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Absorption Spectra of Radical Forms of 2,4-Dihydroxybenzoic Acid, a Substrate for *p*-Hydroxybenzoate Hydroxylase*

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Combined optical and conductimetric measurements in aqueous solution indicate that at high pH (≥10) $^{\bullet}OH$ radicals react with the phenoxide form of 2,4-dihydroxybenzoic acid to form transiently phenoxyl radicals and a small amount of hydroxycyclohexadienyl (HCHD) radicals by 150 ns. The respective yields of 88 and 12% of the total $^{\bullet}OH$ radical yield were deduced from conductance and optical changes as well as from studies using a low potential reductant. The HCHD radical possesses a pK_a of 8.0 ± 0.1 and the constructed spectrum of the deprotonated forms of HCHD has a λ_{max} at 420 nm with a minimum extinction coefficient of ~7250 M^{-1} cm^{-1}. The red shift in λ_{max} and increase in extinction coefficient compared to the revised spectral properties of the protonated form of the HCHD radical (λ_{max} at 390 nm with extinction coefficient of ~4500 M^{-1} cm^{-1}), together with the pK_a of the HCHD radical, provide an explanation for the pH-dependent spectral changes of the so-called highly absorbing intermediate II species, observed in the functioning of the enzyme *p*-hydroxybenzoate hydroxylase. These results add further to the evidence in support of the proposal that intermediate II is composed of species which absorb similarly to the flavin 4(a)-hydroxide and a form of the substrate/product such as the HCHD radical (Anderson, R. F., Patel, K. B., and Stratford, M. R. L. (1987) *J. Biol. Chem.* 262, 17475-17479).

The mechanism by which certain flavoprotein monooxygenases activate molecular oxygen to undergo an oxygen atom addition reaction to specific substrates has received considerable attention. Many proposed mechanisms involve complex structural alterations to the isoalloxazine ring of the FAD coenzyme to account for the distinctive highly absorbing species formed upon oxygen insertion into substrate (see Ref. 1 for a review), the so-called intermediate II. Much mechanistic detail has come from studies on *p*-hydroxybenzoate hydroxylase (1, 2) and phenol hydroxylase (3, 4) where the precursor to intermediate II has been identified as a flavin 4(a)-hydroperoxide (intermediate I). However, none of the proposed mechanisms based on structural changes to the flavin ring alone has been substantiated by comparison with any model systems (5, 6).

We have initiated studies to see if certain modified forms of the substrates (7) can account in part for the absorption properties of intermediate II. A close match is found between intermediate II and the combining of the spectra of the hydroxycyclohexadienyl (HCHD)[1] radicals of the substrates, for both *p*-hydroxybenzoate hydroxylase (8), and phenol hydroxylase (9), with a flavin 4(a)-substituted species (such as the flavin 4(a)-hydroxide, intermediate III). On the basis of these results a biradical mechanism has been proposed (7, 10) in which a protein-induced homolytic cleavage of the O-O flavin hydroperoxide bond occurs in the presence of the substrate to form simultaneously a hydroxycyclohexadienyl radical of the substrate and a flavin 4(a)-alkoxyl radical. Subsequent oxidation of the hydroxycyclohexadienyl radical by a radical form of the flavin leads to hydroxylated product formation.

In this study we extend our previous work on 2,4-dihydroxybenzoic acid (8) to the high pH region to see if the radical chemistry of this substrate can account for the distinctive spectral changes in its respective intermediate II species. A close match is found between the pH-dependent spectral changes of intermediate II and that for the HCHD radical. Extension of the previous work on 4-hydroxybenzoic acid (7) is also included for comparative purposes.

EXPERIMENTAL PROCEDURES

2,4-Dihydroxybenzoic acid (DHBA), 98%, and *N,N,N'N'*-tetramethyl-*p*-phenylenediamine dihydrochloride (TMPD) were obtained from BDH and 4-hydroxybenzoic acid (HBA) was obtained from Aldrich (gold label product). Other reagents were of the highest purity commercially available.

Pulse radiolysis experiments were performed with the Van de Graaff accelerator system of the Gray Laboratory and the methods of optical absorption detection, charge monitoring, and dosimetry employed have been described (7, 11). Absorption measurements are presented as the product of the radiation chemical yield G[2] (M Gy^{-1}) and the extinction coefficient ϵ (M^{-1} cm^{-1}). Transient conductance changes were measured simultaneously with optical changes using a balanced conductivity cell with platinum electrodes (cell constant k_c = 0.27 cm^{-1}) similar to that described previously (11, 12). The cell was symmetrically excited with two out-of-phase 62.5-Hz sine waves; polarizing voltages of 10-70 V peak-to-peak were used with the electron pulse timed to occur on one of the peaks of the sine wave. The large background conductance was eliminated by employing a similar but unirradiated cell with the signals from the two cells combined in a wideband multiport transformer. The risetime of the complete detection electronics was <10 ns and conductance charges of <0.1 μS could be resolved. The symmetry of the detection method ensures that electron pulse charge artefacts are largely eliminated; this rejection is further enhanced by using software to subtract pairs of traces, recorded on a Tektronix 7612D digitizer, taken at opposite polarizing voltage phases (polarities). Typically two to eight pairs were averaged in this way.

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[1] The abbreviations used are: HCHD, hydroxycyclohexadienyl; DHBA, 2,4-dihydroxybenzoic acid; HBA, 4-hydroxybenzoic acid; TMPD, *N,N,N,'N,'*-tetramethyl-*p*-phenylenediamine.

[2] A G value of 1 molecule (100 eV)$^{-1}$ corresponds to a radiation chemical yield, G = 0.1036 μmol J^{-1}.

RESULTS

Reactions initiated by the ·OH radical are conveniently studied in irradiated aqueous solution saturated with N_2O gas to convert (e^-_{aq}) into ·OH radicals as follows.

$$H_2O \rightarrow {}^{\cdot}OH, e^-_{aq}\ H^{\cdot}, H_2O_2, H_3O^+, OH^-$$

$$N_2O + e^-_{aq} \rightarrow {}^{\cdot}OH + OH^- + N_2$$

yielding [·OH] = 0.62 μM Gy^{-1} and [H·] = 0.06 μM Gy^{-1} (13). (The spectrum formed upon H· addition to DHBA has a weak absorption near 380 nm. Since [H·] accounts for <9% of the total radical yield; however, this absorption can be ignored in the present study).

The transient spectra arising from the reaction of ·OH radicals with DHBA (5 mM) following pulse radiolysis (4 Gy (J kg^{-1}) in 30 ns) of a N_2O-saturated solution at pH 11.0 (2 mM sodium orthophosphate adjusted with NaOH) are presented in Fig. 1, together with a typical transient measured at 420 nm, Fig. 1, *insert B*. Three distinct kinetic phases, consistent with Scheme I presented under "Discussion," are detected in that the spectrum measured 150 ns after the pulse (λ_{max} = 430 nm) shifts to longer wavelengths by 2 μs (λ_{max} = 450 nm) followed by an increase in absorbance at all wavelengths to give a third spectrum by 15 μs (λ_{max} = 460 nm). Similar spectral results were also obtained at pH 10.3. Clearly, the spectrum measured at 2 μs is not composed of a single entity but must consist of both a proportion of the spectrum measured at 15 μs and an unresolved spectrum which is less absorbing. Experiments were also performed in the presence of sodium azide to try to identify some of the observed transient species. Azide radicals $N_3^{\cdot}$, formed upon scavenging of ·OH radicals by azide ions, are known to rapidly oxidize hydroxylated aromatic compounds to form phenoxyl radicals (14). The transient spectra observed following pulse radiolysis (4 Gy in 30 ns) of N_2O-saturated solutions containing DHBA (2 mM) and sodium azide (0.1 M) at both pH 6 and 11 are also presented in Fig. 1. It is seen that the phenoxyl radical spectrum measured at pH 11, designated spectrum C, closely matches the previously measured spectrum at 15 μs. The phenoxyl radical spectrum measured at pH 6, designated spectrum B, absorbs less strongly at all wavelengths than the previously measured spectrum at 2 μs. It is tempting to suggest that the spectrum measured at 2 μs contains in part a contribution due to this phenoxyl radical, spectrum B. The initial spectrum measured at 150 ns at pH 11.0 is distinctly different from the initial spectrum measured at pH 6 (λ_{max} = 390 nm (8), *dotted spectrum* in Fig. 1). The pK_a of the species giving rise to the initial spectrum was determined to be 8.0 ± 0.1 from $G\epsilon$ measurements made at 390 nm. Also, the pK_a of the phenoxyl radical (produced using the azide radical) was determined to be 8.1 ± 0.1 from measurements made at 460 nm, Fig. 1, *insert A*.

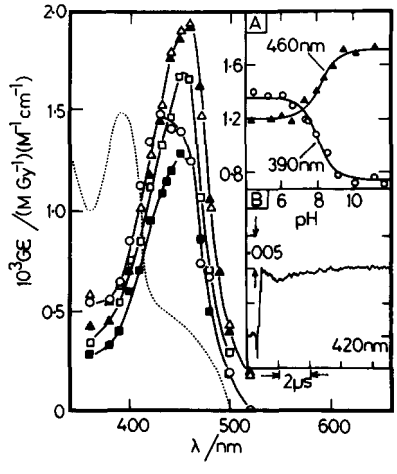


FIG. 1. **Spectral changes observed following the pulse radiolysis (4 Gy in 30 ns) of a N_2O-saturated solution of DHBA (5 mM) at pH 11.0 (NaOH, orthophosphate (2 mM)).** Spectra measured: ○, 150 ns; □, 2 μs; △, 15 μs after the pulse. Spectral changes observed upon the addition of sodium azide (0.1 M) to the above solution at pH 6 (■) and pH 11 (▲) measured 2 μs after the pulse. The immediate radical spectrum (*dotted line*) observed at pH 6 (from Ref. 8) is drawn for comparison. *Insert A*, the dependence of absorption (*ordinate*) on pH (*abscissa*) measured at 150 ns for 390 nm (○) and 15 μs for 460 nm (▲). *Solid lines* represent the best fits using least squares analysis which yield pK_a values of 8.0 ± 0.1 and 8.1 ± 0.1, respectively. *Insert B*, oscilloscope trace at 420 nm of the change in optical density (*ordinate*) against time (*abscissa*) following the pulse in pH 11 solution showing three kinetic phases.

Kinetic transients observed at 380 and 440 nm are similar, and the data obtained at both wavelengths as a function of pH are presented in Fig. 2. A maximum rate constant for the formation of spectrum C of 1.6 × 10^5 s^{-1} from precursor(s) is observed at pH ≥ 9. The lowest rate constants for the formation of spectrum C, measured in the pH region 6 to 7, probably consist of small contributions from both acid and base catalysis rather than catalysis by the phosphate buffer which proceeds at ~2 × 10^6 M^{-1} s^{-1} (data not shown). An enlarged set of data over that for the previous study for HBA (7) is also presented in Fig. 2 for comparison purposes (see "Discussion"). It is seen that the rate constant for conversion of the initially formed hydroxycyclohexadienyl radical of HBA to a second species (the phenoxyl radical) tends towards a maximum of 2 × 10^6 s^{-1} at high pH.

Experiments were also performed at pH 10.3 with DHBA and with added TMPD which is known to undergo a fast electron transfer reaction with phenoxyl radicals to form the $TMPD^{+\cdot}$ radical cation (15), Fig. 3. Kinetic analysis of the observed biphasic transient reveals that 88% of the maximum possible yield of $TMPD^{+\cdot}$ radical cation at this pH is built up with a rate constant of 1.4 × 10^6 s^{-1} and the secondary build-up, accounting for the remaining 12%, occurs at 1.5 × 10^5 s^{-1}. Only the initial rate is dependent on the concentration of TMPD giving a second order rate constant of ~3.5 × 10^9 M^{-1} s^{-1} in agreement with the published rate constant for the oxidation of TMPD by the phenoxyl radical (15). The second rate constant corresponds to the maximum rate for the formation of spectrum C from precursor(s). These results indicate that a large fraction of the ·OH radicals react with DHBA at high pH to form the phenoxyl radical directly and

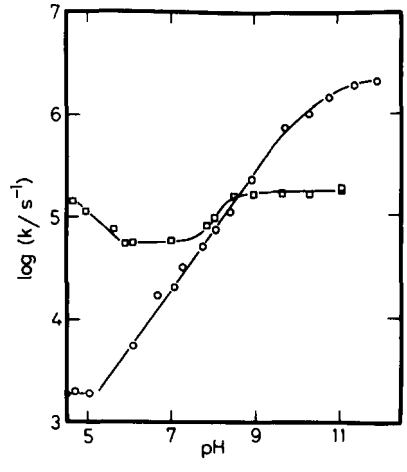


FIG. 2. **The pH dependence of the rate constants for the decay of the initial species formed upon ·OH radical addition to HBA (○, 360 nm) and DHBA (□, 380 and 440 nm).**

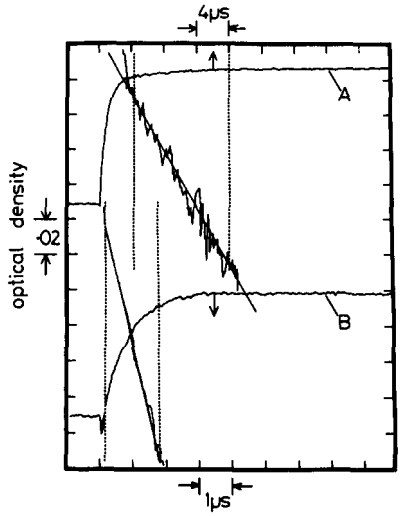


FIG. 3. *A*, oscilloscope trace at 565 nm of the change in optical density (*ordinate*) against time (*abscissa*) following the pulse radiolysis (6 Gy in 30 ns) of a N_2O-saturated solution at pH 10.3 containing DHBA (5 mM) and TMPD (0.4 mM). Also displayed is a plot of ln (A_∞–A) *vs.* time, where *A* is absorbance, of the slow phase of the transient (one half-life equals two divisions), *vertical broken lines* indicate the time frame of analysis from which the rate constant of 1.5×10^5 s^{-1} is derived. *B*, trace constructed from *A* by subtracting out the slow phase. The displayed kinetic analysis on the fast phase yields a rate constant of 1.4×10^6 s^{-1} which accounts for 88% of the absorption of *A*.

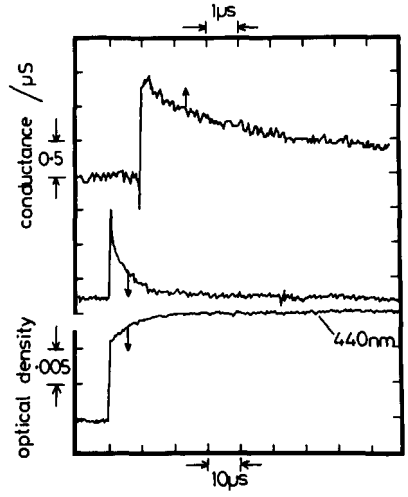


FIG. 4. **Simultaneous conductance and absorption changes following the pulse radiolysis (5.6 Gy in 30 ns) of a N_2O-saturated solution adjusted to pH 10 (NaOH) containing DHBA (2 mM).** *Upper traces* are μS (*ordinate*) against time (*abscissa*), and the *lower trace* is optical density at 440 nm (*ordinate*) against time.

the small secondary formation occurs on a longer timescale from a precursor intermediate(s).

The formation and decay of charged species can also be followed using conductimetric detection to help clarify reaction mechanisms. The optical and conductivity transients observed simultaneously following the pulse radiolysis of a solution at room temperature (23 °C) containing DHBA (2 mM), adjusted to pH 10.0 with NaOH, is displayed in Fig. 4. The rate of the build-up in absorbance at 440 nm ($k = 1.7 \times 10^6$ s^{-1}) parallels the decay of the initial increase in conductance (0.26 μS Gy^{-1}) following the pulse.

DISCUSSION

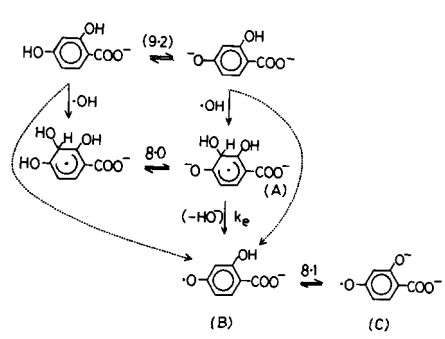
The present results are interpreted in terms of Scheme 1. In high pH solution (pH ≫ pK_a 9.2 of the ground state of DHBA, based on that for resorcinol (14)) the ·OH radical reacts with the phenoxide anion to give both the rapid formation of a phenoxyl radical, species B, and ring addition to form a hydroxycyclohexadienyl (HCHD) radical, species A. The proportion of the ·OH radicals forming phenoxyl radicals by 150 ns (the time by which neutralization of the protons produced by the pulse is completed at pH 10) can be found by comparing the measured increase in conductance $G_c(t)$ with the calculated yield for the maximum yield of ·OH radicals in N_2O-saturated solution (0.62 μM Gy^{-1} (13)) using the expression as follows.

$$G_c(t) \cdot k_c = 10^{-3} \Sigma\, c_i\, |z_i|\, \Lambda_i$$

where the conductance multiplied by the cell constant (k_c) is equal to the summation of the changes in the concentrations (c_i) of all species present multiplied by the modulus of their charges $|Z_i|$ and specific conductances, Λ_i (estimated from similar sized and charged molecules in the literature (16)). Loss of a doubly charged phenoxide ion ($|z|\Lambda = 2 \times 45$ S cm^2 M^{-1}) forms an OH^- ion ($|z|\Lambda = 1 \times 190$ S cm^2 M^{-1}) and a phenoxyl radical ($|z|\Lambda = 1 \times 30$ S cm^2 M^{-1}) leading to a calculated maximum change in conductance of 0.30 μS Gy^{-1}. The measured change in conductance at 150 ns of 0.26 μS Gy^{-1}, Fig. 4, corresponds to 87% of this calculated maximum. Subsequent deprotonation of the phenoxyl radical, species B, is evident from the parallel decrease in conductance and increase in absorbance due to species C (Fig. 4).

Results with added TMPD indicate that by 2 μs 88% of the maximum yield of ·OH radicals have formed phenoxyl radicals and undergone electron transfer to form the $TMPD^{+\cdot}$ radicals. It cannot be excluded however that this yield of phenoxyl radicals is the same as that at 150 ns since a finite time (≤2 μs) is required for the electron transfer to take place under the experimental conditions employed. In fact the conductivity results are consistent with the rapid formation of the phenoxyl radicals and the same concentration being formed by 150 ns. An increase in the percentage of phenoxyl radical formation through direct attack of the ·OH radical on the phenoxide form of the substrate (88%) over the hydroxide form (15%, through *ipso* attack (8)) is expected due to the increased electronegativity of the phenoxide group compared to the hydroxyl group.

We associate the pK_a of 8.0 ± 0.1 of the initial spectrum measured at 150 ns with the deprotonation of the HCHD radical, most probably of the 4-hydroxy substituent. On comparing the results obtained using azide radicals with the spectra obtained following ·OH radical attack, we conclude



SCHEME 1. **Radical species formed upon ·OH radical attack on DHBA.** *Numbers* represent pK_a values. (Conjugate protons omitted for clarity.) The *dotted lines* represent the direct formation of the phenoxyl radical rather than from the HCHD radical.

that species C is the deprotonated phenoxyl radical of DHBA with a pK_a of 8.1 ± 0.1. This pK_a could not be determined accurately from data using the ·OH radical as the HCHD radical decays by other pathways at low pH in competition to the relatively slow formation of the phenoxyl radical.

The conversion of the HCHD radical to the phenoxyl radical by base catalysis has been previously reported for HBA (7) to follow the kinetic description (17) as follows.

$$k_{obs} = k_0 + k_e K[OH^-](K_w + K[OH^-])^{-1}$$

where K is the equilibrium constant for the deprotonation of the HCHD radical, k_e the rate constant for the conversion of the deprotonated HCHD radical to the phenoxyl radical, and k_0 is the uncatalyzed rate constant. Performing regression analysis using the above equation on the extended data for HBA presented in Fig. 2 with $k_0 = 3 \times 10^3$ s^{-1} (7) gives $k_e = 2.0 \pm 0.1 \times 10^6$ s^{-1} and $K = 7.4 \pm 1.3 \times 10^{-11}$. The p$K_a$ value of 10.1 ± 0.1 is in fairly good agreement with that found from optical measurements at 420 nm of 9.8 ± 0.2 (data not shown) but is different from 8.4 ± 0.2 found from preliminary data over a smaller pH range at 360 nm (7). Performing the same analysis on the data for DHBA with $k_0 = 5.6 \times 10^4$ s^{-1} (plateau in rate constants from Fig. 2) gives $k_e = 1.1 \pm 0.1 \times 10^5$ s^{-1} and $K = 7.4 \pm 2.3 \times 10^{-9}$. The p$K_a$ value of 8.1 ± 0.2 is in agreement with that found from optical measurements of 8.0 ± 0.1 (Fig. 1). The rate of OH^- elimination from the deprotonated HCHD radical of the monohydroxylated substrate (HBA) is considerably faster than in the case of the dihydroxylated substrate (DHBA). It must be stressed that the formation of the HCHD radical of both HBA and DHBA at high pH is a minor pathway compared to the rapid formation of the phenoxyl radical from the phenoxide ion.

The spectrum of the deprotonated forms of the HCHD radical of DHBA can be calculated on the basis that the initial radical spectra measured 150 ns after the pulse consists of 88% of the protonated phenoxyl radical (*i.e.* the spectrum measured at pH 6, using sodium azide, Fig. 1, species B) and 12% HCHD radical (species A) relative to the initial yield of ·OH radicals, Fig. 5. It is seen that the spectrum of the deprotonated HCHD radical has a λ_{max} = 420 nm with an extinction coefficient of ~7250 M^{-1} cm^{-1}. The spectrum of the protonated HCHD radical (8), was recalculated using the accurately determined protonated phenoxyl radical spectrum, has a λ_{max} = 390 nm with an extinction coefficient of ~4500 M^{-1}cm^{-1}, and is also displayed in Fig. 5. The magnitude of the extinction coefficients must be considered as approximate given the fact that they are markedly affected by small errors in measurement. The protonated and deprotonated forms of the phenoxyl radical possess the same λ_{max} (near 450 nm) and the smaller extinction coefficients of 2100 M^{-1} cm^{-1} and 3200 M^{-1} cm^{-1}, similar to that reported for the phenoxyl radical of resorcinol (18). The HCHD radical of HBA has also been shown to absorb more strongly than its phenoxyl radical (10).

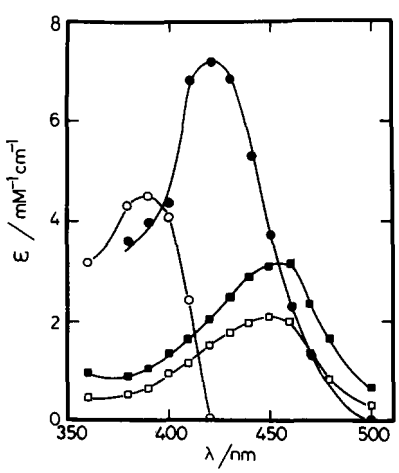
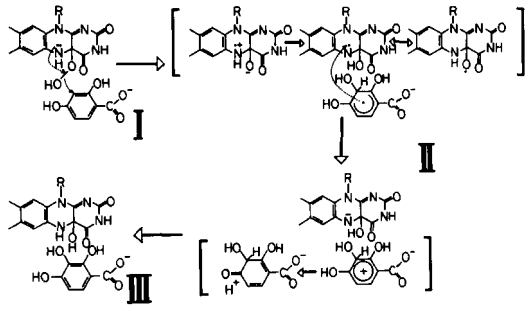


FIG. 5. **Constructed radical spectra for DHBA (see text).** Spectra of the protonated HCHD radical (○), the deprotonated HCHD radical (●), the protonated phenoxyl radical (□), and the deprotonated phenoxyl radical (■).

A comparison between the absorbance of intermediate II and a composite consisting of the HCHD radical and a flavin species, that is assumed to have a similar absorption spectrum to intermediate III, can be made. Combining the extinction coefficients of the protonated and deprotonated HCHD radicals at their wavelengths of maximum absorption (390 and 420 nm) with those of intermediate III at the same wavelengths (9500 and 7250 M^{-1} cm^{-1} (1)) yields 14000 and 14500 M^{-1} cm^{-1}, respectively. While the composite extinction coefficient for the protonated HCHD radical matches within 10% that reported for intermediate II (15500 M^{-1} cm^{-1} (1)), a poorer fit to the observed extinction coefficient of intermediate II at high pH (18500 M^{-1}cm^{-1} (1)) is found. If the postulated flavin species is indeed the flavin 4(a)-alkoxyl radical, then part of the discrepancy between the composite spectrum and that observed for intermediate II may well be due to a radical absorption band of the alkoxyl radical. While use of the spectrum of intermediate III at low pH to form a composite spectrum may be a valid approximation to the absorption spectrum of the flavin 4(a)-alkoxyl radical (*i.e.* both species have an oxygen atom substituted at the 4(a) position and the same flavin ring chromophores intact), this may not be the case at high pH. Deprotonation of the flavin ring of such a species at high pH could well result in an increase in its absorbance.

A large source of error in the calculated spectrum of the deprotonated HCHD radical could arise from the assumption that all of the ·OH radicals which do not react with DHBA to directly form the phenoxyl radical add to the 3 position of the benzene ring. This is unlikely based on previous results obtained at low pH where at least two fast kinetic phases contribute to the formation of the protonated phenoxyl radical by ~2 μs after the pulse (8). These phases most probably originate from ·OH radical addition *ipso* to ring substituents followed by fast dehydration to form the phenoxyl radical. As it has been deduced previously that intermediates formed upon *ipso* addition of the ·OH radical do not absorb significantly ≥ 320 nm (7), any decrease in the fraction of ·OH radical yield adding to the 3 position of DHBA (12%) will give rise to a considerable underestimation of the calculated extinction coefficient of the deprotonated HCHD radical. For example if, as in the case of low pH, approximately half of the ·OH radical yield which add to the benzene ring do so *ipso* to the 1-carboxy or the 2-hydroxy groups (8), then the calculated extinction coefficient of the deprotonated HCHD radical is doubled and its composite spectrum would fully account for the spectrum of intermediate II at high pH. Unfortunately, due to the fast rates of dehydration, it is not possible, at high pH, to use oxidants to trap the various isomeric intermediates formed upon ·OH radical addition to determine the exact distribution through product analysis.

The pK_a of the HCHD of 8.0 ± 0.1 is in good agreement with that reported for the spectral changes of intermediate II of 7.8 (1). A small shift in the pK_a (and spectrum) of the HCHD radical might well occur in the relatively hydrophobic active site of the enzyme compared to that in aqueous solution. Importantly, the pK_a of the HCHD radical is over one pH unit removed from that of DHBA. This means that the radical



SCHEME 2. **Proposed conversion of intermediate I to intermediate II via the formation of a biradical pair.** The absorption spectrum of intermediate II may be represented by the summation of the absorption spectra of the transient flavin and substrate/product species.

pK_a is unrelated to changes in the reaction specificity of the ·OH radical which changes with the protonated and deprotonated forms of DHBA. Although the phenoxyl radical of DHBA possesses a similar pK_a of 8.1 ± 0.1, it can be disregarded as a candidate to account partially for intermediate II because of its small extinction coefficient and the fact that its λ_{max} at 450 nm is unchanged upon deprotonation.

The summation in absorptions of the HCHD radical and the flavin 4(a)-hydroxide species accounts well for the absorption of intermediate II. The proposed reaction scheme for the formation of intermediate II from intermediate I is presented in Scheme 2. Homolysis of the O-O bond in the enzyme results in the initial formation of the HCHD radical and the flavinium radical cation. This form of the flavin pseudo base could rearrange to the neutral α-hydroxyaminyl radical with the radical center partially spread to form the flavin 4(a)-alkoxyl radical. The flavinium radical cation species exhibits an absorption band in the visible region of the spectrum[3] and hence must be disregarded for forming a composite spectrum. It can be argued that both the neutral α-hydroxyaminyl radical and the flavin 4(a)-alkoxyl radical absorb similarly to the flavin 4(a)-hydroxide since the reduced flavin chromophores are unaltered.

However, no electron spin resonance evidence at 6 °C or at 24 K has been found to support the existence of a radical pair.[4] This might be due to a number of reasons; (i) spin-spin coupling problems between the two free electrons, (ii) the radicals coalesce at low temperatures to form a covalent bond, or (iii) rapid-freezing stops second-order reactions which involve the diffusion together of the reactive species, but might not prevent the reaction of closely bound species taking place (19). Rapid oxidation of the HCHD radical or the phenoxyl radical form of the substrate by the flavin radical, could give rise to the keto form of the product or a phenoxonium ion (Scheme 2, Ref. 9). The unequivocal spectra and the lifetimes of these possible intermediates have yet to be determined, but their spectra would have to be remarkably similar to that of the HCHD radical. It is interesting that oxidation of the HCHD by oxygen did not yield the final product directly but gave rise to a species absorbing in the same region of the spectrum as the HCHD radical (8). This species, in contrast to the HCHD radical, decayed on the millisecond timescale.

The proposed biradical mechanism has been instrumental in reassessing the possible absorbing forms of the substrate/product to help account for the absorption properties of intermediate II. Further evidence that intermediate II is composed of the flavin 4(a)-hydroxide (or a similar absorbing species) and some form of the substrate/product is presented in the accompanying paper of Schopfer *et al.* (20). Our present results offer the first satisfactory explanation for the pH-dependent spectral shifts of intermediate II in terms of the deprotonation of the HCHD radical. The results, when taken together with the body of evidence to account for the absorption spectra of intermediate II for two enzymes and eight substrates (8, 9), give further support to the operation of a biradical mechanism (8, 10) in flavoprotein hydroxylases.

Acknowledgments—We wish to thank Professor Vincent Massey and his group at Michigan University for continual helpful discussions on this topic. We also thank Brian Hall and colleagues for operating the Van de Graaff accelerator and Rosalind Locke for development of the computer software.

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