The Roles of Superoxide Anion and Methylene Blue in the Reductive Activation of Indoleamine 2,3-Dioxygenase by Ascorbic Acid or by Xanthine Oxidase-Hypoxanthine*

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To clarify the roles of superoxide anion (O$_2^-$) and methylene blue in the reductive activation of the heme protein indoleamine 2,3-dioxygenase, effects of xanthine oxidase-hypoxanthine used at various oxidase concentration levels as an O$_2^-$ source and an electron donor on the catalytic activity of the dioxygenase have been examined in the presence and absence of either methylene blue or superoxide dismutase using L- and D-tryptophan as substrates. In the absence of methylene blue, initial rates of the product N-formylkynurenine formation are enhanced in parallel with the xanthine oxidase level up to ~100 and ~50% of the apparent maximal activity (~2 s$^{-1}$) for L- and D-Trp, respectively. Superoxide dismutase effectively inhibits the reactions by 80–98% for both isomers. Additions of methylene blue (25 μM) help to maintain the linearity of the product formation that would be rapidly lost a few minutes after the start of the reaction without the dye, especially for L-Trp. Additions of methylene blue also enhance the activity to the maximal level for D-Trp. In the presence of methylene blue, the inhibitory effects of superoxide dismutase are considerably decreased with the increase in xanthine oxidase concentration, and at near maximal dioxygenase activity levels superoxide dismutase is totally without effect. In separate anaerobic experiments leuco-methylene blue, generated either by photoreduction or by ascorbate reduction, is shown to be able to reduce the ferric dioxygenase up to 25–40%. Substrate Trp and heme ligands (CO, n-butyl isocyanide) help to shift a ferric ferrous form equilibrium to the right. Thus, under aerobic conditions leuco-methylene blue might similarly be able to reduce the dioxygenase in the presence of an electron donor with the aid of substrate and O$_2$. These results strongly suggest that indoleamine 2,3-dioxygenase can be activated through different pathways either by O$_2^-$ or by an electron donor-methylene blue system. For the latter case, the dye is acting as an electron mediator from the donor to the ferric dioxygenase.

The apparent involvement of superoxide anion (O$_2^-$) in the activation and catalytic processes of the heme protein, indoleamine 2,3-dioxygenase, has been an intriguing property of this dioxygenase (1–3) since its discovery in Hayashi's laboratory (4, 5). This unique feature of the enzyme along with its molecular (mononeric glycoprotein, Mr ~ 41,000) (6) and immunogenic (7) properties, relatively wide substrate specificity for several indoleamines (6, 8), and ubiquitous mammalian tissue distribution except the liver (3, 9–11), clearly distinguish indoleamine 2,3-dioxygenase from another similar heme-containing protein, tryptophan 2,3-dioxygenase (12). The catalytic reaction of indoleamine 2,3-dioxygenase can be initiated by O$_2^-$ that is added directly using KO$_2$ (14) or generated either chemically (1, 2) or enzymatically (2). Superoxide dismutase was reported to significantly inhibit the reaction not only at the initial stage but also at the steady state (1). The observed dioxygenase activation by O$_2^-$ is directly related to the fact that the enzyme is in the ferric (inactive) state when purified from tissues (5) and needs to be reduced to the ferrous (active) state to perform the catalytic reaction (15). Unlike several other O$_2$-binding heme proteins such as hemoglobin (16, 17), myoglobin (16), and cytochrome P-450 (18, 19), the ferric native form of indoleamine 2,3-dioxygenase readily binds O$_2^-$ to form the dioxygen complex of the ferrous enzyme, which is also catalytically active but highly autoxidizable in the presence of substrate (18). However, superoxide anion alone is not sufficient to maintain the fully activated state of the dioxygenase, and another co-factor, an artificial dye, methylene blue, is also required for maximal activity (14). Methylene blue has been used in the following two typical assay systems for the dioxygenase: (a) ascorbate-methylene blue (5) and (b) KO$_2$-methylene blue (14). In system a, the more convenient assay system, the dye is absolutely required for the catalytic reaction since ascorbate alone can hardly activate the enzyme. Methylene blue here is considered to be an electron carrier from ascorbate to molecular oxygen to generate O$_2^-$ (1, 2). In system b, methylene blue is required to maintain the initial linearity of the reaction that is quickly lost in 1–2 min after the start of the reaction (14). The roles of methylene blue in these two systems thus seem to differ. Yet, no clear explanations for the actual role(s) of the dye in relation to the role of O$_2^-$ have been offered.

To address this question in the present study an alternative O$_2^-$-generating enzymatic system, xanthine oxidase-hypoxanthine, has been used for the activation of indoleamine 2,3-dioxygenase. The advantages of using this system are that the rate of O$_2^-$ generation can be easily controlled by changing the concentration of the oxidase and that the catalytic reaction can be continuously monitored by following absorbance...
Changes due to the product formation. Xanthine oxidase can also be used as an electron donor. Since methylene blue is a good electron acceptor from xanthine oxidase (19, 20), as is molecular oxygen, it is necessary to carry out experiments both in the presence and absence of the dye in order to examine separately the effects of $O_2$ and the actual role of methylene blue in the xanthine oxidase–methylene blue co-factor system. In view of these points, effects of a relatively wide range (1.7–40 milliunits/ml) of xanthine oxidase levels on catalytic activity of indoleamine 2,3-dioxygenase has been examined in detail in this study in the presence and absence of either methylene blue or superoxide dismutase using L-Trp and D-Trp as dioxygenase substrates. This study has allowed quantitative analysis of the correlation between the extent of the $O_2$ participation and the reductive activation of indoleamine 2,3-dioxygenase and has provided new evidence that methylene blue acts as an electron mediator from donors to the ferric dioxygenase.

### Experimental Procedures

**Enzymes**—Indoleamine 2,3-dioxygenase was purified from rabbit small intestine by the method of Shimizu et al. (6), except that the final step of microfiltration was omitted and instead step 6 (Sephadex G-100 chromatography) was repeated 2–4 times. The purified native ferric enzyme exhibited an $A_{280}/A_{390}$ value of 1.7–1.8 in 20 mM potassium phosphate buffer at pH 6.0 and 24 °C and was 60–70% pure as judged by sodium dodecyl sulfate gel electrophoresis (21). The amount of the enzyme was expressed in terms of its heme content based on the absorbance at 406 nm ($ε = 159$ mM$^{-1}$ cm$^{-1}$) at pH 6.0 and 25 °C (21)). Xanthine oxidase from cow milk and catalase from beef liver were products of Boehringer Mannheim. Xanthine oxidase activity was determined by the method of Kalocker with hypoxanthine as substrate (22, 23). One unit is defined as the amount of enzyme which catalyzes the formation of 1 nmol of uric acid/min in a total volume of 1.0 ml at 24 °C and pH 7.7 using an $ε_295$ value of 12.2 mM$^{-1}$ cm$^{-1}$ (23). Superoxide dismutase purified from bovine erythrocytes with a specific activity of 3300 units/mg of protein (15, 24) was a generous gift of Professor Osamu Hayaishi (Kyoto University).

**Materials**—Sperm whale myoglobin (Type II) was purchased from Sigma. All of the following chemicals of reagent grade obtained from the companies indicated were used without further purification: ascorbic acid and hypoxanthine from Aldrich; L- and D-Trp from Sigma; methylene blue, isopropyl alcohol, and acetoephonene from Fisher; methanol from ICN Pharmaceuticals.

**Photoreduction Method**—Anaerobic reduction of artificial electron carriers (methylene blue and methyl viologen) or heme proteins (indoleamine 2,3-dioxygenase and myoglobin) was performed by photoreduction under argon according to the method reported by Ward and Chang (25). Trace amounts of dioxygen in the samples were removed by bubbling argon through an acidic aqueous Cr(CO)$_4$$_2$ solution containing amalgamated zinc (26). Samples to be photoreduced were prepared as follows: buffer (0.1 mM potassium phosphate, pH 7.0) containing 2% (v/v) isopropyl alcohol and 0.1% (v/v) acetoephonene was placed in a rubber septum-stoppered cuvette (path length 0.2 cm) and made anaerobic by gently bubbling with argon gas on ice for at least 30 min through a syringe needle inserted through the rubber septum. Next, a microliter volume of a sample from its concentrated aqueous stock solution (0.5–1 mM) was placed in a small side reservoir attached near the top of the cuvette, and the bubbling was further continued for another 10–15 min. Then, the sample was mixed in the buffer by tilting the cuvette. The anaerobic solution of the sample thus prepared was subjected to photoreduction in a circulating ice-water bath to protect the sample from heat while keeping slight positive pressure with argon in the cuvette. The photoreduction apparatus consisted of a 400-watt quartz mercury arc immersion UV lamp (Ace Glass Inc.) and a power supply. The reduction of the sample was monitored from its spectral changes after each photoradiation.

**Spectrophotometric Measurements**—The measurements were carried out with either a Unicam SP 1800 spectrophotometer or a Varian Cary 219 spectrophotometer each of which was equipped with a circulator for temperature control (25 ± 1 °C). Except for the photoreduction experiments (see above), all measurements were done in a cuvette with a 1-cm light path.

### Results

Fig. 1 shows typical time courses for the formation of the product, N-formylkynurenine ($A_{360}$ = 321 nm) using the lower two xanthine oxidase concentration levels (1.7 and 3.4 milliunits/ml) examined in this study. Throughout the text, concentrations of xanthine oxidase and of superoxide dismutase are expressed as their catalytic activity values rather than ordinary molar concentration values. Catalase is also added to prevent the dioxygenase from decomposition by the hydrogen peroxide that is produced in the system as a by-product (18). Even though both L-Trp (0.2 mM) and D-Trp (5 mM) are used in sufficient concentrations ($>50$ $K_m$) (6, 27), one notices that the L-isomer yields considerably higher initial rates ($V_o$) for the product formation than the D-isomer by a factor of about 1.7. This trend is consistently observed for higher oxidase concentrations. It should be pointed out that the $V_o$ values for L-Trp and D-Trp under these conditions account for only 6–10 and 4–6%, respectively, of the apparent maximal turnover number (2 s$^{-1}$) (6, 15). Interestingly, however, the linearity of the time course for L-Trp is gradually lost after 3–4 min following the start of the reaction, while relatively good linearity is held for 25 min for the D-isomer. Results similar to those for L-Trp were observed with DL-Trp using $K_{O_2}$ as an $O_2$ source (14), but D-Trp was not previously examined separately from the L-isomer in the absence of methylene blue. When methylene blue (25 $\mu$M) is present, different effects are seen for the two Trp isomers. The dye helps to maintain the initial linearity for L-Trp without significantly changing the initial rate of the product formation (not shown), while for D-Trp, methylene blue not only improves the linearity but also markedly enhances the catalytic rate by a factor of 2–4 (see below).

In Fig. 2, the inhibitory effects of varying concentrations (1–880 units/ml) of superoxide dismutase on the $V_o$ values for L- and D-Trp using the lowest xanthine oxidase concentration level (1.7 milliunits/ml) examined are shown in the absence (A) and presence (B) of methylene blue. In the absence of the dye, superoxide dismutase effectively inhibits the product formation; 1 unit of dismutase causes 55–65% inhibition, and nearly complete (~98%) inhibition is achieved with 350 units of the enzyme. Both L- and D-Trp exhibit similar results. Thus, $O_2^*$ is the sole and key activator under these conditions. However, when methylene blue (25 $\mu$M) is added under the same conditions, the maximal inhibition by superoxide dismutase is noticeably reduced to 45–48%, and 1 unit of dismutase causes only ~7% (D-Trp) and ~17% (L-
previously been reported, extents of superoxide dismutase inhibition in the presence of the dye is further examined as a function of xanthine oxidase concentration. Results with L- and D-Trp are demonstrated in Fig. 3. Although the rate of the product formation (open circles) increases with the increase in xanthine oxidase concentration, the extent of inhibition by dismutase becomes significantly smaller at higher oxidase concentrations. At 10 milliunits/ml xanthine oxidase concentration shown in the presence and absence of methylene blue. Boiled superoxide dismutase (100 °C; 5 min) was also examined for A (1 unit/ml) and B (20 units/ml).

This indicates that about one-half of the activation is attributed to O2 and the remaining half to an O2-independent reduction pathway(s). Boiled superoxide dismutase exhibits no inhibition with or without methylene blue.

Since such effects of methylene blue are observed in this study in a xanthine oxidase and its substrate system have not previously been reported, extents of superoxide dismutase inhibition in the presence of the dye is further examined as a function of xanthine oxidase concentration. Results with L- and D-Trp are demonstrated in Fig. 3. Although the rate of the product formation (open circles) increases with the increase in xanthine oxidase concentration, the extent of inhibition by dismutase becomes significantly smaller at higher oxidase concentrations. At 10 milliunits/ml xanthine oxidase concentration shown in the presence and absence of methylene blue. Boiled superoxide dismutase (100 °C; 5 min) was also examined for A (1 unit/ml) and B (20 units/ml).

It is also noted that even in the absence of methylene blue, superoxide dismutase-insensitive activity (closed squares) is almost linearly enhanced for both L- and D-Trp cases when xanthine oxidase concentration is raised. When 40 milliunits/ml oxidase is used, ~20% of the maximal activity remains uninhibited by superoxide dismutase for both substrates, indicating that direct reduction of the dioxygenase by xanthine oxidase is occurring to some extent under these conditions. The addition of methylene blue (25 μM) completely diminishes
the inhibitory effects of superoxide dismutase (solid circles) at xanthine oxidase concentrations of 10 millimoles/ml for L-Trp and 30 millimoles/ml for D-Trp, where ~40% and ~90% of the apparent maximal activity are attained. These results strongly suggest that either in the presence or absence of methylene blue, some portions of the dioxygenase activation are not mediated by O2. The activation process is entirely independent of O2, especially when the dioxygenase is turning over at its near-maximal rate (~2 s\(^{-1}\)) for both substrates in the presence of methylene blue. The most likely reductant of the dioxygenase in such cases is the reduced methylene blue, i.e. leuco-methylene blue; the dye is known to be a good electron acceptor from xanthine oxidase under both anaerobic and aerobic conditions (19, 20).

To test this possibility, the following two series of experiments have been carried out where leuco-methylene blue is mixed with the ferric dioxygenase under anaerobic conditions. In the first series, methylene blue is reduced by a photoreduction method as described under "Experimental Procedures." Spectral changes during the course of the experiments are shown in Fig. 5. Photoirradiation for 7–10 min is sufficient to completely convert the dye (blue color) to its reduced form (colorless) (spectrum a → b). When ferric indoleamine 2,3-dioxygenase is mixed in the leuco-methylene blue solution thus prepared under argon, a spectral change (Fe\(^{III}\) → c) occurs that is indicative of the partial reduction of the ferric enzyme as judged from an isosbestic point (~416 nm) for the ferric and ferrous enzyme. The extent of the reduction under these conditions reaches only about 25%. An addition of L-Trp (0.25 mM) somewhat but significantly raises the extent of the reduction from ~25 to ~40% (c versus d). In both the presence and absence of the substrate, bubbling CO through the samples almost completely converts the enzyme to its ferrous-CO form (not shown); an addition of dithionite to ensure the reduction of the enzyme causes only a small further spectral change. The resulting ferrous-CO enzyme exhibits a Soret peak at 420 nm (~Trp) or 418 nm (+0.5 mM L-Trp). Similar spectral conversion to near complete ferrous enzyme-ligand complex is also observed with n-butyl isocyanide, a heme ligand which binds tightly to the ferrous enzyme (K\(_D\) = 10\(^{-10}\) M). For comparison, when sperm whale myoglobin (110 mM) is placed in place of the dioxygenase in the presence of 25 \(\mu\)M leuco-methylene blue, over 95% reduction is achieved without adding any heme ligand under the same experimental conditions (results not shown). In separate experiments, when methylene blue is replaced by methyl viologen (25 mM), the reduction of the dioxygenase is complete within a few minutes.

In the second series of experiments, methylene blue is chemically reduced by ascorbic acid (10 mM) under anaerobic conditions. Spectral changes during the experiments are shown in Fig. 6. Since the reduction of the ferric dioxygenase in the presence of L-Trp with 25 \(\mu\)M leuco-methylene blue does not exceed 40% in the experiments described above, the concentration of the dye is raised to 125 \(\mu\)M. Ten millimolar ascorbate solution was prepared first in anaerobic 0.1 M potassium phosphate buffer, pH 7.0, at 25 °C, to which 125 \(\mu\)M methylene blue was anaerobically added. Spectrum a (dashed line) was recorded at about 1 h following the mixing when the spectral changes due to the reduction of methylene blue by ascorbate reached equilibrium (~98% reduction). Then ferric indoleamine 2,3-dioxygenase was added to the leuco-methylene blue-ascorbate solution (final 5.0 \(\mu\)M). After the spectral change reached equilibrium in about 30 min, spectrum b (dashed-dotted line) was recorded. Addition of 0.33 mM L-Trp to this sample caused the spectrum b to change to c (dashed-double-dotted line, 45 min after the L-Trp addition) to d (dotted line, 85 min) to e (solid line, 10.5 h). The relatively high absorbance values, especially notable in the 620–720-nm region as compared with those in Fig. 5, are due to the formation of undissolved particles of leuco-methylene blue in the sample. This was caused by the conversion of the relatively high concentration (125 \(\mu\)M) of methylene blue that was soluble (monocationic form) in aqueous solution, to leuco-methylene blue that was less soluble (neutral form) under these conditions.

FIG. 6. Spectral changes upon reduction of ferric indoleamine 2,3-dioxygenase with ascorbate-reduced methylene blue (leuco-methylene blue) under anaerobic conditions in the presence of ascorbate. Ten millimolar ascorbate solution was prepared first in anaerobic 0.1 M potassium phosphate buffer, pH 7.0 at 25 °C, to which 125 \(\mu\)M methylene blue was anaerobically added. Spectrum a (dashed line) was recorded at about 1 h following the mixing when the spectral changes due to the reduction of methylene blue by ascorbate reached equilibrium (~98% reduction). Then ferric indoleamine 2,3-dioxygenase was added to the leuco-methylene blue-ascorbate solution (final 5.0 \(\mu\)M). After the spectral change reached equilibrium in about 30 min, spectrum b (dashed-dotted line) was recorded. Addition of 0.33 mM L-Trp to this sample caused the spectrum b to change to c (dashed-double-dotted line, 45 min after the L-Trp addition) to d (dotted line, 85 min) to e (solid line, 10.5 h). The relatively high absorbance values, especially notable in the 620–720-nm region as compared with those in Fig. 5, are due to the formation of undissolved particles of leuco-methylene blue in the sample. This was caused by the conversion of the relatively high concentration (125 \(\mu\)M) of methylene blue that was soluble (monocationic form) in aqueous solution, to leuco-methylene blue that was less soluble (neutral form) under these conditions.

FIG. 5. Spectral changes upon reduction of ferric indoleamine 2,3-dioxygenase with photoreduced methylene blue under anaerobic conditions. An anaerobic 25 \(\mu\)M methylene blue solution (spectrum a, dotted line) was prepared first in a total volume of 300 \(\mu\)l of 0.1 M potassium phosphate buffer, pH 7.0, at 25 °C in a 0.2-cm cuvette as described under "Experimental Procedures." This solution was photoirradiated at ~55 °C for 7 min to completely reduce methylene blue to leuco-methylene blue (spectrum b, short dashed line). Then, several microliters of a concentrated (~450 \(\mu\)M) stock solution of indoleamine 2,3-dioxygenase (final 5.5 \(\mu\)M) were anaerobically added to the leuco-methylene blue solution at 25 °C. The spectrum c (dashed-dotted line) was recorded when the spectral changes reached equilibrium at about 45 min after mixing the solution. Spectrum d (solid line) corrected for 5.5 \(\mu\)M heme concentration was obtained from separate experiments in which 0.25 mM L-Trp was added to the reaction mixture after the ferric dioxygenase had been mixed with the leuco-methylene blue solution. Spectra of the native ferric dioxygenase (Fe\(^{III}\), dashed-dotted-dotted line) and its reduced form (Fe\(^{II}\), dashed line) overlapped in the Soret region after correction to 5.5 \(\mu\)M heme concentration were also obtained from separate experiments. The former was converted to the latter by photoreduction in 2 min.
418 nm, spectrum not shown); only a small further spectral change is seen upon addition of dithionite.

**DISCUSSION**

A significant finding in this study is that, under certain conditions, O$_2^+$ is not an absolute requirement for the maximal catalytic activity of indoleamine 2,3-dioxygenase. This conclusion is supported by the results shown in Figs. 3 and 4 which indicate that in the presence of methylene blue (25 μM) the near-maximal activity that is attained using xanthine oxidase is hardly inhibited by superoxide dismutase added in sufficient high concentrations (880 units/ml). It is unlikely that methylene blue directly prevents the superoxide dismutase activity since the dismutase can still inhibit the dioxygenase catalytic reaction when used with low levels of xanthine oxidase even in the presence of the dye at the same concentration (25 μM) (Fig. 2B). In the ascorbate-methylene blue co-factor system, inhibitory effects of superoxide dismutase (maximum about 50%) were reported to be significantly diminished in parallel with the increases in the dye concentration and in parallel with the increase in the catalytic activity of the dioxygenase (2). When 25 μM methylene blue is used in the presence of 10 μM ascorbate, no inhibition is detected in the present study even with 880 units/ml superoxide dismutase (not shown). Obviously, methylene blue is by-passing the O$_2^+$-mediated dioxygenase activation pathway in both ascorbate-methylene blue and xanthine oxidase-methylene blue systems.

The present study has also revealed that in the absence of methylene blue O$_2^+$ is the key activator of indoleamine 2,3-dioxygenase. Even in the presence of methylene blue, superoxide anion still contributes to about 50% of the dioxygenase activation at relatively low levels of xanthine oxidase (1.7 milliunits/ml) where less than 10% of the maximal activity is detected in the ascorbate-methylene blue system. At higher xanthine oxidase concentrations, O$_2^+$-mediated activation diminishes to negligible extents. To explain these results, various O$_2^+$- and methylene blue-mediated reactions that are relevant to the present study are shown below.

\[
\begin{align*}
\text{Xanthine oxidase} & \rightarrow O_2^- + H_2O_2 \\
\text{Xanthine oxidase or ascorbate} & \rightarrow S-MB. + L-MB \\
\text{L-MB + IDO(III)} & \rightarrow S-MB. + IDO(II) \\
\text{S-MB. + IDO(III)} & \rightarrow MB + IDO(II) \\
\text{L-MB + O$_2^+$} & \rightarrow S-MB. + O$_2^+$ \\
\text{S-MB. + O$_2^+$} & \rightarrow MB + O$_2^+$ \\
\text{O$_2^+$ + IDO(III)} & \rightarrow IDO(II) + O$_2$ \\
\text{O$_2^+$ + O$_2^+$ + 2H$^+$} & \rightarrow O$_2$ + H$_2$O$_2$ \\
\text{O$_2^+$ + MB} & \rightarrow O$_2^- + S-MB. \\
\text{S-MB. + S-MB.} & \rightarrow L-MB + MB
\end{align*}
\]

where MB represents methylene blue, L-MB leuco-methylene blue (2-electron-reduced MB), S-MB - a short-lived semi-methylene blue radical (1-electron-reduced MB) (28-30), and IDO indoleamine 2,3-dioxygenase. Without methylene blue, all of the electrons derived from a substrate of xanthine oxidase are used for the univalent and divalent reduction of O$_2$ to generate O$_2^+$ and H$_2$O$_2$ (i) (31-33). Thus, O$_2^+$ is the sole activator of the dioxygenase (v). When methylene blue (25 μM) is added, the dye and O$_2$ compete for electrons from xanthine oxidase. Methylene blue can be reduced to leuco-methylene blue most likely involving semi-methylene blue (ii). The lack of the inhibitory effects of superoxide dismutase at above 10 milliunits/ml xanthine oxidase (Fig. 3A) strongly suggests that methylene blue at 25 μM concentration might well predominate over O$_2$ at any oxidase level in accepting electrons that would be used for the univalent reduction of O$_2$ in the absence of the dye. An analogous case was reported in a past study by Muraoka et al. (34) where menadione (33 μM) dominates over O$_2$ as an electron carrier from xanthine oxidase to ferricytochrome c. When xanthine oxidase concentrations are low, i.e. fewer electrons are available, small amounts of leuco-methylene blue and semi-methylene blue are generated. The reduced dye thus formed can donate electrons either to the ferric dioxygenase (70 nM) (iiia, iiib) or to O$_2$ (250 μM) (iic, iib). The latter leads to reaction v. Apparently, the reactions iiia, iib and iic or iib or v have comparable rates under these conditions. At higher xanthine oxidase levels, leuco-methylene blue and semi-methylene blue are generated in higher concentrations (ii). This leads to a considerable increase in the rate constant (k$_{app}$ = k$_a$[MB]$_{sus}$ [IDO] $\ll$ [MB]$_{tot}$) for reaction vii while that for the reaction vi (k$_{app}$ = k$_a$[O$_2$]) $\ll$ [O$_2$]) is less affected, where [MB]$_{tot}$ refers to the total concentration of leuco-methylene blue and semi-methylene blue. Even if an O$_2^+$ concentration increases through reactions iiv and iivb, disproportionation of O$_2$ (vii) would become more significant. As a result of this, the reduction of the dioxygenase by reduced methylene blue (iiia, iib) might predominate over that by O$_2^+$ (v). Similar situations were reported by McCord and Fridovich (35) where ferricytochrome c was reduced totally by reduced methylene blue (1-10 μM) rather than by O$_2^+$ that was generated by the autoxidation of the reduced dye under aerobic conditions (2).

Although the actual O$_2^+$ concentrations under the various conditions used in this study can not easily be determined because of its quite unstable nature, i.e. disproportionation reaction vii, and although no attempts to determine O$_2^+$ generation rates in the xanthine oxidase-hypoxanthine system have been done in this study, it is possible to estimate these values. Based on a previous study by Fridovich on the percent O$_2^+$ generation per total electrons donated from xanthine (2 electrons/xanthine) and its pH dependence (31), and considering that 1 unit of xanthine oxidase using hypoxanthine as substrate (the unit used in this study) corresponds to approximately 2 units with xanthine being used as substrate (36), one can estimate the rates of O$_2^+$ production by 1.7 and 40 milliunits/ml xanthine oxidase at pH 8.0 and 25 °C to be 1.2-1.4 and 28-32 nmol/min/ml, respectively. The value with 40 milliunits/ml xanthine oxidase is in reasonable agreement with the O$_2^+$ (KO$_2$) infusion rate of 5 nmol/min/0.2 ml, i.e. 25 nmol/min/ml which was reported by Ohnishi et al. (14), to yield the maximal enzyme activity. These rates of O$_2^+$ production (V) by 1.7 and 40 milliunits/ml xanthine oxidase would result in -0.46 and -2.2 μM steady state O$_2^+$ concentrations ([O$_2^+$])$_{ss}$, using the constant for reaction vii as ~10$^4$ M$^{-1}$ s$^{-1}$ at pH 8.0 (37); [O$_2^+$]$_{ss}$ = (V/k$_a$)$^{-1}$. Leuco-methylene blue is shown to be able to reduce ferric indoleamine 2,3-dioxygenase (reaction iiia) to the extremes of 25-40% under the anaerobic conditions employed, using ~6 μM enzyme and 25-125 μM dye (Figs. 5 and 6). The incomplete reduction is most likely due to a relatively low oxidation-reduction potential of the dioxygenase as compared with that of methylene blue (E'_o = 0.011 V at pH 7.0 and 30 °C (38)). The present results suggest that the E'_o value for a ferric-ferrous pair of indoleamine 2,3-dioxygenase is considerably lower than that of sperm whale myoglobin (E'_o = 0.05 V (39)) but higher than that of methyl viologen (E'_o = -0.44 V (38)). The significant increases in the extent of the dioxygenase
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Trp and O$_2$ will help shift the ferric form to ferrous form equilibrium to the right.

The present study has not answered the questions as to (a) why O$_2$ alone, even at its sufficiently high concentrations (5 x 10$^{-6}$ M) cannot maintain the linearity of the catalytic reaction and (b) why methylene blue together with O$_2$ can restore the activity of the "O$_2$-inactivated" enzyme. Superoxide anion can reduce methylene blue according to reaction viii with a relatively high second order rate constant ($k$ = 10$^5$ - 10$^6$ M$^{-1}$ s$^{-1}$) at pH 7.0 and 25°C. Hence, the leuco-methylene blue thus generated may reactivate the "O$_2$-inactivated" enzyme. The cause and mechanism of the reversible "O$_2$-inactivation" of the dioxygenase remain to be answered. The reduction of the ferric enzyme with leuco-methylene blue is so slow (Figs. 5 and 6) as compared with the catalytic rate constant (~2 s$^{-1}$). To explain the reason for this, the inclusion of the semi-methylene blue radical as a potent reductant of the ferric dioxygenase (cf. reactions iii) might be necessary, since semi-methylene blue is a more reactive and stronger reductant than leuco-methylene blue (29). Semi-methylene blue might be preferably generated under aerobic rather than the anaerobic conditions employed in the present study. In fact, in the ascorbic acid-methylene blue co-factor system under air, when the reduction of the dioxygenase is monitored by trapping the reduced enzyme with CO to generate the stable ferrous-CO enzyme, much faster reduction rates ($\tau$ = 25 and 70 s with and without L-Trp, respectively) are obtained.

Under similar conditions, superoxide dismutase has no inhibitory effect on the catalytic reaction of indoleamine 2,3-dioxygenase (see above).

Xanthine oxidase is one of the likely candidates for the physiological electron donors to indoleamine 2,3-dioxygenase either via O$_2$ or other carriers, since the oxidase has been shown to be abundant in the small intestine and lung (40, 41) where relatively high dioxygenase activity is found in rabbit (3, 7), mice (9), and rats (10). However, it would be unrealistic to assume that over 10$^{-6}$ M steady state concentrations of O$_2^-$ are available (42, 43) in the cytosol of the tissues where the dioxygenase is located (5-7). As indicated in this study, low levels of O$_2^-$ (5 x 10$^{-5}$ M) can directly activate the dioxygenase up to about 10% of its maximal activity. Since $K_a$ values for L-Trp ($K_a$ = 10$^{-5}$ M) at pH 7.0 and 25°C (6, 27) and for O$_2^-$ ($K_a$ = 5 x 10$^{-6}$ M at pH 7.0 and 25°C (14)) are relatively low, O$_2^-$ may be utilized for L-Trp metabolism in vivo by the dioxygenase, assuming that (a) the enzyme can compete with superoxide dismutase (42) for O$_2^-$ or that (b) the dioxygenase normally operates at low percent levels of its maximal activity under physiological conditions. If, however, we expect near-maximal activity of the dioxygenase in tissues, some physiological electron carrier(s) (44, 45) between the donor(s) and the dioxygenase might exist. Such an activation process may not require O$_2^-$. In either event, the physiological significance of the uniquely high reactivity of ferric indoleamine 2,3-dioxygenase toward O$_2^-$ remains an intriguing question.

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REFERENCES

1. Hirata, F., and Hayaishi, O. (1971) J. Biol. Chem. 246, 7825-7826
2. Hirata, F., and Hayaishi, O. (1975) J. Biol. Chem. 250, 5960-5966
3. Hayaishi, O. (1976) J. Biochem. (Tokyo) 79, 13-21
4. Higuchi, K., and Hayaishi, O. (1987) Arch. Biochem. Biophys. 240, 397-403
5. Yamamato, S., and Hayaishi, O. (1967) J. Biol. Chem. 242, 5290-5296
6. Shimizu, T., Nomiyama, S., Hirata, F., and Hayaishi, O. (1978) J. Biol. Chem. 253, 4700-4706
7. Watanabe, Y., Yoshida, R., Sonoda, M., and Hayaishi, O. (1981) J. Histochem. Cytochem. 29, 623-632
8. Hirata, F., and Hayaishi, O. (1972) Biochem. Biophys. Res. Commun. 47, 1112-1119
9. Yoshida, R., Nukiwa, T., Watanabe, Y., Fujiwara, M., Hirata, F., and Hayaishi, O. (1980) Arch. Biochem. Biophys. 203, 343-351
10. Cook, J. S., Pogson, C. I., and Smith, S. A. (1980) Biochem. J. 189, 461-466
11. Yamazaki, F., Kuroiwa, T., Takikawa, O., and Kido, R. (1985) Biochem. J. 230, 635-638
12. Feigelson, P., and Brady, F. O. (1974) in Molecular Mechanisms of Oxygen Activation (Hayaishi, O., ed) pp. 87-133, Academic Press, New York
13. Ishimura, Y., Makino, R., Ueno, R., Sakaguchi, K., Brady, F. O., Feigelson, P., Aisen, P., and Hayaishi, O. (1980) J. Biol. Chem. 255, 3835-3837
14. Ohnishi, T., Hirata, F., and Hayaishi, O. (1977) J. Biol. Chem. 252, 4643-4647
15. Taniguchi, T., Sonoda, M., Hirata, F., Hayaishi, O., Tamura, M., Hayaishi, K., Iizuka, T., and Ishimura, Y. (1978) J. Biol. Chem. 254, 3289-3294
16. Ito, Y. A., Rabani, J., and Czapski, G. (1976) Biochim. Biophys. Acta 449, 277-286
17. Sutton, H. L., Robert, P. B., and Winterbourn, C. C. (1976) Biochem. J. 155, 503-510
18. Hirata, F., Ohnishi, T., and Hayaishi, O. (1977) J. Biol. Chem. 252, 4637-4642
19. Fridovich, I., and Handler, P. (1962) J. Biol. Chem. 237, 916-921
20. Murakoa, S., Enomoto, H., Sugiyama, M., and Yamasaki, H. (1976) Biochim. Biophys. Acta 143, 408-415
21. Sonoda, M., and Dawson, J. H. (1984) Biochim. Biophys. Acta 789, 170-187
22. Kalcker, H. M. (1947) J. Biol. Chem. 167, 429-443
23. Worton and Ohnishi, T. (1972) p. 30, Worton Biochemical Corporation, Freehold, NJ
24. McCord, J. M., and Fridovich, I. (1969) J. Biol. Chem. 244, 6049-6055
25. Ward, B., and Chang, C. K. (1982) Photochem. Photobiol. 25, 757-759
26. Cordon, A. J., and Ford, R. A. (1973) Chemists’ Companion, p. 438, Wiley Interscience, New York
27. Sonoda, M., Taniguchi, T., Watanabe, Y., Hayaishi, O. (1980) J. Biol. Chem. 255, 1339-1345
28. Michaelis, M. P., and Granick, S. (1940) J. Am. Chem. Soc. 62, 294-291
29. Parker, C. A. (1959) J. Phys. Chem. 63, 26-30
30. Hay, D. W., Martin, S. A., Ray, S., and Lichtin, N. N. (1981) J. Phys. Chem. 85, 1474-1479
31. Fridovich, I. (1970) J. Biol. Chem. 245, 4053-4057
32. Hille, R., and Massey, V. (1981) J. Biol. Chem. 256, 9090-9095
33. Porras, A. G., Olson, J. S., and Palmer, G. (1981) J. Biol. Chem. 256, 9096-9100
34. Murakoa, S., Sugiyama, M., and Yamasaki, H. (1965) Biochem. Biophys. Res. Commun. 19, 346-350
35. McCord, J. M., and Fridovich, I. (1970) J. Biol. Chem. 245, 1374-1377
36. Biochemical and Organic Compounds for Research and Diagnostic Clinical Reagents (1988) p. 1504, Sigma Chemical Co., St. Louis, MO
37. Metz, D., Czapski, G., Rabani, J., Dorfman, L. M., and Schwarz, H. A. (1970) J. Phys. Chem. 74, 3209-3213
38. Dawson, R. M. C., Elliott, D. C., Elliott, W. H., and Jones, K. M. (1986) Data for Biochemical Research, 3rd Ed., pp. 350-359, Oxford University Press, New York
39. Brunori, M., Saggese, U., Rotilio, G. C., Antonini, E., and Wyman, J. (1971) Biochemistry 10, 1604-1609
40. Roussos, G. G. (1963) Biochim. Biophys. Acta 73, 338-340
41. Stirpe, F., and Corte, E. D. (1969) J. Biol. Chem. 244, 3855-3865
42. Fridovich, I. (1981) in Oxygen and Living Processes (Gilbert, D. L., ed) pp. 250-272, Springer-Verlag, Berlin
43. Chance, B., Sies, H., and Boveris, A. (1979) Physiol. Rev. 59, 527-605
44. Nishikimi, N. (1975) Biochem. Biophys. Res. Commun. 63, 92-96
45. Ozaki, Y., Nichol, C. A., and Duch, D. S. (1987) Arch. Biochem. Biophys. 257, 207-216