MOLECULAR MECHANISMS OF OXYGEN ACTIVATION

EDITED BY

OSAMU HAYAISHI

MOLECULAR BIOLOGY
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PREFACE

"Oxygenases," a comprehensive treatise on these and related enzymes, was first published in 1962. During the past decade, considerable progress has been made regarding the basic mechanisms involved in activation of molecular oxygen and the physiological functions of various oxygenases. Information and data acquired from the detailed analyses of a large number of isolated and highly purified enzyme systems, together with results and conclusions derived from biological and medical investigations *in vivo*, constitute a body of knowledge from which generalized concepts and basic principles of oxygenases and related enzymes have emerged. Advances in this field of research have indeed been so rapid and diverse that keeping abreast of the progress has presented difficulties even for the experts. In view of these considerations, I have undertaken the editing of "Molecular Mechanisms of Oxygen Activation" covering the major new advances that have been made in our understanding of this vital process.

In the previous volume, the emphasis was primarily on the nature of the reactions rather than on the enzymes and mechanisms of catalysis, and in a sense this side-stepped the most interesting questions which relate to the activation process itself. However, the availability of new physical and chemical techniques has made possible remarkable advances in our knowledge of the structure of many of the enzymes and the development of sophisticated models to account for this activity. One particularly notable advance was the recognition of ternary complexes containing enzyme, substrate, and oxygen as obligatory intermediates in many of the di- or monooxygenase-catalyzed reactions. In addition, entirely new areas of research have been opened up with the discovery of important new biological products such as the prostoglandins and new classes of oxygen-activating enzymes such as the cytochromes P-450, the flavoprotein oxygenases, and the α-ketoglutarate-requiring oxygenases. These oxygenases were unknown

or unrecognized in 1962. Superoxide, superoxide dismutase, and superoxide-utilizing enzymes provided additional insight into the mechanism by which molecular oxygen is activated and reduced.

The new discoveries have highlighted the important and widespread role of oxygen activation in contemporary biological processes. It is my hope that a review of this current knowledge of various aspects of oxygenases will be of value to investigators already working in the field and will provide a foundation for other researchers who wish to become involved in aspects of this important and exciting problem.

I express my appreciation to Dr. B. L. Horecker for his assistance with the editorial work and to the staff of Academic Press for their cooperation. The writing and editing of this volume were carried out during my tenure as a Fogarty International Scholar in the summers of 1972 and 1973.

OSAMU HAYAISHI

1

GENERAL PROPERTIES AND BIOLOGICAL FUNCTIONS OF OXYGENASES

OSAMU HAYAISHI

I. HISTORICAL BACKGROUND

A. Early History

Oxygen, one of the most abundant elements on the earth and directly or indirectly essential for almost all forms of life, has been the subject of

intensive studies by biochemists and physiologists ever since Lavoisier initiated the study of biological oxidation processes some 200 years ago. Since that time, the mechanisms by which various nutrients are oxidized by living organisms have remained among the most important and interesting problems in biological science.

Lavoisier and his contemporaries defined the term "oxidation" as the addition of oxygen atoms to a substrate, (X), while the opposite process, that of reduction, was regarded as the removal of oxygen from an oxide [Eq. (1)].

$$X + O \underset{\text{reduction}}{\overset{\text{oxidation}}{\rightleftharpoons}} XO \tag{1}$$

As early as 1896, Bertrand observed that living organisms contain a number of enzymes which catalyze the oxidation of various biological compounds, and these were designated "oxydases." The early workers generally assumed that oxygen was affected and modified by "oxydases" in such a way that stable oxygen molecules were activated then bound to substrates. The nature of the so-called activated oxygen, however, was unknown for many years, although organic peroxides and ozonides were postulated as active forms by a number of investigators. At the turn of the twentieth century, Bach and his co-workers proposed that oxygen reacted with an acceptor, A, in the primary reaction to produce an organic peroxide, which then reacted with a substrate, (X), to form an oxide (1).

$$A + O_2 \xrightarrow{\text{oxygenase}} A\begin{smallmatrix}O\\|\\O\end{smallmatrix} \tag{2}$$

$$A\begin{smallmatrix}O\\|\\O\end{smallmatrix} + X \xrightarrow{\text{peroxidase}} AO + XO \tag{3}$$

The enzymes which catalyzed reactions (2) and (3) were named "oxygenase" and "peroxidase," respectively. This hypothetical mechanism, however, failed to gain general acceptance and was eventually abandoned, chiefly because experimental evidence for the formation of organic peroxides during general oxidative processes was lacking.

About 20 years later, Otto Warburg proposed a theory of cell respiration which was to considerably influence students of biological oxidation in subsequent years. According to this theory, the essential process in cell respiration is the activation of oxygen catalyzed by heme-containing enzymes, "the respiratory enzyme" (*das Atmungsferment*) (2). In many ways this theory was reminiscent of that of Bach, since Warburg assumed that the primary reaction in cell respiration is between molecular oxygen

and heme iron (Fe) as follows:

$$\text{Enz–(Fe)} + O_2 \rightarrow \text{Enz–(Fe)–}O_2 \tag{4}$$

The oxidation of the organic molecule then follows according to Eq. (5).

$$\text{Enz–(Fe)–}O_2 + 2\,X \rightarrow \text{Enz–(Fe)} + 2\,XO \tag{5}$$

This role of oxygen molecules per se in biological oxidation processes was vigorously challenged and questioned following the discovery by Schardinger of an enzyme in milk which catalyzed the conversion of aldehydes to acids in the presence of methylene blue but in the total absence of oxygen. The oxidation of aldehyde was accompanied by the concomitant reduction of methylene blue under anaerobic conditions. This finding prompted Wieland to investigate the nature of biological oxidation processes and eventually to propose a generalized mechanism which could work out in the total absence of oxygen (3). According to this scheme the essential characteristic of biological oxidation processes is the removal or transfer of electrons from the substrate molecule, (XH_2), to an appropriate acceptor, A, [Eq. (6)].

$$XH_2 + A \underset{\text{reduction}}{\overset{\text{oxidation}}{\rightleftharpoons}} X + AH_2 \tag{6}$$

This "dehydrogenation" theory was supported by the ingenious experiments of Thunberg, who was able to demonstrate enzymic oxidation of a variety of substrates in the presence of methylene blue under completely anaerobic conditions. Furthermore, in the last several decades many dehydrogenases, which do not utilize oxygen, have been isolated, purified, and crystallized from animal and plant tissues as well as from microorganisms. Pyridine nucleotides, flavin nucleotides, and cytochromes have been found to act as electron acceptors for various dehydrogenases. In the occasional case when oxygen molecules serve as the immediate electron acceptor, the enzymes have been called "oxidases."

According to our present knowledge, classic oxidases can be divided into three categories. In the first category, the enzyme catalyzes the transfer of one electron to one molecule of oxygen forming superoxide anion as shown in Eq. (7). Xanthine oxidase is an example of this type of enzyme.

$$XH + O_2 \rightarrow X + O_2^- + H^+ \tag{7}$$

In the second group, two electrons are transferred to one molecule of oxygen to produce hydrogen peroxide as a product [Eq. (8)]. D-Amino acid oxidase is a classic example of this type of enzyme.

$$XH_2 + O_2 \rightarrow X + H_2O_2 \tag{8}$$

The enzymes of the third class catalyze the transfer of four electrons to a molecule of oxygen producing water as a product [Eq. (9)]. In this case the bond between the two oxygen atoms must be cleaved, whereas in the first and second cases the bond between the two oxygen atoms is preserved. Cytochrome oxidase and ascorbate oxidase are examples of the third class of oxidases.

$$XH_4 + O_2 \rightarrow X + 2H_2O \tag{9}$$

Thus, the early concept of oxygen incorporation by Lavoisier was almost completely replaced and molecular oxygen was considered to serve as a terminal electron acceptor in cellular respiration. In fact, in 1932, Wieland made the following statement in his famous book, "On the Mechanism of Oxidation" (3): "Limiting ourselves to the chief energy-supply foods, we have in this class carbohydrates, amino acids, the higher fatty acids, and glycerol. There is no known example among them of an unsaturated compound in the case of which it is necessary to assume direct addition of oxygen, that is, additive oxidation."

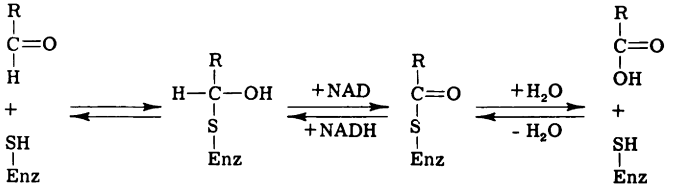
According to this theory, when the overall reaction can be formulated as an addition of oxygen, it was assumed that hydration or hydrolysis is involved, and that the oxygen atoms are derived from the water molecule rather than from atmospheric oxygen. An example of this mechanism is shown in Eqs. (10) and (11), where the substrate is hydrated in the primary reaction, followed by dehydrogenation in a second reaction. The sum [Eq. (12)] is an addition of oxygen to the substrate, but the oxygen is derived from the water molecule rather than from atmospheric oxygen.

$$\text{Sub} + H_2O \rightarrow \text{SubH}_2\text{O} \tag{10}$$

$$\text{SubH}_2\text{O} + A \rightarrow \text{SubO} + AH_2 \tag{11}$$

$$\text{Sum: Sub} + H_2O + A \rightarrow \text{SubO} + AH_2 \tag{12}$$

The enzymic transformation of aldehydes to acids catalyzed by aldehyde dehydrogenases is an example of an oxidation in which the oxygen atom is derived from the water molecule. In this case acyl thioesters formed in the oxidation step are converted to acids by hydrolysis [Eq. (13)].



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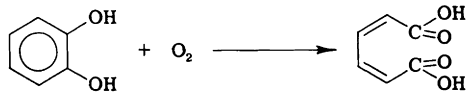
B. Discovery of Oxygenases

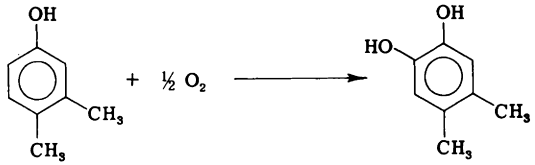
In 1950, during a study of tryptophan metabolism, an enzyme was isolated from cells of a pseudomonad which catalyzed oxidative ring cleav-

age of the benzene ring of catechol forming *cis,cis*-muconic acid as the reaction product [Eq. (14)] (4). This enzyme, which was termed "pyro-



(14)

catechase," exhibited properties unlike those of an "oxidase" or "dehydrogenase," for it was not associated with any of the previously known coenzymes or electron carriers, and none of the dyes or artificial electron acceptors tested could replace oxygen as an oxidant. According to the then generally accepted belief, biological oxidation proceeded exclusively by the removal of electrons or hydrogen atoms from substrates, and direct addition of molecular oxygen was excluded from consideration. Oxygen might still be incorporated into substrates by hydration reactions involving water, but prior or subsequent dehydrogenation process would remove hydrogen or electrons as in the case of aldehyde oxidases. In 1955, however, when a heavy oxygen isotope was used as a tracer in $^{18}O_2$ and $H_2{}^{18}O$, it was demonstrated that the two oxygen atoms incorporated into the product of the above reaction were derived exclusively from molecular oxygen rather than from water (5). Concurrently and independently, Mason and his collaborators, using the same isotope, found that during the oxidation of 3,4-dimethylphenol to 4,5-dimethylcatechol catalyzed by a phenolase complex, the oxygen atom incorporated into the substrate molecule was derived exclusively from molecular oxygen but not from the oxygen of water [Eq. (15)] (6).



(15)

These findings conflicted sharply with the then current concept that oxygen could act only as an ultimate electron acceptor in biological oxidation and that all oxygen atoms incorporated into substrates are derived from the oxygen atoms of water. These two newly discovered reactions may be schematically represented by Eqs. (16) and (17).

$$X + O_2 \rightarrow XO_2 \tag{16}$$

$$X + \tfrac{1}{2}O_2 \rightarrow XO \tag{17}$$

The overall reaction in Eq. (16) may be visualized as the addition of both atoms of an oxygen molecule to a molecule of substrate, (X). It was soon acknowledged that in the case of Eq. (17), one of the atoms of molecular

oxygen is incorporated into a substrate molecule and the other reduced to H_2O in the presence of an appropriate electron donor, (DH_2), such as NADH, NADPH, tetrahydrofolic acid, or ascorbic acid [Eq. (18)].

$$X + O_2 + DH_2 \rightarrow XO + H_2O + D \qquad (18)$$

These two types of reactions both involve "oxygen fixation" into a substrate molecule, and they are therefore different from the classic oxidase reactions shown in Eqs. (7), (8), and (9). They are similar to the oxygenation reactions known to occur in chemical or photochemical processes, and we therefore proposed a new term "oxygenase" to designate enzymes which catalyze such oxygen fixation reactions (7).

II. NOMENCLATURE, CLASSIFICATION, AND GENERAL PROPERTIES OF OXYGENASES

From the historical point of view as well as from the preceding discussion, it is evident that the term "oxygenase" may be appropriately assigned to a group of enzymes presumably catalyzing the activation of oxygen and the subsequent incorporation of either one or two atoms of oxygen per mole of various substrates. The terms "mono" and "di" oxygenases are generally assigned, respectively, to the enzymes catalyzing these two types of reactions (8).

When the substrate, the acceptor of oxygen, is hydrogen, the enzyme has been called an oxidase. In that sense, oxidases may be envisaged as a special class of oxygenases for reactions in which hydrogen atoms serve as the oxygen acceptor. These enzymes will, however, not be considered extensively here, as evidence indicates that enzymic reduction of oxygen to HO_2, H_2O, or H_2O_2 would involve the activation of hydrogen rather than oxygen, and in addition this subject has been extensively covered in a number of recent reviews.

Because information concerning the mechanism of action of oxygenases is still limited even though the field is progressing rapidly, any classification scheme would necessarily be arbitrary and perhaps temporary. Two subclasses, di- and monooxygenases, have been employed by the majority of workers in this field, although the distinction between the di- and monooxygenase subclasses, discussed below, is phenomenological and historical and may not be so permanent and meaningful.

A. Dioxygenases

Dioxygenases are defined as enzymes catalyzing reactions in which both atoms of molecular oxygen are incorporated into substrates. In the many

instances where one substrate can act as the oxygen acceptor [Eq. (16)], the term "*intra*molecular dioxygenases" may be used. The dioxygenases acting upon two acceptor substrates, which have recently been reported from a number of laboratories (9), may be referred to as "*inter*molecular dioxygenases." One of the two substrates for the latter type has so far been invariably α-ketoglutarate, and the overall reaction may be schematically shown by Eq. (19). More detailed discussions of this type of enzyme will be found in a later chapter by Abbott and Udenfriend (9).

$$\alpha\text{-Ketoglutarate} + O_2 + X \rightarrow \text{succinate} + CO_2 + XO \tag{19}$$

A third class of dioxygenases include various enzymes which require NADH or NADPH as an electron donor. Although it is quite possible that reactions of this class may involve a simple dioxygenation reaction followed by a reductive step, these two processes may be coupled in such a way as to justify a separate category. The formation of catechol from anthranilate (10) is an example of this type of reaction. The overall reaction may be represented as follows:

$$X + O_2 + NADH + H^+ \rightarrow X\begin{matrix}OH\\ \\OH\end{matrix} + NAD \tag{20}$$

Some dioxygenases, such as tryptophan 2,3-dioxygenase, contain heme as the sole prosthetic group, while others, such as pyrocatechase, contain nonheme iron, or like quercetinase, contain copper as the prosthetic group.

B. Monooxygenases

Monooxygenases are defined as a group of enzymes which catalyze the incorporation of one atom of molecular oxygen into a substrate while the other is reduced to water. These enzymes are sometimes referred to as mixed function oxygenases or mixed function oxidases since they are apparently bifunctional, carrying out oxidase activity on one site and oxygenase activity on the other. Recently, the term "hydroxylase" has been used by some investigators; however, this term is misleading and would be better avoided because not all hydroxylation reactions are catalyzed by monooxygenases. In some cases, dihydroxy compounds are formed by the introduction of two oxygen atoms derived from the same oxygen molecule. The reaction is presumably catalyzed by a dioxygenase. Alternatively, the oxygen atom of a newly formed hydroxyl group may be derived from water rather than from molecular oxygen. The formation of 6-hydroxynicotinic acid from nicotinic acid (11) and that of barbituric acid from uracil (12)

TABLE I

CLASSIFICATION OF OXYGENASES

Oxygenase	EC No.[a]
A. Dioxygenase	
1. Intramolecular dioxygenase	1.13.11
a. Hemoprotein	
b. Iron-sulfur protein	
c. Copper protein	
2. Intramolecular dioxygenase	1.14.11
3. Miscellaneous	1.13.99
B. Monooxygenase	
1. Internal monooxygenase	1.13.12
2. External monooxygenase	1.14
a. Pyridine nucleotide-linked flavoprotein	1.14.13
b. Flavin-linked hemoprotein	1.14.14
c. Iron-sulfur protein-linked hemoprotein	1.14.15
d. Pteridine-linked monooxygenase	1.14.16
e. Ascorbate-linked cupper protein	1.14.17
f. With another substrate as reductant	1.14.18

[a] EC number refers to the new numbering system introduced in 1972 by the International Union of Biochemistry, Enzyme Nomenclature Commission.

and 6-hydroxylation of the pteridine ring (13) are examples of the latter type of reaction.

Monooxygenases may be classified on the basis of the electron donor involved. Since in some cases the primary reductant is unknown, these may eventually be reclassified.

1. Internal Monooxygenases

The simplest type of monooxygenase catalyzes the incorporation of a single atom of molecular oxygen concomitant with the reduction of the other oxygen atom by electrons derived from the substrate. Thus, the overall reaction may be expressed by the following equation:

$$XH_2 + O_2 \rightarrow XO + H_2O \qquad (21)$$

Since the reducing agent is internally supplied, these enzymes may be referred to as internal monooxygenases. The first of these to be crystallized was the lactate oxidative decarboxylase from *Mycobacterium phlei* (14). This enzyme catalyzes the conversion of lactate to acetate with the in-

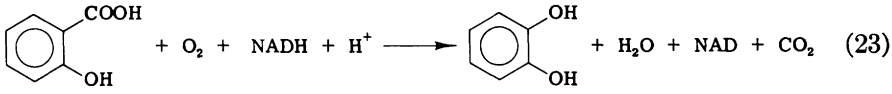
corporation of one atom of oxygen into acetate, the evolution of one mole of CO_2, and the reduction of one atom of oxygen to water as follows (15):

$$CH_3CHOHCOOH + O_2 \rightarrow CH_3COOH + CO_2 + H_2O \quad (22)$$

2. External Monooxygenases

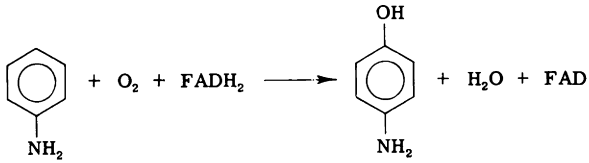
While the internal monooxygenases do not require external reducing agents, more common types of monooxygenases require various kinds of electron donors. The overall reactions are schematically represented by Eq. (18). The electron donor (DH_2) serves as a basis for the subclassification of external monooxygenases. Some examples are shown below.

a. Flavoprotein Monooxygenases with Reduced Pyridine Nucleotides as DH_2. Salicylate 1-monooxygenase (16), a flavoprotein, is an example of this type of enzyme and catalyzes the reaction shown below.



(23)

b. Heme-Containing Monooxygenases. Aryl 4-monooxygenase (liver microsomal cytochrome P-450) (17, 18) catalyzes hydroxylation of a variety of substrates with reduced flavin as DH_2.



(24)

c. Heme-Containing Monooxygenases with a Reduced Iron-Sulfur Protein as DH_2. Camphor 5-monooxygenase (19, 20) is a typical example of this type of enzyme.

$$\text{Camphor} + O_2 + 2 \text{ reduced putidaredoxin} \xrightarrow{+2\,H^+} \text{5-}exo\text{-hydroxycamphor} + H_2O + 2 \text{ oxidized putidaredoxin} \quad (25)$$

d. Pteridine-Linked Monooxygenases. Phenylalanine-4-monooxygenase (21) catalyzes the formation of tyrosine from phenylalanine as follows:

$$\text{L-Phenylalanine} + O_2 + \text{tetrahydropteridine} \rightarrow \text{L-tyrosine} + H_2O + \text{dihydropteridine} \quad (26)$$

This group of enzymes will be discussed in detail by Kaufman in a separate chapter (22).

e. With Ascorbate as DH_2. Dopamine β-monooxygenase (23, 24) is an example of this type of enzyme.

3,4-Dihydroxyphenylethylamine + O_2 + ascorbate

$$\rightarrow \text{norepinephrine} + H_2O + \text{dehydroascorbate} \quad (27)$$

f. With Another "Substrate" as DH_2. Monophenol monooxygenase (25) is an example of this type. In this case dopa may be considered as electron donor in the reaction.

$$\text{Tyrosine} + O_2 + \text{dopa} \rightarrow \text{dopa} + H_2O + \text{dopa quinone} \quad (28)$$

A brief summary of the above classification scheme is shown in Table I.

III. CHEMICAL ASPECTS OF OXYGEN FIXATION REACTIONS

A. General Comments

As mentioned above, biological oxidation processes may occur in three apparently different ways: (1) by the removal of an electron, as when a ferrous ion (Fe^{2+}) is converted to a ferric ion (Fe^{3+}); (2) by the removal of hydrogen, as when alcohol is oxidized to aldehyde; or (3) by the addition of oxygen to a molecule, as when an aromatic compound (Ar) is oxygenated to a hydroxy derivative.

$$Fe^{2+} \rightarrow Fe^{3+} + e^- \quad (29)$$

$$CH_3CH_2OH \rightarrow CH_3CHO + H_2 \quad (30)$$

$$ArH + \tfrac{1}{2}O_2 \rightarrow Ar\text{–}OH \quad (31)$$

Although these three types of reactions appear to be dissimilar, they all involve a transfer of electrons. Oxidation is defined as removal of electrons with an increase in valency, whereas reduction is defined as a gain in electrons with a decrease in valency. When carbon and oxygen interact to form covalent C–O bonds [Eq. (31)], electrons are shared between both atoms. Since these electrons are somewhat closer to the oxygen nucleus than to the carbon nucleus, the oxygen has partially gained electrons and is reduced while the carbon has partially lost electrons and is oxidized.

Therefore, the fundamental principle governing the above three types of reaction is basically the same; however, they are catalyzed by different types of enzymes in the cell. The enzymes which catalyze dehydrogenation of primary substrates [Eqs. (29) and (30)] are designated "dehydrogenases" and the enzymes which catalyze the addition of oxygen have been called "oxygenases."

B. Molecular Oxygen as a Substrate

Oxygenases utilize two different species of substrate, namely, molecular oxygen and the oxygen acceptor, which is called the substrate and may be either an organic or an inorganic compound. It has not yet been clarified whether gaseous oxygen or oxygen dissolved in water is utilized by these enzymes. It is generally believed that the latter type of oxygen is different from the former. In water, oxygen is considered to exist largely in a dimerized form, O_2–O_2, because of its biradical nature, and probably forms a charge transfer complex with a water molecule (26). Whatever form the oxygen dissolved in water takes, the type of oxygen that serves as substrate for oxygenases is in rapid equilibrium with gaseous oxygen under normal experimental conditions and can be clearly distinguished through the use of $^{18}O_2$ from the oxygen in water molecules or other compounds in the reaction mixture.

For the details of the isotope technique involving $^{18}O_2$ and $H_2{}^{18}O$, readers are referred to a previous article in "Oxygenases" (27). It is not always possible, however, to demonstrate $^{18}O_2$ incorporation into products after reaction in an $^{18}O_2$-containing atmosphere for the following reasons. (1) Oxygen atoms in carbonyl groups are easily exchanged with the oxygen of water molecules; therefore, analytical results are not always stoichiometric. In order to avoid this difficulty, carbonyl groups in the reaction product may be further reduced, oxidized, or conjugated in some way to stabilize the oxygen. (2) The oxygenated intermediate may be labile or transient and not susceptible to trapping in a stable form. Desaturation of fatty acids (28) and the side chain of heme (29), which will be discussed in detail in separate chapters, are examples of this case. In these reactions, the oxygenated intermediate appears to be extremely labile or enzyme-bound and is presumed to dehydrate immediately. Therefore a demonstration of the incorporation of molecular oxygen in this transient intermediate is impossible [Eqs. (32)–(34)].

$$R{-}CH_2{-}CH_2{-}R' + O_2 + H_2X \rightarrow [R{-}\underset{\displaystyle H}{\overset{\displaystyle OH}{\overset{|}{\underset{|}{C}}}}{-}CH_2{-}R'] + H_2O + X \quad (32)$$

$$[R{-}\underset{\displaystyle H}{\overset{\displaystyle OH}{\overset{|}{\underset{|}{C}}}}{-}CH_2{-}R'] \rightarrow R{-}CH{=}CH{-}R' + H_2O \quad (33)$$

$$\text{Sum: } R{-}CH_2{-}CH_2{-}R' + O_2 + H_2X \rightarrow R{-}CH{=}CH{-}R' + 2H_2O + X \quad (34)$$

The overall reaction [Eq. (34)], therefore, represents desaturation of the

substrate with the concomitant reduction of both atoms of molecular oxygen to water.

C. The Role of Substrate Which Acts as Oxygen Acceptor

As for the acceptor for molecular oxygen, a great variety of both organic and inorganic compounds can be oxygenated. In general, oxygen-rich compounds, such as carbohydrates, are not favorable substrates for oxygenases since these usually have many reactive groups containing oxygen such as the hydroxyl, carbonyl, or formyl, and their biochemical function does not require further oxygenation. On the other hand, lipids and aromatic compounds are often metabolized by oxygenases. These oxygen-deficient compounds generally require oxygenation in order to become biologically active or more soluble in water. Because of the hydrophobic nature of lipids and aromatic compounds, molecular oxygen is the preferred hydroxylating agent rather than water. In contrast, purines and pyrimidines with their hydrophilic ring systems are usually hydroxylated by the addition of water, followed by dehydrogenation.

In addition to its role as an oxygen acceptor, the substrate usually acts also as an allosteric regulator. Only in the presence of the substrate does molecular oxygen bind to the active center of the enzyme and become catalytically active. Because this phenomenon appears to be a common and unique feature of many oxygenases, it will be discussed further in a following section.

D. Reaction Mechanisms and the Nature of Active Oxygen

One of the most intriguing and challenging problems in the field of oxygenases has been the mechanism whereby the two substrates interact with the enzyme and become activated; e.g., does molecular oxygen bind to the enzyme first to form EO_2 or does the acceptor first bind to the enzyme to form an ES complex? Is it molecular oxygen, or the substrate, or both, which are activated by the oxygenase? Is there any evidence for the activation of molecular oxygen, and, if so, what is the nature of the so-called active oxygen?

These mechanisms will be dealt with in detail in separate chapters; however, essentially speaking, recent work from a number of laboratories has indicated rather conclusively that the enzyme binds oxygen only in the presence of substrate to form the ternary complex, ESO_2, in which oxygen and substrate interact to form a product. Such a ternary complex was postulated in 1964 on the basis of binding experiments (8), but more direct experimental evidence was not available until 1967 when a ternary complex

of tryptophan 2,3-dioxygenase–tryptophan-O_2 was demonstrated by spectrophotometric experiments. This oxygenated form of the enzyme was later shown by the stopped-flow technique to be the so-called obligatory intermediate of the reaction (30–33). Since that time similar oxygenated intermediates have also been observed with protocatechuate 3,4-dioxygenase (an iron-sulfur protein dioxygenase) (34, 35), lysine monooxygenase (a flavoprotein) (36), and also with cytochrome P-450 (37–39). In each case, the enzyme has to bind the organic substrate first before it can be oxygenated, in contrast to oxygen-carrying pigments such as hemoglobin and hemoerythrin which are freely and reversibly oxygenated in the absence of any effector or substrate. In fact, several lines of evidence indicate that the substrate, tryptophan, binds specifically to the heme coenzyme of tryptophan 2,3-dioxygenase; as a consequence, the state of the heme is altered in such a way that its reactivity toward ligands is increased by several orders of magnitude (31–33).

In contrast, Tai and Sih (40) have reported that in the case of steroid dioxygenase molecular oxygen is bound first to the enzyme and the EO_2 complex then reacts with the organic substrate. Their conclusion is based on steady-state kinetics data, and further studies are necessary to elucidate possible differences between this enzyme and others in the class.

The nature of so-called active form of oxygen in the above-mentioned ternary complex has been one of the most important as well as the most difficult questions in this field. All oxygenase-catalyzed reactions are exothermic and are therefore irreversible. Nevertheless, molecular oxygen is a rather inert compound and at room temperature reacts slowly with the substrate compounds in the absence of enzymes. This low kinetic reactivity is usually explained on the basis that molecular oxygen is in a triplet ground state. The direct reaction of a triplet molecule with organic molecules in the singlet state is electronically spin forbidden so that a substantial activation energy is required. For this reason singlet oxygen has been suggested as a likely intermediate in many oxygenase-catalyzed reactions (41, 42); however, definitive evidence is so far unavailable. It has recently been discovered that during enzymic hydroxylation of aromatic substrates the substituent (deuterium, tritium, chlorine, bromine, etc.) displaced by the entering hydroxyl group migrates to an adjacent position in the aromatic ring (43). On the basis of extensive studies of this phenomenon, called the "NIH shift," the active oxygen species involved in certain monooxygenases was postulated to be oxenoid (44). These reactions will be discussed in greater detail in a separate chapter by Hamilton (45).

On the other hand, evidence has appeared indicating that O_2^-, superoxide anion, may be the active form of oxygen in the case of intestinal tryptophan 2,3-dioxygenase (46), and similar experimental results reported by Coon

and co-workers indicated that superoxide anion may be involved in the hydroxylation reactions catalyzed by hepatic cytochrome P-450 (47). It is, however, uncertain whether or not superoxide anion is the general form of active oxygen in all oxygenase-catalyzed reactions or whether these enzymes represent a new class of enzyme which utilizes superoxide anion rather than molecular oxygen as an oxygenating agent. It is still quite feasible that intestinal tryptophan 2,3-dioxygenase will prove to be the first example of such a new group of enzymes which may be referred to as "superoxygenases."

E. Uncoupling of Oxygenase Activity

Certain oxygenases, particularly monooxygenases, have exhibited oxidase rather than oxygenase activity under certain conditions. Such a phenomenon has often been referred to as "uncoupling." In 1968, Okamoto *et al.* (48) reported that when imidazoleacetate monooxygenase was treated with varying amounts of mercurials or silver nitrate, the enzyme was almost completely inactivated with respect to oxygenase activity at a ratio of approximately 2 moles of reagent per mole of enzyme. The oxidase activity of the enzyme, however, was unaffected by this treatment. It is of particular interest that some mercurials even enhanced oxidase activity. The reaction product of oxidase activity was identified as hydrogen peroxide, while that of the oxygenase reaction was water. Other oxygenases are known to exhibit oxidase activity when allowed to react with certain substrate analogs. Thus, salicylate monooxygenase was shown to catalyze the oxidation of NADH forming H_2O_2 when benzoate was substituted for salicylate (49). Lysine monooxygenase could act as an amino acid oxidase and produce α-ketoacid analogs of certain substrate analogs such as ornithine and 2,8-diaminooctanoate with the stoichiometric formation of ammonia and H_2O_2 (50).

One possible interpretation of the above findings is that only the substrates with an appropriate size or structure fit the active center of the enzyme molecule forming an enzyme–substrate complex, after which the substrate can be oxygenated by the attack of molecular oxygen. On the other hand, substrates which do not exactly fit the active center of the enzyme may form another type of enzyme–substrate complex, in which the dehydrogenation of the substrate by FAD does not couple with oxygen activation, and the reduced FAD is then merely oxidized by oxygen to form H_2O_2. Such an interpretation is substantiated by a more recent observation by Yamamoto *et al.* (51) that the "fragmented substrate" such as L-alanine plus propylamine is not oxygenated but oxidized. L-Alanine alone is completely inactive as substrate; however, when propylamine is present,

oxidation, but not oxygenation, of alanine was demonstrated in the presence of a crystalline preparation of L-lysine monooxygenase. Other α-monoamino acids are also oxidized in the presence of alkylamines with various carbon chain length, but the highest oxidase activity is observed when the total chain length of both amino acid and amine is nearly identical with that of lysine.

IV. BIOLOGICAL FUNCTION OF OXYGENASES

A. General Comments

The significance of biological oxygen fixation in medicine, agriculture, microbiology, and also in food technology, cosmobiology, public health problems, and biochemistry in general has now been well established. Oxygenases play important roles in biosynthesis, transformation, and degradation of essential metabolites such as amino acids, lipids, sugars, porphyrins, vitamins, and hormones. They also play a crucial role in the metabolic disposal of foreign compounds such as drugs, insecticides, and carcinogens. Furthermore, they participate in the degradation of various natural and synthetic compounds by soil and airborne microorganisms in nature and are therefore of great significance in environmental science.

Many monooxygenases catalyze hydroxylation of both aromatic and aliphatic compounds. Monooxygenases also catalyze a seemingly diverse group of reactions including epoxide formation, dealkylation, decarboxylation, deamination, and N- or S-oxide formation. Although the overall reactions catalyzed by various monooxygenases appear grossly unlike each other, the primary chemical event is identical since these processes are all initiated by the incorporation of one atom of molecular oxygen into the substrate. After the initial monooxygenation reaction the compounds become more soluble in water or become biologically more reactive in the sense that they are susceptible to the action of various dioxygenases. The major reaction catalyzed by dioxygenases is the cleavage of an aromatic double bond, which may be located (a) between two hydroxylated carbon atoms, (b) adjacent to a hydroxylated carbon atom, or (c) in an indole ring. However, a similar type of reaction also occurs with aliphatic substrates such as β-carotene, which yields vitamin A as the product (52).

B. Oxygenases in Amino Acid Metabolism

The important role played by oxygenases in the metabolism of aromatic amino acids such as tryptophan, phenylalanine, tyrosine, and various phenolic compounds has long been recognized; for example, tryptophan

undergoes a variety of metabolic transformations. Among these, two of the most important are initiated by a monooxygenase and a dioxygenase (Fig. 1). The black arrows in this figure denote oxygenase reactions, and the white arrows indicate reactions catalyzed by enzymes other than oxygenases. The oxygen atoms shown in heavy print represent molecular oxygen which has been incorporated into these substrates by the action of various oxygenases. It can be seen that essentially all the oxidative steps are catalyzed by oxygenases rather than by oxidases and dehydrogenases.

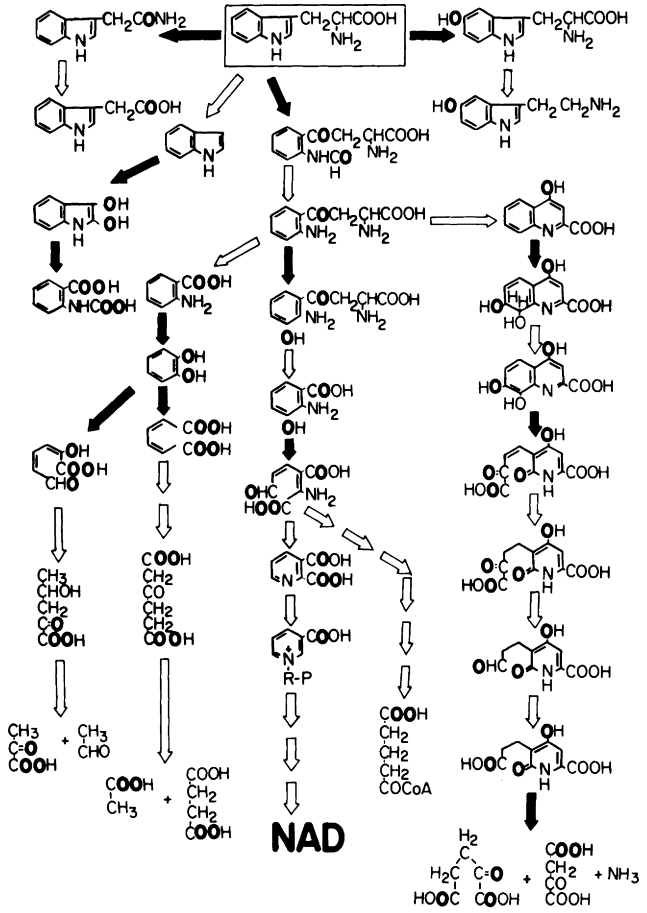


FIG. 1. Oxygenase reactions involved in the pathway of tryptophan metabolism. The black arrows denote oxygenase reactions and the white arrows represent reactions catalyzed by enzymes other than oxygenases.

Tryptophan serves as a precursor for the biosynthesis of serotonin, a potent vasoconstrictor substance and a neurohumoral agent. The enzyme tryptophan 5-monooxygenase catalyzes the initial step of this metabolic transformation and has been purified from the brain stem of various species of mammals (53–55). One atom of molecular oxygen is introduced into tryptophan to form 5-hydroxytryptophan, which is then decarboxylated to serotonin. Tryptophan 2,3-dioxygenase (56, 57) catalyzes the insertion of two atoms of molecular oxygen into the pyrrole moiety of tryptophan, forming formylkynurenine as a product; hence, it is sometimes referred to as tryptophan pyrrolase. This reaction is the initial step in a metabolic sequence which leads to the biosynthesis of a coenzyme, nicotinamide-adenine dinucleotide (NAD), from tryptophan in mammals and in some microorganisms and is probably a rate-limiting step and a likely site of regulation of this pathway. Formylkynurenine thus formed is converted to kynurenine which is then hydroxylated to 3-hydroxykynurenine by the specific action of kynurenine 3-monooxygenase. This enzyme is localized in the outer membrane of rat liver mitochondria (58) and contains flavin adenine dinucleotide (FAD) as its prosthetic group (59). It is induced by oxygen in *Saccharomyces cerevisiae*; the biosynthesis of pyridine nucleotides therefore starts mainly from tryptophan in aerobic cultures but begins from aspartate and a C_3 fragment under anaerobic conditions (60). In mammals, administration of L-thyroxine causes a decrease of about 50% in kynurenine 3-monooxygenase activity, presumably because of decreased biosynthesis of this enzyme, thus providing a reasonable biochemical explanation for the low NAD(P) level in hyperthyroid animals (61).

3-Hydroxykynurenine is transformed to 3-hydroxyanthranilic acid, which is then cleaved by a specific dioxygenase. This reaction is physiologically of great importance since the resulting compound can either be converted to acetyl-coenzyme A through various intermediates including glutaryl-coenzyme A, or it can form a new pyridine ring yielding picolinic acid and quinolinic acid as reaction products. Quinolinic acid has been shown to yield nicotinic acid ribonucleotide, the precursor of NAD (62).

Similarly, the metabolism of phenylalanine and tyrosine is catalyzed by a number of oxygenases; for example, the enzymic formation of tyrosine from phenylalanine and the formation of epinephrine, norepinephrine, melanin, and thyroxine are catalyzed by a number of consecutive reactions involving various mono- and dioxygenation reactions. Regarding many hereditary metabolic disorders as studied in humans, the biochemical anomaly has been traced to the absence of activity of specific oxygenases in these pathways. In normal individuals, almost all of phenylalanine metabolism is channeled through tyrosine by the action of phenylalanine 4-monooxygenase. Kaufman and co-workers have shown that tetrahydropteridine

derivatives serve as the direct hydrogen donor in this process (21, 22). Hereditary deficiency of phenylalanine 4-monooxygenase results in phenylketonuria, an inborn error of metabolism and a common cause of mental retardation. Similarly, alkaptonuria is the result of the hereditary deficiency of homogentisate 3,4-dioxygenase, which catalyzes the conversion of homogentisic acid to 4-maleylacetoacetate.

Hydroxyprolines, primarily 4-hydroxyproline, together with a small amount of the 3-hydroxy isomer, are unique constituents of collagen, a major component of cartilage and other connective tissues, and the most abundant protein in the body. Early isotopic studies indicated that free hydroxyproline is not incorporated into collagen but that hydroxylation of peptidylproline yields collagen hydroxyproline. This reaction is catalyzed by a dioxygenase which has been partially purified and requires ferrous ion, ascorbate, and α-ketoglutarate (63–65). The hydroxylation of peptidyllysine appears to proceed in a similar manner (65, 66). These reactions will be reviewed in more detail in a separate chapter (9).

A novel type of amino acid decarboxylase, lysine monooxygenase, which yields the corresponding acid amide instead of the amine, was initially isolated from a pseudomonad and crystallized in our laboratory (67). Although the physiological significance of this type of reaction is not yet clear, two different enzymes have been discovered since then which act specifically on L-arginine (68) and L-tryptophan (69).

The formation of hypotaurine and cystine sulfinate from cysteamine and L-cysteine, respectively, are catalyzed by two distinct dioxygenases (70, 71), which will be discussed in detail later.

C. Oxygenases in Lipid Metabolism

Oxygenases play a versatile and ubiquitous role in the metabolism of lipid and related compounds. They also play a major role in the biosynthesis, transformation, and degradation of steroids, fatty acids, prostaglandins, vitamin A, bile acids, etc. As early as 1867, Pasteur made the classic observation that anaerobically growing yeast cells degenerated in structure unless periods of aerobiosis were interspersed, although yeast had been known to be capable of maintaining life under strictly anaerobic conditions (72). This observation suggested that molecular oxygen is required for the biosynthesis of some compounds essential to morphogenesis. In fact, subsequent work from a number of laboratories indicated that molecular oxygen is vital for the biosynthesis of some essential lipid components of cell membrane (73, 74). Oxygenases in lipid and steroid metabolism will be dealt with in a separate chapter by Hamberg *et al.* (75).