INTERMEDIATES IN FLAVOPROTEIN CATALYZED HYDROXYLATIONS

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Summary - The reaction of molecular oxygen with the complex of reduced p-hydroxybenzoate hydroxylase and 2,4-dihydroxybenzoate has been followed by rapid reaction techniques. During the reaction, which produces stoichiometric amounts of oxidized enzyme and the hydroxylated product, 2,3,4-trihydroxybenzoate, three spectroscopically distinguishable intermediates have been detected and characterized.

INTRODUCTION

One of the major problems in the elucidation of the mechanisms of biological hydroxylation reactions is the way in which molecular oxygen is inserted into the hydroxylated product. While most enzyme systems carrying out such reactions are complex and difficult to isolate, a number of hydroxylation reactions catalyzed by simple flavoproteins is now known (1). Some of these enzymes prepared from bacteria are stable and relatively simple to purify, and offer interesting possibilities for detailed study of hydroxylation mechanisms. With two such enzymes, p-hydroxybenzoate hydroxylase from *Pseudomonas fluorescens* (2,3) and melilotate hydroxylase from a Pseudomonad species (4), evidence has been reported of new spectral species of the flavoproteins on reaction of the reduced enzyme-substrate complexes with O_2. It was suggested that these spectral species were peroxydihydroflavin intermediates. One puzzling feature of this earlier work was the pronounced difference in the spectral characteristics of the intermediates. Thus, with p-hydroxybenzoate hydroxylase the spectrum of the intermediate formed when p-hydroxybenzoate was the substrate (3) was quite different from that formed when 2,4-dihydroxybenzoate was the substrate (2). The intermediate formed with melilotate hydroxylase had spectral characteristics (4) resembling those of the p-hydroxybenzoate hydroxylase-p-hydroxybenzoate system, but nevertheless

with well marked differences. A possible resolution of this puzzle comes from the present work, in which the reaction with O_2 of the reduced p-hydroxybenzoate hydroxylase-2,4-dihydroxybenzoate complex was studied at a lower temperature than previously. Under these conditions, three distinct intermediates have been characterized spectroscopically. Their properties suggest that the observation of different intermediates in the hydroxylation reactions mentioned above can be explained by differences in the rate constants for the steps in the mechanism of hydroxylation, rather than differences in mechanism.

MATERIALS AND METHODS

p-Hydroxybenzoate hydroxylase was prepared by the method of Howell *et al.* (4). 2,4-Dihydroxybenzoic acid was purchased from Aldrich Chemical Co. and 2,3,4-trihydroxybenzoic acid from J.T. Baker Chemical Co. They were purified by recrystallization. All other reagents were the highest quality commercially available. All reactants were dissolved in glass-distilled water.

Rapid reaction spectrophotometric studies were performed with a modified Gibson-Milnes (6) apparatus. Sample handling under anaerobic conditions was as described previously (2); specific details are given in the figure legends. The acid-quench experiments were performed with the rapid mixing apparatus previously described (7). These experiments employed a three-syringe system. Reduced enzyme-substrate complex from one syringe was reacted with oxygenated buffer plus substrate from a second syringe; after given times, the reaction was quenched by mixing with 0.17 M HCl from the third syringe. The final pH of the quenched reaction mixture was 1.8. The amount of 2,3,4-trihydroxybenzoate present in such reaction mixtures was determined as follows. The mixture, containing soluble, but inactive enzyme, was passed through a column of Bio-Gel P-2 (polyacrylamide gel beads from Bio-Rad Laboratories) in a solution of HCl at pH 1.5. The product (2,3,4-trihydroxybenzoic acid) was quantitatively separated from all the other components of the mixture except traces of 2,4-dihydroxybenzoic acid. The amount of 2,3,4-trihydroxybenzoic acid present was determined from the ultraviolet absorption spectrum of the

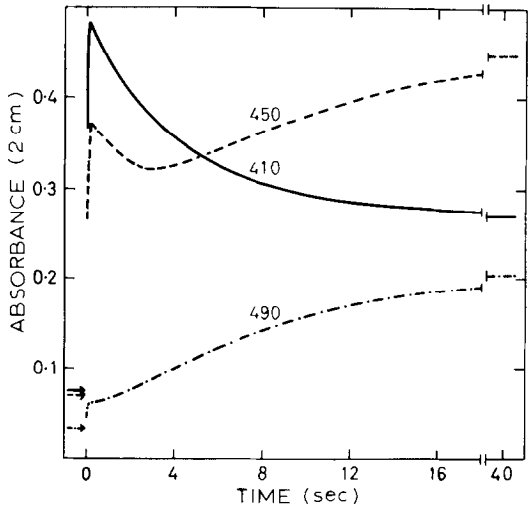


Fig. 1. Time course of overall absorbance changes at selected wavelengths after mixing oxygenated buffer with the complex of reduced p-hydroxybenzoate hydroxylase and 2,4-dihydroxybenzoate. A buffered solution of reduced enzyme (produced by irradiation of the enzyme solution containing 50 mM EDTA with visible light under an atmosphere of nitrogen) plus 2,4-dihydroxybenzoate from one syringe was rapidly mixed with an equal volume of buffer plus 2,4-dihydroxybenzoate (previously equilibrated with air at room temperature) from another syringe at 1.5°C. The final reaction mixture contained 17.6 μM enzyme, 10 mM 2,4-dihydroxybenzoate, 130 μM O_2, 25 mM EDTA, and 34 mM Tris-Cl at pH 8.55. The arrows indicate the absorbance of the reduced enzyme complex at the selected wavelengths.

solution, by making use of the distinctive differences in the spectra of the two components present.

RESULTS

Fig. 1 shows the overall absorbance changes at selected wavelengths when reduced enzyme complexed with 2,4-dihydroxybenzoate was reacted at 1.5° with buffer containing oxygen and 2,4-dihydroxybenzoate. Records such as these were made at 5 to 10 nm intervals from 340 to 600 nm, and at different final oxygen concentrations. The results are qualitatively very similar to those found previously at 25° (2), except that the overall process is slower. The absorbances reached after 40 sec are those of the oxidized enzyme— 2,4-dihydroxybenzoate complex, since the 2,4-dihydroxybenzoate is present in very large excess over both the enzyme and the stoichiometric amount of 2,3,4-trihydroxybenzoate formed in the reaction with O_2. From the results (Fig. 1) it

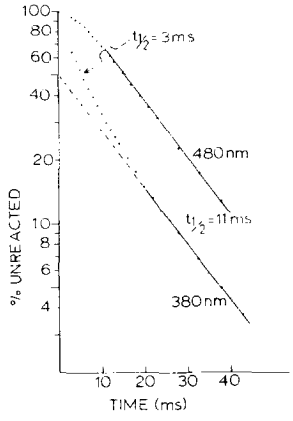


Fig. 2. Absorbance increases at selected wavelengths during the rapid phase of the reaction commenced by mixing oxygenated buffer with the complex of reduced p-hydroxybenzoate hydroxylase and 2,4-dihydroxybenzoate. Conditions in this experiment were identical to those described in the legend to Fig. 1, except that the reaction mixture had a final oxygen concentration of 330 μM. The absorbance changes are presented as the logarithmic plot of the unreacted percent of the fast phase of the reaction.

is clear that several consecutive reactions are proceeding at different rates and with different spectral characteristics. After the initial rapid burst of absorbance increase from 340 to 510 nm, analysis of the slower changes show that there are two steps with rate constants of 21 min^{-1} and 8.3 min^{-1}. For example, at 410 nm, after the initial burst, there is a biphasic decrease in absorbance with the above rate constants. At 450 nm, the faster of these two steps is associated with an absorbance decrease, the slower with an absorbance increase. At 490 nm, the faster step results in no absorbance change (giving rise to a pronounced lag period), while the slower step is associated with an absorbance increase. The rates of these two steps are independent of O_2 concentration.

Analysis of the rapid burst phase reveals that this is also made up of at least two steps. This is illustrated in Fig. 2, which shows the logarithmic absorbance changes plotted against time at two selected wavelengths occurring during this rapid phase of the reaction. At 380 nm there is a biphasic increase in absorbance, and at the O_2 concentration employed in this experiment, the $t_{1/2}$ values of the two steps are respectively 3 msec and 11 msec, with

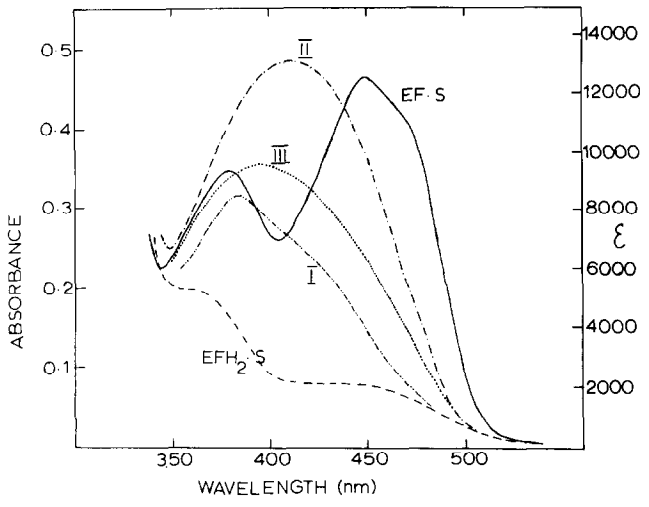


Fig. 3. Spectral intermediates observed during the reaction commenced by mixing oxygenated buffer with the complex of reduced p-hydroxybenzoate hydroxylase and 2,4-dihydroxybenzoate. The spectrum of the reduced enzyme-substrate complex was recorded on a Cary 17 spectrophotometer before reaction with oxygen, and the spectrum of the oxidized enzyme-substrate complex was similarly recorded after exposing the reduced enzyme to air. The intermediate spectra were obtained from experiments carried out under the conditions described in the legend to Fig. 1. Curve I was calculated from absorbance changes in the fast phase of the reaction (like those in Fig. 2), at a final oxygen concentration of 330 μM. Curve II represents the absorbance of the reaction mixture at the end of the fast phase of the reaction at all concentrations of oxygen used. Curve III was calculated from absorbance changes in the slow phase of the reaction (like those in Fig. 1).

approximately equal absorbance changes being associated with each step. At 480 nm there is a pronounced lag in the absorbance increase, indicating little absorbance change in the fastest step, followed by the majority of the absorbance increase occurring with a $t_{1/2}$ value of 11 msec. Because of the high rates involved, it is not possible to decide whether the first observable step involves a second order reaction with oxygen or if the reaction saturates at high oxygen concentrations. The rate of the second step is saturated at high O_2 concentration, with a limiting rate constant of 5,300 min^{-1}.

Analysis of the reaction observed at different wavelengths permits us to determine the spectra of the three intermediates observed. These are shown in Fig. 3, together with the spectra of the starting reduced enzyme-and final oxidized enzyme-2,4-dihydroxybenzoate complexes. Intermediate I is formed first and has a maximum at 385 nm with an extinction coefficient of approximately

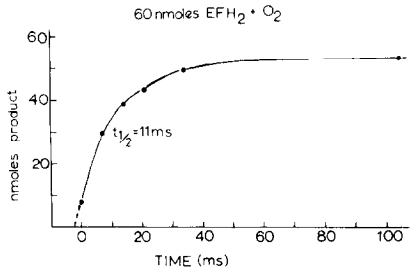


Fig. 4. The amount of 2,3,4-trihydroxybenzoate present in solution after reactions between oxygen and the complex of reduced p-hydroxybenzoate hydroxylase and 2,4-dihydroxybenzoate were quenched with acid at various times after the reaction commenced. The reactions were carried out under the same conditions as described in the legend to Fig. 1, using the technique described in Materials and Methods. The final concentration of enzyme in the reaction solutions was 66.7 μM and oxygen 180 μM. After the reaction was quenched with 0.17 M HCl, a total of 1.5 ml was collected which contained 60 nmole of denatured enzyme. The mean quantity of 2,3,4-trihydroxybenzoate recovered after analysis from four completed reactions was 55 nmole. The time coordinate represents the calculated time for the reaction mixture to travel from the reaction mixer to the quenching mixer.

8,500 $M^{-1}cm^{-1}$. Intermediate II, identical with that previously observed at 25°, is maximal at 410 nm with ε = 13,600 $M^{-1}cm^{-1}$. Intermediate III has a maximum at 395 nm with ε a little less than 10,000 $M^{-1}cm^{-1}$.

It was of obvious interest to try to define at what stage of the reaction the actual transfer of an oxygen atom to the substrate had occurred. For this purpose experiments such as that shown in Fig. 4 were performed. In this experiment, carried out under conditions similar to those of Figs. 1 and 2, reduced enzyme-substrate complex and oxygen were reacted together for varying times. The reaction was then quenched rapidly by mixing with acid, and the amount of 2,3,4-trihydroxybenzoate formed was determined. From Fig. 4 it is readily seen that the formation of product, or at least a species in a form which is liberated as product on acid denaturation of the enzyme, occurs at the same rate as the conversion of intermediate I to intermediate II.

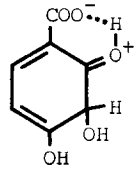
DISCUSSION

Formally, the reaction of the reduced enzyme-2,4-dihydroxybenzoate complex with O_2 is described by the following sequence.

$$E\text{-}FH_2 + O_2 \xrightarrow[\text{very fast}]{k_1} I \xrightarrow[5{,}300\ \text{min}^{-1}]{k_2} II \xrightarrow[21\ \text{min}^{-1}]{k_3} III \xrightarrow[8.3\ \text{min}^{-1}]{k_4} E\text{-}F + \text{product}$$

While more work clearly remains to be done, plausible chemical identification of the intermediates is possible. Intermediate I, with an absorption maximum at 385 nm and extinction coefficient of ~8,500 $M^{-1}cm^{-1}$, together with the fact that its rate of formation is very dependent on O_2 concentration, would most logically represent a covalent adduct of reduced flavin and O_2.

The rapid-quench experiments indicate that oxygen atom transfer from the first intermediate to the protein-bound substrate occurs coincidentally with the formation of intermediate II. As only one oxygen atom of molecular oxygen is transferred in the hydroxylation reaction, it is tempting to postulate that intermediate II represents a complex of hydroxydihydroflavin and a precursor of the product which could well be some quinoid type intermediate, such as



which by deprotonation would yield the trihydroxybenzoate product. Such a species could be expected to transform spontaneously to 2,3,4-trihydroxybenzoate on denaturation of the enzyme with acid, accounting for the results of Fig. 4. In the unquenched reaction, however, the deprotonation may occur while still enzyme-bound, resulting in the formation of intermediate III, a complex of 2,3,4-trihydroxybenzoate and enzyme with the flavin in the form of a hydroxydihydroflavin. This, by elimination of H_2O and the release of product, would then revert to the oxidized enzyme. Previous work (2) has shown that oxidized enzyme and substrate form a complex extremely rapidly; hence, the final spectrum of Fig. 3 would be that of the enzyme-2,4-dihydroxybenzoate complex. Comparison of the spectra of the intermediates (Fig. 3) with the spectral properties of model compounds (8) suggests that the intermediates may have the structure of C(4a)-N(5) dihydroflavins. Attention is also drawn to

the similarity of the spectrum of intermediate I with that recently reported for the oxygenated luciferase flavin intermediate (9).

It is interesting to note that the spectrum of the intermediate detected when O_2 is reacted with reduced enzyme complexed with p-hydroxybenzoate (3) resembles that of intermediates I and III of Fig. 3 and is formed at a comparable rate to that for intermediate I under the same conditions. No other intermediates were detected in that case. Similarly, only one intermediate was detected with melilotate hydroxylase (4). The present results suggest that in these cases, while the same reaction sequence as that described in this communication may occur, the subsequent intermediates may not be visualized because of kinetic reasons. Thus if k_2 were slower than k_3 and k_4, only intermediate I, or a mixture of intermediates that would resemble I, would be detected It is only because of favorable rate constants that three intermediates can be readily detected in the reaction with 2,4-dihydroxybenzoate as substrate. The possibility of even more intermediates in this reaction is of course obvious.

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