$ reduction of $\text{E}_3(\text{S-S})\cdot\text{FAD}$ (step 4), which in turn is reoxidized back with NAD^+ (step 5). [From (115), with permission.]

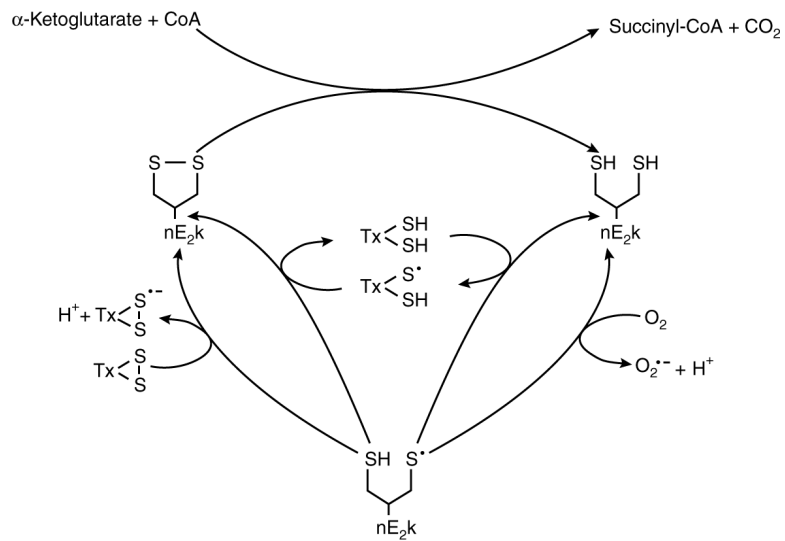


Figure 3. Formation of KGDHC-dependent radical species and the thioredoxin-catalyzed dismutation of KGDHC-bound dihydrolipoyl radicals. For a detailed discussion, see the text. [Modified from (82).]

TABLE 1

Carbanion-Forming Enzymes (Excluding PLP-Containing Enzymes) That Catalyze Oxygenase/Oxidase Side Reactions

Enzyme ^a	Oxygenase or Oxidase	Reaction Catalyzed
Ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco)	Oxygenase	Ribulose 1,5-bisphosphate + O ₂ → phosphoglycolate + D-3-phosphoglycerate
Acetolactate synthase	Oxygenase	Pyruvate + O ₂ → peracetate + CO ₂ nonenzymatic: pyruvate + peracetic acid → 2 acetate + CO ₂
Pyruvate decarboxylase	Oxygenase	Pyruvate + O ₂ → peracetate + CO ₂ non-enzymatic: pyruvate + peracetic acid → 2 acetate + CO ₂
Rhamnulose 1-phosphate aldolase	Oxidase	1,3-Dihydroxyacetone phosphate + O ₂ → hydroxypyruvaldehyde phosphate + H ₂ O ₂
Fructose 1,6-bisphosphate aldolase	Oxidase	1,3-Dihydroxyacetone phosphate + O ₂ → hydroxypyruvaldehyde phosphate (or 3-phosphoglycerate) + H ₂ O ₂
α-Ketoglutarate dehydrogenase	Oxidase	α-Ketoglutarate → succinate + CO ₂ + 2e ⁻ (with various electron acceptors)

^aThe enzymes in this list have been purified from various sources. For simplicity the source is not identified. The only case where species differences appear to make a difference is fructose 1,6-bisphosphate aldolase. The enzyme isolated from *Staphylococcus aureus* catalyzes a side reaction with O₂, whereas the enzyme isolated from rabbit muscle does not. For original references, see the text.

TABLE 2

Carbanion-Forming PLP Enzymes That Catalyze Oxidation Reactions

Enzyme ^a	Oxygenase or Oxidase	Reaction Catalyzed
<i>Escherichia coli</i> glutamate decarboxylase (GAD)	Oxidase	Glutamate + O ₂ → succinic semialdehyde + NH ₄ ⁺ + CO ₂ + H ₂ O ₂
<i>E. coli</i> glutamate decarboxylase (GAD)	Uncertain	α-Methyl-L-glutamate + $\frac{1}{2}$ O ₂ → levulinate + NH ₄ ⁺ + CO ₂
Pig kidney dopa decarboxylase (DDC)	Uncertain	Aromatic amine + $\frac{1}{2}$ O ₂ → aromatic aldehyde + NH ₄ ⁺
Pig kidney dopa decarboxylase (DDC)	Uncertain	α-Methyl L-dopa + $\frac{1}{2}$ O ₂ → dihydroxyphenylacetone + NH ₄ ⁺ + CO ₂
<i>Lactobacillus</i> ornithine decarboxylase (ODC)	Uncertain	α-Methyl L-ornithine + $\frac{1}{2}$ O ₂ → 2-methyl-Δ ¹ -pyrroline + NH ₄ ⁺ + CO ₂ + H ₂ O
<i>Lactobacillus</i> ornithine decarboxylase (ODC)	Uncertain	Putrescine + $\frac{1}{2}$ O ₂ → Δ ¹ -pyrrolone + NH ₄ ⁺ + H ₂ O
<i>Hafnia alvei</i> ornithine decarboxylase (ODC)	Oxidase	L-Ornithine + O ₂ → Δ ¹ -pyrroline + NH ₄ ⁺ + CO ₂ + H ₂ O ₂
Petunia phenylacetaldehyde synthase (PAAS)	Oxidase	L-Phenylalanine + O ₂ → phenylacetaldehyde + NH ₄ ⁺ + CO ₂ + H ₂ O ₂
Petunia phenylacetaldehyde synthase (PAAS)	Oxidase	α-Methyl-L-phenylalanine + O ₂ → phenylacetone + NH ₄ ⁺ + CO ₂ + H ₂ O ₂

^aIn each case except PAAS, the reactions shown are side reactions (paracatalytic) with O₂. With PAAS the reaction shown is the dominant reaction. For *E. coli* GAD, *H. alvei* ODC, and petunia PAAS, oxidative decarboxylation results in stoichiometric formation of H₂O₂. The enzymes catalyzing these reactions are thus clearly oxidases. For pig heart DDC and *Lactobacillus* ODC, where no H₂O₂ has been directly detected in the oxidation reaction, the situation is not yet clear. For original references, see the text.

TABLE 3

Relative Nonoxidative Decarboxylation and Oxidation Reactions Catalyzed by Various PLP-Containing Amino Acid Decarboxylases^a

Reaction	Enzyme					
	<i>Escherichia coli</i> GAD	Native Pig Kidney DDC	Pig Kidney DDC Y332F Mutant	<i>Lactobacillus</i> ODC	<i>Hafnia alvei</i> ODC	Petunia PAAS
Nonoxidative decarboxylation of L-amino acid substrate	+++	+++	-	+++	+++	-
Oxidative decarboxylation of L-amino acid substrate	+	-	+++	+	+	+++
Amine oxidation	+/-	+	+	-	-	-
Oxidative decarboxylation of α -methyl L-amino acid substrate	+	+	ND ^b	+	ND ^a	+++

^aFor details, see the text.

^bNot determined.

TABLE 4
Paracatalytic Reactions of KG Oxidation Catalyzed by E₁k Purified from Different Sources^a

Enzyme Source	Artificial Electron Acceptor	Relative KG Oxidation Rates (%)	K _m KG (mM)	K _m acceptor at Saturating KG (mM)	Assay Conditions				λ _{max} and e	
					pH	Concentration of the Acceptor (mM)	Potassium Phosphate (M)	λ _{max} (nm)		
Pigeon breast muscle	Hexacyanoferrate(III) DCIP NBD-Cl ^b	100 10 3	0.046 ± 0.013 0.050 ± 0.02 —	0.084 ± 0.009 0.058 ± 0.012 0.3	6.3 6.8 6.8	0.8 0.1 1	0.05 0.1 0.2	420 600 540	1,000 20,800 12,500 ^c	(2) (2) (73)
<i>Escherichia coli</i>	Tetranitromethane 4-Chloronitrosobenzene	200 100	0.043 ± 0.017 —	0.6 ± 0.2 <i>d</i>	6.3 7.5	1 0.1	0.1 0.05	350 320	14,400 —	(2) (72)
Bovine Porcine	4-Chloronitrosobenzene 4-Chloronitrosobenzene	230 380	— 0.1	— 0.1	— —	— —	— —	— —	— —	— —

^aThe initial reaction rates were determined and corrected for the non-specific reduction of oxidants in the absence of KG. The data obtained with pigeon breast muscle E₁k are from (71). The data with 4-chloronitrosobenzene are from (72). λ_{max} and e are the absorbance maximum and extinction coefficient, respectively, of the reduced species obtained after reaction of the redox indicator (artificial electron acceptor) with carbanion intermediate.

^bKG oxidation by NBD-Cl is highly dependent on conditions; in particular, it is stimulated by increasing concentration of potassium phosphate, is light-sensitive in MOPS buffer, and does not take place in imidazole buffer.

^cDetermined under the indicated conditions from the maximal ΔD₅₄₀ with limiting KG at an excess of E₁k and NBD-Cl. Linear increase in ΔD₅₄₀ was observed with 1 to 4μM KG.

^dExceeds the solubility of 4-chloronitrosobenzene.

TABLE 5

Maximal Reaction Rate (V_{\max}) and Apparent Rate Constant for Irreversible Inactivation Associated with Turnover (k_{in}), Characteristic of the Paracatalytic Reaction of E_1k Oxidizing KG with Hexacyanoferrate(III)^a

Enzyme Source	E_1k Preparation	KG: Hexacyanoferrate(III) Oxidoreduction V_{\max} ($\mu\text{mol}/\text{min}\cdot\text{mg}$)	k_{in} (min^{-1})
Pigeon breast muscle	Isolated E_1k	0.2	0.12
	Complex-bound E_1k	4.2	0.05
Pig heart	Complex-bound E_1k	3.0	0.04
<i>Azotobacter vinelandii</i>	Complex-bound E_1k	2.0	0.08

^aThe data for the mammalian and pigeon E_1k are from (88). The data for the *Azotobacter* E_1k are from (126).