Rapid Reaction Studies on the Oxygenation Reactions of Catechol Dioxygenase*

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The reaction of oxygen with catechol 1,2-dioxygenase from Pseudomonas arvilla ATCC 23974 in complex with catechol, 4-methylcatechol, and 4-fluorocatechol has been studied using single turnover stopped flow spectrophotometry. Two sequential enzyme intermediates have been resolved and their visible spectra characterized by computer-assisted methods. These intermediates are spectrally similar to those observed in a similar study with protocatechuate dioxygenase (Bull, C., Ballou, D. P., and Otsuka, S. J. Biol. Chem. 256, 12681-12686 (1981), although the first intermediate seen with the latter enzyme was not observed in this study. The rate of formation of intermediate I is oxygen-dependent and also accelerated by electron-donating substituents on the C-4 of the substrate. This is consistent with the proposed substrate reduction of dioxygen to form a hydroperoxide. Intermediate I is thus suggested to be a 6-hydroperoxycyclohexa-3,5-diene-1-one. The decay of intermediate I is also accelerated by electron donors and is consistent with the rearrangement of intermediate hydroperoxide via an acyl migration mechanism. It is inconsistent with mechanisms involving nucleophilic attack at the carbonyl carbon. Intermediate II is proposed to be an enzyme-product complex based on the resemblance of its visible spectra to those of the benzoate complex of Pseudomonas arvilla protocatechuate dioxygenase. Careful 18O2-labeling experiments have shown that no label is lost to the solvent, implying that no free hydroxide forms during catalysis.

Catechols are the central metabolites in the breakdown by soil bacteria of a wide variety of naturally occurring, complex aromatic compounds such as lignins, tannins, terpenes, etc. (Chapman, 1972; Dagley, 1975) as well as many xenobiotic pollutants (Wood, 1982). Catechols are cleaved to form ring-opened products by non-heme iron containing catechol dioxygenases. Catechol 1,2-dioxygenase from Pseudomonas arvilla ATCC 23974 is one such enzyme, and is a member of the intradiol-cleaving group of catechol dioxygenases that typically contains high spin Fe3⁺ in the active site (Nozaki, 1978; Que, 1980). These enzymes are burgundy-red in color due to a visible absorbance band at ~460 nm (ε ~ 3000–4000 M⁻¹ cm⁻¹ Fe₃⁺) arising from tyrosinate to iron charge transfer interactions (Keyes et al., 1978; Tatsuno et al., 1978; Felton et al., 1978; Bull et al., 1979; Que and Heistand, 1979). The spectra of these enzymes can be characteristically perturbed by the anaerobic addition of substrates, or by substrate and product analogues (Que et al., 1980; Que and Epstein, 1981; Bull and Ballou, 1981; Walsh and Ballou, 1983).

Current ideas for the mechanism of catalysis by the Fe³⁺-containing catechol dioxygenases focus around the observation that no Fe²⁺ species has yet been detected in the catalytic cycle. Thus, it has been proposed that, rather than the more classical activation of oxygen by Fe²⁺, the iron acts essentially as a Lewis acid activating the substrate towards O₂ attack and producing a peroxy adduct of the substrate (Hamilton, 1974; Que et al., 1977). This adduct may then rearrange to form product, conserving the two atoms of molecular oxygen in the resulting dicarboxylate. Rapid kinetic studies by Bull et al. (1981) on protocatechuate dioxygenase in which the enzyme-catalyzed oxygenation of the catechol, protocatechuic acid, was monitored, revealed two sequential enzyme intermediates in the reaction. It is not known to which chemical species in the proposed scheme these intermediates should be attributed, as the extremely transient nature of the intermediates has prevented any further characterization beyond that of their visible absorption spectra.

In this study, we have investigated the reaction of oxygen with catechol dioxygenase from P. arvilla in complex with the native substrate, catechol, and the two substrate analogues, 4-methylcatechol and 4-fluorocatechol. These analogues have relative turnover rates that are 76 and 19%, respectively, of that using catechol as substrate. Using stopped flow spectrophotometry, we have detected two sequential enzyme intermediates in single turnover reactions with each of these substrates. The rates of formation and decay of these intermediates vary according to the nature of the C-4 substituent of the substrate, enabling correlations to be made between the spectral forms and their possible chemical structures, in light of the current mechanistic proposals. Comparisons of these intermediates to those observed in the reaction of protocatechuate dioxygenase with protocatechuic acid and oxygen leads to tentative assignments of chemical structures to the species. The use of these substrates also enables a more reliable determination of the sequence in which these intermediates are formed.
Materials and Methods

Results

Enzyme-Substrate Complexes—Anaerobic titrations of catechol dioxygenase with either catechol, 4-methylcatechol, or 4-fluorocatechol produce similar changes in the spectrum of the enzyme that result in the formation of a broad absorbance band centered around 700 nm (Fig. 1, a and b; the spectrum of the enzyme in complex with 4-fluorocatechol is almost identical with that seen with the native substrate, and is not shown). These are typical of the spectral changes associated with the formation of ES2 in the non-heme, ferric-ance band centered around 700 nm (Fig. 1, a and b; is not shown). These are typical of the spectral changes almost identical with that seen with the native substrate, and produce a decrease in absorbance and hence a distinct isosbestic point at 540 nm.

Attempts at measuring the rate of formation of the enzyme-catechol complex were unsuccessful as the reaction was complete within the dead time of the stopped flow apparatus (~2.5 ms), even using concentrations of substrate approaching stoichiometry with that of enzyme (~40 μM). This implies that the second order rate constant for ES formation is greater than $10^6$ M$^{-1}$ s$^{-1}$. However, using 4-methylcatechol, the reaction was somewhat slower and hence more tractable. A plot of the observed rate of ES formation (as monitored at 700 or 440 nm) versus concentration of 4-methylcatechol was linear and passed through the origin; the slope of the line yielded a second order rate constant of $1.1 \times 10^5$ M$^{-1}$ s$^{-1}$. This lower rate could reflect some steric hindrance to binding due to the methyl group at the 4-position. It is interesting to note that both pyrogallol and 3-methoxycatechol behave like catechol in that the rate of ES formation with these substrate analogues is too fast to monitor in the stopped flow apparatus. Thus, compared to the 4-position, the 3-position seems relatively unhindered.

Reaction of ES with Oxygen—Single enzyme turnover events using catechol as substrate were monitored at wavelengths between 320 and 720 nm in the stopped flow apparatus, as described under "Materials and Methods." A selection of reaction traces recorded at various wavelengths is shown in Fig. 2a. A total of three kinetic phases can be resolved, corresponding to the formation and decay of two intermediates in the reaction. This is best seen at 620 nm as at this wavelength, the absorbance changes due to each phase are opposed. The decay of the second intermediate, i.e. the final phase, results in the formation of resting enzyme and free product. The rates of the three phases were determined to be 240, 36, and 15 s$^{-1}$ at an oxygen concentration of 0.96 mM (Table I). The rate of the first phase was linearly dependent

1 The "Materials and Methods" are presented in miniprint at this end of the paper. Miniprint is easy to read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 83M-972, cite the authors, and include a check or money order for $2.40 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

2 The abbreviations used are: ES, enzyme-substrate complex; ESO$_2$, first observed intermediate after mixing O$_2$ with ES; ESO$_2^*$, second intermediate observed after mixing O$_2$ with ES; EP, enzyme-product complex.

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Figure 1: Spectra of catechol 1,2-dioxygenase (---) and the enzyme in complex with (----) (a) catechol and (b) 4-methylcatechol. The spectrum of the enzyme in complex with 4-fluorocatechol is similar to that seen with catechol (a), on the oxygen concentration, whereas the following two rates were independent of the oxygen concentration. A pseudo-first order plot of the observed rate of the first phase versus oxygen concentration showed the reaction to be essentially irreversible, and yielded a second order rate constant of $2.5 \times 10^5$ M$^{-1}$ s$^{-1}$.

The turnover number of the enzyme with catechol as substrate can be calculated from the rates determined in the stopped flow experiments by the following relationship:

$$1/\text{turnover number} = 1/k_i + 1/k_j + 1/k_k$$

At 0.24 mM oxygen, where the observed rate of $k_i$ is 60 s$^{-1}$, the turnover number from the rapid kinetics data can be calculated to be 10.9 s$^{-1}$. This compares favorably with the value of 11.0 s$^{-1}$ determined under the same conditions by steady state kinetics techniques using catalytic amounts of enzyme and saturating amounts of catechol.

Using the computer analysis techniques described under "Materials and Methods," it is possible to obtain the extinction coefficients of the two intermediates in the reaction from the kinetic time courses taken at 20-nm intervals in the 320–720 nm region. Computer-generated time courses are shown overlaying the data collected in the stopped flow experiments in Fig. 2. The fit is excellent at all wavelengths, indicating that the determined rates and extinctions are reasonable. In computer simulations of a given kinetic record, it was found that adjustment of more than 5–10% of either the rate constants or the extinctions determined by the fitting routine gave poorer fits. Another useful test of both the kinetic scheme, and the extinction values and first order rate constants derived from the analysis of data obtained using 1 mM oxygen was the following. Experimental data recorded at different oxygen concentrations (0.3 and 0.45 mM) were simulated using the same scheme and values determined previously. The oxygen addition step, $k_i$, was altered to account for the difference in oxygen concentration. Excellent fits were obtained, providing confidence that the scheme and the rate constants are reasonable.

Fig. 3a shows the resulting spectra of the two intermediates. The spectrum of the first intermediate lacks the absorbance band centered at 700 nm typical of the ES and also shows a significant decrease in extinction at 460 nm, to produce a low shoulder at 480 nm. Note, however, the large increase in extinction at lower wavelengths (340–380 nm). The second intermediate is characterized by a poorly resolved peak at 480 nm ($\epsilon = 3000$ M$^{-1}$ cm$^{-1}$) and an increased extinction (over that of resting enzyme) between 580 and 700 nm. However, this absorbance does not take the form of a distinct new band as in the ES.
It was of interest to compare both the kinetics and spectra of the intermediates seen in the oxygenation of substrate analogs with those of the native substrate, in order to gauge the effect of ring substituents on the reaction. The reaction of oxygen with the 4-methylcatechol complex of the enzyme was qualitatively similar to that using catechol in that triphasic kinetics were again observed. However, the rates of the first two processes were significantly faster than those with the native substrate, while the final decay to resting enzyme and free product was marginally slower (Table I). Computer simulations of the reaction curves using extinctions for the two intermediates as shown in Fig. 3b, and the appropriate rate constants, gave excellent fits (Fig. 2b).

Intermediate I with 4-methylcatechol as substrate has a pronounced shoulder at 480 nm (ε = 2600 M⁻¹ cm⁻¹) and intermediate II has a distinct peak at 500 nm (ε = 2600 M⁻¹ cm⁻¹). Thus, both intermediates have somewhat more resolved spec-ra than those found using catechol as substrate. The analysis of the data from the experiments using 4-methylcatechol is somewhat more facile since the rates of the second and third kinetic phases are separated by a factor of 4.4, as compared to only 1.9 in the catechol case. However, in both cases, the closeness of fit of the simulated curves to the experimental data makes us confident that the spectra of the intermediates are accurate.

The kinetic course of the reaction of oxygen with the

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**TABLE I**

**Kinetic constants for the reaction of three substrates with catechol dioxygenase**

The kinetic constants correspond to the following scheme:

\[
E + S \xrightleftharpoons[k_{-a}]{k_a} ES \xrightarrow{k_1} \text{Intermediate I} \xrightarrow{k_2} \text{Intermediate II} \xrightarrow{k_3} E + P
\]

Dissociation constants were determined by anaerobic spectral titration of the enzyme with the appropriate compound. The values for \(k_\text{on}\) were determined by anaerobically mixing the enzyme with the substrate in the stopped flow apparatus. The values of \(k_1, k_2,\) and \(k_3\) were obtained by analysis of kinetic records following the reaction of oxygen with the enzyme in complex with substrate in the stopped flow apparatus. Experimental details are in the text. The turnover numbers calculated from the rapid kinetic data were derived from the following relationship:

\[
\frac{1}{\text{turnover number}} = \frac{1}{k_2} + \frac{1}{k_3} + \frac{1}{k_3}
\]

The value for \(k_1\) was taken to be that at 21% oxygen saturation. The turnover numbers obtained from steady state kinetic data were derived from Lineweaver-Burk plots using catalytic amounts of enzyme (~1 μM) and at 21% oxygen saturation. Oxygen equilibration was performed at 25°C.

| Substrate          | \(K_d\) | \(k_{-a}\) | \(k_1\) | \(k_2\) | \(k_3\) | Turnover number |
|--------------------|---------|------------|--------|--------|--------|----------------|
| Catechol           | 2.1     | >10⁷       | 2.5 × 10⁵ | 36     | 19     | 10.3           | 11.0           |
| 4-Methylcatechol    | 3.2     | 1.1 × 10⁶  | 3.8 × 10⁵ | 75     | 17     | 12.1           | 8.4            |
| 4-Fluorocatechol    | 2.3     | ND         | 2.1 × 10⁶ | 4.7    | 1.42   | 1.1            | 2.1            |

* ND, not determined.

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**FIG. 2.** Stopped flow kinetic traces (monitored at various wavelengths) of the reaction of oxygen with catechol dioxygenase in complex with (a) catechol, (b) 4-methylcatechol, and (c) 4-fluorocatechol. For a and b, solutions of 138 μM enzyme in the presence of 94 μM substrate were mixed in the stopped flow apparatus with a buffer solution containing 1.96 mM oxygen. In the case of c, the enzyme and substrate concentrations were 84 and 68 μM, respectively. Each division on the ordinate axis corresponds to 0.04 absorbance unit in a and c and to 0.05 absorbance unit in b. The smooth curves represent computer-generated simulations of the data using the scheme, rate constants, and extinction values as described in the text. In many cases, the fit is so close that the experimental data and the simulated curves are almost indistinguishable. It should be noted that the experimental data consist of four sets of 100 points and that the time scale over which each of these sets of points is collected is user-determined so that in the records above, for example, the first set was collected over the first 40 ms of the reaction. Thus, accurate data are obtained for every phase of the reaction so that accurate determinations of the observed rate constants for each of the processes can be made from a single data record.
enzyme in complex with 4-fluorocatechol was also triphasic (Fig. 2c). This is rather less apparent than with the other two substrates as there is no wavelength where all three phases are distinctly opposed. However, careful analysis of the reaction traces (in the 360–440 nm region especially) shows a biphasic return to resting enzyme and free product after the initial bimolecular reaction with oxygen. The oxygenation of the phases are shown in Table I. With the halogenated compound, all three rates are slower than with the native substrate. The oxygen reaction is slowed by ~25%, but the subsequent two reactions are very much more affected. Computer simulation of the reaction traces using the extinctions of the intermediates showed in Fig. 3c and the appropriate rate constants again gave excellent fits (Fig. 2c). Intermediate I in this case is almost identical to that found with catechol as substrate although intermediate II is rather different, particularly in having a similar extinction to resting enzyme in the 600 nm region and above (thus explaining the lack of wavelengths where all three phases are opposed).

Previous 18O-labeling studies (Hayashi et al., 1957) have shown that both atoms of molecular oxygen are incorporated into the product. This implies that little or no exchange of label occurs during catalysis. Since mechanistic interpretations rely heavily on this observation, we undertook careful 18O2-labeling experiments to determine if under some conditions any exchange of label with solvent could occur. It was expected that the conversion of I to II would be the most likely step in which oxygen exchange might occur. A larger difference in the rates of formation and decay of intermediate I was observed for 4-fluorocatechol than for catechol. Therefore, we reasoned that this halogenated substrate would afford a better opportunity for observing exchange of labeled oxygen with the solvent. However, 18O2-labeling experiments with both catechol and 4-fluorocatechol showed complete retention of both atoms of 18O2 in the products (>99%). This is consistent with earlier studies using catechol (Hayashi et al., 1957).

**Discussion**

These studies show that catechol, 4-methylcatechol, and 4-fluorocatechol are oxygenated by catechol 1,2-dioxygenase via a common mechanism. The ES for the substrates are similar, displaying a long wavelength absorption band arising from a catecholate to iron charge transfer interaction (Fig. 1) (Felton et al. 1978; Que and Heistand, 1979). Moreover, in rapid mixing experiments two comparable sequential intermediates are seen when each of the ES complexes is mixed with oxygen. The rapid oxygen-dependent disappearance of the catecholate charge transfer band concomitant with the formation of intermediate I strongly suggests that the substrate has become oxygenated in the process. Studies with the related enzyme, protocatechu ate dioxygenase, have also shown a similar reaction scheme in which ES reacts with oxygen to form sequentially, ESO2, ESO2*, and E + P (Bull et al., 1981). However, the first intermediate seen with catechol 1,2-dioxygenase is spectrally more similar to the second intermediate observed with protocatechu ate dioxygenase (ESO2*) that to the first (ESO2).

We have no clear evidence that a species analogous to ESO2 occurs in the present study although the mechanisms of the two enzymes appear to be very similar in all other respects. ESO2 may not be observable in the scheme for catechol 1,2-dioxygenase because ESO2 is transformed into ESO2* (intermediate I) as fast as it is formed. Given that intermediate I is formed at 240 s−1, the conversion of ESO2 to ESO2* would have to be >1000 s−1 to satisfy this hypothesis (This rate is 450 s−1 for protocatechu ate dioxygenase.) With 4-methyl catechol as substrate, for which the rate of oxygen addition is significantly greater than for the other two substrates (making kinetic resolution more favorable), the spectrum of intermediate I has higher absorbance at ~440 nm and lower absorbance at ~550 nm (Fig. 2) than those for the other two substrates. This type of spectral difference could be accounted for by a slight contribution of an ESO2 species as seen with protocatechu ate dioxygenase. Alternatively, the variations in the spectra for intermediate I could arise from substituent effects rather than from unresolved kinetic processes.

The spectra of intermediate II obtained with the three substrates are characterized by a shift of the absorption maxima to ~490 nm and show some variability with the different reactions. The spectrum of this intermediate very strongly resembles that of the benzote complex of catechol dioxygenase (Que et al., 1980). Also, protocatechu ate dioxygenase forms complexes with dicarboxylates, particularly at pH values below ~7 (Que and Epstein, 1981; Ballou and Bull, 1978). Its natural product, β-carboxy-cis,cis-muconate, as well as the product analogs, β-carboxy-cis,trans-muconate, terephthalate, and glutarate form complexes with spectra which are similarly red-shifted, but have variable extinctions and λmax values. Therefore, we believe that intermediate II is an EP. Unfortunately, catechol 1,2-dioxygenase has such a low affinity for product that we have been unsuccessful in generating an EP for comparison (even with concentrations of cis,cis-muconate up to 5 mM).

No reduced iron species has as yet been detected in the catalytic cycle of any of the catechol dioxygenases. Therefore, current mechanistic proposals for the reaction catalyzed by the intradiol catechol dioxygenases involve Fe2+-assisted activation of the substrate to electrophilic attack by oxygen. Fraser and Hamilton (1982) and Jefford and Cadby (1981) have proposed that essentially any reasonable mechanism for this reaction has, as a first step, the formation of an α-hydroperoxide ketone. It is proposed that the intermediate formed is the 8-hydroperoxycyclohexa-3,5-dien-1-one (species...
2 in Scheme 1) which rearranges to form the dicarboxylate product.

Our results show how substituents in the 4-position of catechols affect the rates of formation and decay of intermediate I. This allows us to examine postulated mechanisms for consistency with the results using the enzyme. We find that both the first and second processes in the reaction of oxygen with catechol 1,2-dioxygenase ES are accelerated by electron donation. The rate of the first process is dependent on oxygen concentration and thus would be expected to be enhanced by substituents which increase electron density on the substrate and make it more susceptible to electrophilic attack by oxygen. Indeed, Walsh and Ballou (1983) have shown that, in the reaction of oxygen with the protocatechuate dioxygenase-6-chloroprotocatechuate complex, the bimolecular step involving the initial attack on oxygen is 25-fold slower than that for the natural ES, again demonstrating that the presence of an electron-withdrawing group retards the oxygen reaction (Walsh and Ballou, 1983).

The assignment of intermediate I to the hydroperoxy species described in Scheme 1 is an attractive hypothesis and its formation would be favored by electron donors. Considerable work on the mechanism of rearrangement of hydroperoxy species such as these has recently been reported. The work reported here is the first that directly bears on this mechanism and its assignment to the hydroperoxy species via acyl migration (pathway 3) is predicted to apply to the acid-catalyzed rearrangement of hydroperoxy ketones. These reactions are accelerated by electron-donating substituents, which stabilize the incipient carboxonium ion (Sawaki and Ogata, 1978).

In our studies we have found that the decay of intermediate I is accelerated by electron donors. The data are inconsistent with either pathway 1 or 2, since both mechanisms involve nucleophilic attacks on the acyl carbon, which would be expected to be enhanced by electron-withdrawing substituents. It is possible, however, that a nucleophilic attack by an enzyme group is not a rate-limiting step in the rearrangement so that the appropriate substituent effect would not be observed. The data are consistent, however, with the rearrangement of an intermediate α-hydroperoxyketone via acyl migration (pathway 3). In the proposed mechanism, the Fe” in the active site may serve as a Lewis acid both in the activation of the substrate toward oxygen and in the subsequent Criegee (1948) rearrangement of the peroxide to form products. The 18O studies imply that these interesting acid-catalyzed rearrangements occur extremely efficiently since no exchange with solvent occurs.

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Oxygenated Intermediates of Catechol Dioxygenase

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Materials and Methods

Catechol 1,2-dioxygenase was purified from Paracoccus denitrificans ATCC 21655 following the procedure of Ballou et al. (1985) with some modifications. An extinction value of 3.500 mL/mg at 400 nm was used to determine the concentration of enzyme solutions. All experiments were performed in 50 mM potassium phosphate buffer, pH 7.5 at 25°C.

Catechol and 4-methylcatechol were obtained from Aldrich Chemical Co. and purified by sublimation. 4-Fluorocatechol was synthesized from 4-fluorotoluene (Ceva and Zaghmout, 1971).

Oxidative labeling studies with catechol 1,2-dioxygenase were performed in the following manner: 14C-labeled arsine, was introduced into one compartment of a dual-walled reaction vial. A reaction flask was charged with a suitable amount of immobilized enzyme, thoroughly degassed, and then filled with He gas. Four ml of a 0.5 mM substrate solution (2.5 mM substrate in phosphate buffer, pH 7.5) were added to the immobilized enzyme. After the reaction of the flask was frozen and the flask evacuated, He was introduced into the flask and condensed with liquid N2. The labeled flask was then allowed to stand at room temperature for the period until the reaction was complete. The system was then removed by sublimation through an amber TM-1000 manifold. The labeled product was obtained as follows: The solution was filtered to remove EC and collected in a sodium hydrate, evaporated at 80°C, and extracted with ether. The organic extract was dried over sodium sulfate and the mixture removed. The product obtained was subjected to preparative thin-layer chromatography by the classical method used at the Cornell University Chemistry Research Laboratory. All experiments were performed under identical conditions and recovery procedures. Each peak for the double-label 3-fluorocatechol-decanoic acid was observed at m/e 159 corresponding to the molecular ion, and for the doubly-labeled 3-fluorocatechol-decanoic acid was observed at m/e 159 corresponding to a loss of H2PO from the molecular ion. No loss of labeled was detected in either case.

Optical spectra and stopped-flow experiments were carried out by the same procedures as described by Walsh and Ballou (1985). (Received for publication: January 1987.)

Single turnover events were monitored by titrating the enzyme enzymatically with a stoichiometric amount of the appropriate substrate (typically 0.5 mM of the enzyme concentration) and rapidly mixing this with a solution of oxygen in the stopped-flow apparatus. Because of the relatively low 02 values for all of the nonstoichiometric solution (see Table I), there is minimal free substrate present in the enzyme solutions at titration because the scavenging rates in the experiments were in excess of that of the substrates and were more than two-fold greater than the 02 values. This alleviates the problems associated with the interpretation of multiple-turnover events and greatly simplifies the analysis.

Analysis of the kinetic constants for the two-substrate reaction

The reaction was monitored in the stopped-flow apparatus at 20 µM intervals over the wavelength range 320 - 100 nm. Inspection of these kinetic records revealed three phases in the reaction, especially in the region around 620 nm, the absorbance change due to each phase was observed (Fig. 1A). It is essential to select the data recorded at several wavelengths because the relative proportions and the absorbance change values with wavelength. For example, the approximate data for one process may be distorted by preceding or following process involving a large change in absorbance, or reaction rates involving a single process may be too rapidly apparent. It is further apparent from these graphs that the effect of uneven illumination on the reaction rates, and for these reasons we used in Table I, the rates of the phases involving the major change in absorbance are more accurately, as there is little interference from the other steps in the reaction. Thus, at 650 nm, b, predominates. At 600 nm, b, and a, are not affected by b, while at 450 nm the absorbance changes due to a, are more slowly seen (see traces in Fig. 1D).

Three substrate values at these three concentrations can be determined, either by direct semi-logarithmic analysis or by subtraction of sequential exponential decay in the case of multiple-phase traces. This survey, performed at different concentrations of oxygen, will effectively demonstrate which reactions are order zero with respect to oxygen.

Having determined the rates of the processes involved in the reaction, the rates of the two intermediates can be determined. The spectra of the two enzymes and the enzyme-substrate complex as well as the appropriate 02 values for each substrates were known from earlier stopped irradiations. The absolute concentrations of each of the species during the course of the reaction can be calculated by computerized numerical integration techniques. At any given time-point along the course of the reaction, the sum of square concentration values, multiplied by the appropriate weighting factors, by the traces for the absorbance, must equal the observed absorbance. Thus, one has a large number of simultaneous equations at each given wavelength with only two unknowns, the initial values for the two intermediates.

Computer simulations of reaction time-courses were performed using a SOR I minicomputer (Data General) employing a FORTRAN-based fourth order Runge-Kutta method (Bover and Bissell, 1969). The following scheme was used:

where a is a free enzyme, b is the enzyme-substrate complex, b is the enzyme-substrate complex, and c is a free product. The oxygen addition, step a, is treated as pseudo-first order at any given oxygen concentration. The simulations were performed at several appropriate wavelengths to ascertain that with a given set of rate constants the fit is good even for cases where the proportions and directions of absorbance change are different. Major adjustments in the rate constants for a particular step are occasionally necessary (less than the uncertainty in the original determination by semi-logarithmic analysis) to obtain good fits for all wavelengths with a single set of rate constants. The parameters at all wavelengths is then fitted to give extinction values of the intermediates at 30 µM intervals from 400 to 750 nm.

One point should be stressed here. A regio change in absorbance followed by a slower change does not necessarily imply that the rate constant governing the first process is faster than that of the second (Wang and Passow, 1981). In the simple example: 

where the extent of C to D, or B to D, in column values and two apparent rates can be determined from the biphasic absorbance-change pattern due to the formation and decay of D, we equally appropriate solutions can be derived with the faster observed process occurring either first or second, i.e., the observed rates for the two oxygen-sensitive processes are compatible with the extinction values of D is adjusted to compensate. In practice, one of the two solutions to x can usually be eliminated as being unlikely or impossible (a negative value for example). In cases where x and y are not well separated (less than a factor of 2), it may be difficult to determine the true solution. We have tested our data by treating x, and y, in our kinetic scheme and fitting the analysis of the simulations and I and II. In the cases using 4-methylcatechol and 4-fluorocatechol as substrates, the ratios of 02/4 are relatively large (4.2 and 2.5 respectively). For the solution where x is greater than y, the extinction values of each of the intermediates remain reasonably well for the two substrates. If the value used for x is the smaller apparent 'apparent constant,' this resulting spectra of intermediates 1 and II for the two substrates are very distinguishable with considerably high or low extinction values (e.g., 60,000 cm⁻¹ and 2600 cm⁻¹). With catechol as substrate b, and c, are only separated by a factor of 1.9 and by ordering the order of the reactions in the simulated scheme give a somewhat more plausible spectrum for intermediate II with extinction values in the 400-700 nm range that are 1.2 times larger than those shown in Fig. 1B. However, if these were the correct solutions as discussed above, the intermediates would be significantly different from those calculated for the other substrates, and hence we may avoid the faster process as the one occurring first. This is a classical approach to solving a difficult kinetic problem.
Rapid reaction studies on the oxygenation reactions of catechol dioxygenase.
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