OXYGEN REACTIONS OF PARA-HYDROXYBENZOATE HYDROXYLASE CONTAINING 6-HYDROXY-FAD

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Introduction

It is now recognized that the reaction mechanisms of flavoprotein oxygenases are known in some detail, principally because of the research with p-hydroxybenzoate hydroxylase (EC 1.14.13.2) from Pseudomonas fluorescens (1). However, major gaps remain in our knowledge. For example, during catalysis by aromatic hydroxylases, the transfer of oxygen to the substrate involves an unidentified transient state of flavin. Modified flavins provide one experimental tool to probe flavoprotein mechanisms, by perturbing the active site. With the naturally occurring flavin, 6-hydroxy-FAD (2), apo-para-hydroxybenzoate hydroxylase forms an active enzyme which hydroxylates p-hydroxybenzoate (A. Claibourne, unpublished result), in contrast to some other modified flavins (3). We chose to examine the oxygen reactions of the 6-OH-FAD enzyme in some detail, as the 6-hydroxy group changes the chemistry of the isoalloxazine ring.

Results and Discussion

Dr L.M. Schopfer kindly prepared 6-OH-FAD using enzymes from

Flavins and Flavoproteins
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Brevibacterium ammoniagenes. Four compounds which are hydroxylated efficiently by native enzyme were tested with 6-OH-FAD enzyme. The same products were formed as with native enzyme. Enzyme containing ≤ 0.2% FAD was used to quantify product formation. Rates of NADPH oxidation in turnover were approximately an order of magnitude lower than with native enzyme at pH 7, 25°. The ratio of moles product formed to NADPH oxidised was calculated - 0.69 for p-hydroxybenzoate, 0.25 for p-aminobenzoate, 0.29 for 2,4-dihydroxybenzoate, and 0.52 for p-mercaptobenzoate. Thus, 6-OH-FAD enzyme was an effective catalyst, but not as efficient as native enzyme.

The oxygen half-reaction of enzyme was studied as described in the Table and (4). Complex reaction traces were interpreted in terms of transient chemical species of flavin. The most

Figure. Spectra of flavin species in the reaction E_R-p-aminobenzoate + 0_2 (see Table) - reduced enzyme (—); intermediate I (□—□); intermediate II (O-O); intermediate III (●-●); oxidised enzyme (— — —).

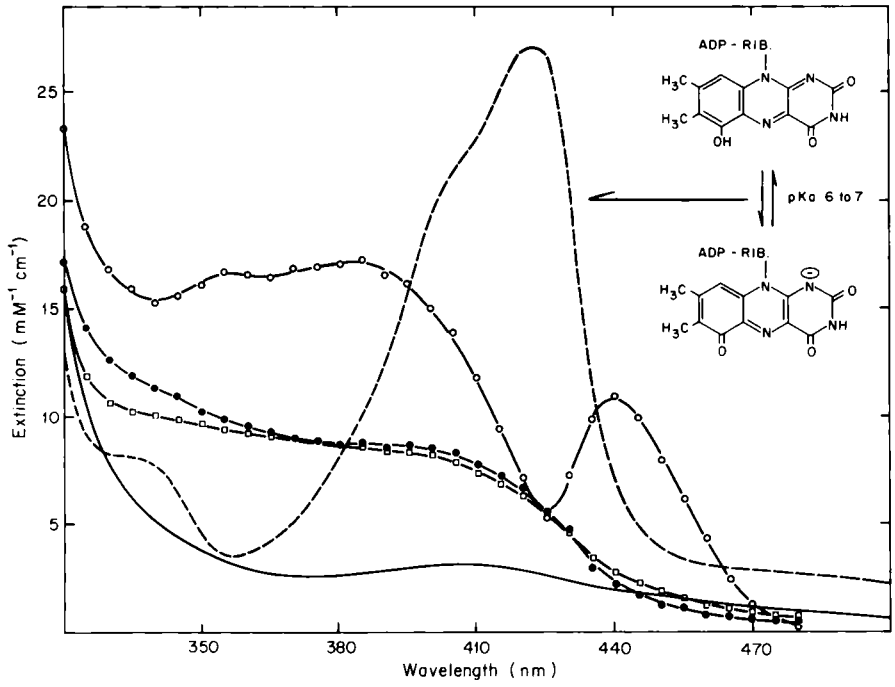

Table. RATE CONSTANTS AND INTERMEDIATES IN REACTIONS OF REDUCED ENZYME WITH OXYGEN

The 6-OH-FAD enzyme was mixed with substrate to saturate the reduced enzyme, based upon known dissociation constants for native enzyme. This assumption was justified for p-hydroxybenzoate and p-aminobenzoate, but probably not for 2,4-dihydroxybenzoate. After the solution was made anaerobic, the enzyme was reduced with buffered sodium dithionite. The reduced enzyme complex was reacted with oxygenated buffer in a stopped-flow spectrophotometer with computerized data aquisition. The reactions were followed at many wavelengths and different oxygen concentrations. Reaction solutions finally contained 16μM enzyme, substrate, and oxygen in 50mM phosphate (K salt) and 10mM EDTA (Na salt), pH6.55 at 3 to 4°, or 20mM phosphate (K salt with Tris) and 4mM EDTA, pH8.5 at 3 to 4°.

Substrate	Minimum Scheme for each Reaction from Observed Kinetics
p-hydroxybenzoate (pH6.55)	$E_RS + O_2 \xrightarrow[2.6 \times 10^{5b}]{3.5 \times 10^{5a}} I \xrightarrow[48]{14} (II)^c \xrightarrow[Fast]{Fast} III \xrightarrow[14.5]{2.0} E_{OX}S + P + H_2O$ $I \xrightarrow[<1]{7} E_{OX}S + H_2O_2$
p-aminobenzoate (pH6.55)	$E_RS + O_2 \xrightarrow[3.3 \times 10^{5}]{2.9 \times 10^{5}} I \xrightarrow[4.9]{1.4} II \xrightarrow[.031]{.63} III \xrightarrow[Fast]{.020} E_{OX}S + P + H_2O$ $I \xrightarrow[<.2]{5} E_{OX}S + H_2O_2$
2,4-dihydroxybenzoate (pH8.5)	$E_RS + O_2 \xrightarrow[5.0 \times 10^{5}]{4.5 \times 10^{4}} (I) \xrightarrow[123]{} II \xrightarrow[.145]{} (III) \xrightarrow[Fast]{} E_{OX}S + P + H_2O$ $(I) \xrightarrow[<5]{Fast} E_{OX}S + H_2O_2$

[a] Numbers in this column are second order rate constants ($M^{-1}s^{-1}$), and in other columns are first order constants (s^{-1}).

[b] Numbers in italics are constants previously obtained for native (FAD) enzyme.

[c] Parentheses indicate that an intermediate is kinetically invisible.

important spectral data is illustrated in the Figure, and appropriate rate constants in the reactions are compared to native enzyme under the same conditions in the Table.

By analogy with native enzyme, intermediate I (Figure and Table) is C(4a)-peroxyflavin and intermediate III is C(4a)-hydroxyflavin. The 6-hydroxy group has changed these spectra relative to native enzyme (4). The C(4a)-peroxyflavin decays partly to H_2O_2 (Table). A third transient species (II) was clearly observed between intermediates I and III with p-aminobenzoate as substrate. This species has not been detected yet in the hydroxylation of other substrates. On the basis of known spectral data, there does not appear to be any known flavin structure responsible for intermediate II. An inspiration is needed to establish the chemical change in C(4a)-peroxyflavin which results in the observed spectrum.

The interaction between 2,4-dihydroxybenzoate and 6-OH-FAD enzyme shows little similarity to the native enzyme. No transient intermediates were detected upon reaction with oxygen (Table). Our tentative explanation is that the Kd for reduced enzyme with substrate is very large ($\geq$ 0.1M), compared to 1.8×10^{-4}M for native enzyme (4). Results suggest that the 6-hydroxy group on the flavin interferes with the binding of this substrate, which places the substrate below and appressed to the flavin ring in the reduced conformation.

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