

Reaction of Reduced Flavins and Flavoproteins with Diphenyliodonium Chloride*

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The reaction of diphenyliodonium chloride with free reduced flavins has been studied by stopped flow spectrophotometry under anaerobic conditions, and second order rate constants were determined as a function of pH. The reactive flavin species was identified as the reduced anion, based on an observed reaction pK of 6.7. The product mixture was independent of the initial concentration of reactant and contained ~20% oxidized flavin. The results can be modeled quantitatively on a modification of the mechanism proposed by Tew (Tew, D. G. (1993) *Biochemistry* 32, 10209–10215). The composition of the complex reaction mixture has been analyzed, and four flavin-phenyl adducts with distinctive absorbance and fluorescence characteristics have been identified, involving substitution at the flavin C4a, N5, and C8 positions. Inactivation of flavoprotein enzymes by diphenyliodonium has also been studied, and several examples were found where inactivation occurs readily, despite noninvolvement of radical intermediates in their reaction mechanisms. It can be concluded that inactivation by phenyliodonium species is not a valid indicator of catalytic mechanism involving radical intermediates. One of the several factors determining inactivation is maintenance of the enzyme flavin in the reduced form in the steady state of catalysis, the other factors being redox potential and accessibility of the inhibitor to the flavin active site.

Recent studies have provided many examples of inhibition of flavoprotein enzymes by diphenyliodonium salts and similar iodonium compounds. The known capability of iodonium compounds to undergo radical reaction (1–4), the fact that most of the known enzymes to be severely inhibited, such as neutrophil NADH oxidase (5, 6), cytochrome P-450 reductase (7), xanthine oxidase (8), nitric-oxide synthase (9), and sulfite reductase (10), involve radical chemistry in their mechanisms has led to the concept that inhibition by iodonium compounds is a marker of flavoprotein enzymes functioning by radical mechanisms, particularly because other flavoproteins, such as D- and L-amino acid oxidases, glucose oxidase, and glutathione reductase, which do not involve radicals in their reaction, are not inhibited (8). Previous work has demonstrated that the enzyme flavin needs to be reduced for inhibition to occur, and that the reaction of the reduced flavin with iodonium compounds results in

phenyl adducts of the flavin (8, 10). Tew has proposed the mechanism shown in Scheme 1 for the formation of the flavin N5-phenyl adduct, involving the one electron oxidation of the reduced flavin to form the semiquinone, at the expense of reduction of diphenyliodonium to the corresponding diphenyliodo radical. The latter is known to fragment rapidly to give iodobenzene and a phenyl radical, and the flavin semiquinone and phenyl radical are proposed to undergo a radical recombination to yield a flavin-phenyl adduct (7).



SCHEME 1

We have reinvestigated the reaction of a number of flavoproteins with Ph_2I^+ , as well as the reaction of reduced FAD and FMN with Ph_2I^+ . Ph_2I^+ has been demonstrated to inhibit catalysis of several enzymes that do not operate by radical mechanisms. The main criteria for inhibition appear to be maintenance of the enzyme flavin in the reduced form during the steady state of catalysis and a system redox potential sufficiently negative to act as a reductant for Ph_2I^+ . Most flavoproteins studied formed phenyl adducts when they were reduced prior to reaction with Ph_2I^+ under anaerobic conditions. Our results with reaction of free reduced flavin with Ph_2I^+ indicate that, in addition to the simple radical pathway of Tew (7), a substantial portion of flavin adducts formation proceeds either by direct attack of Ph_2I^+ on the reduced flavin or via radical recombination from a primary caged radical pair. A series of flavin-phenyl adducts have been isolated and characterized, involving the flavin C4a, N5, and C8 positions.

MATERIALS AND METHODS

Diphenyliodonium chloride and FAD were obtained from Sigma. The enzymes used were prepared and assayed as described in the literature: Old Yellow Enzyme (OYE; Ref. 11), glucose oxidase (12), pig kidney D-amino acid oxidase (13), bovine milk xanthine oxidase (14), pig heart lipoamide dehydrogenase (15), melilotate hydroxylase (16), anthranilate hydroxylase (17), MHPC oxygenase (18), phenol hydroxylase (19), and *p*-hydroxybenzoate hydroxylase (20). Human red blood cell glutathione reductase was a gift from Dr. Georg Schulz (University of Freiburg, Freiburg, Germany) and *E. coli* thioredoxin reductase and thioredoxin reductase were gifts from Dr. Charles Williams (University of Michigan, Ann Arbor, MI). Flavodoxin from *Clostridium beijerinckii* was a gift from Dr. Richard Swenson (Ohio State University, Columbus, OH) and protocatechuate dioxygenase was a gift from Dr. David P. Ballou (University of Michigan, Ann Arbor, MI).

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¹ The abbreviations used are: Ph_2I^+ , diphenyliodonium chloride; $\text{Ph}_2\text{I}^\bullet$, diphenyliodo radical; $\text{Ph}^\bullet$, phenyl radical; PhI , iodobenzene; Fl_{red} , reduced flavin; Fl_{ox} , oxidized flavin; Fl_{sq} , semiquinone flavin; $\text{FlH}^\bullet$, neutral flavin radical; MHPC, 2-methyl-3-hydroxypyridine-5-carboxylate; OYE, Old Yellow Enzyme; HPLC, high performance liquid chromatography; Fld_{ox} , Fld_{sq} , and Fld_{red} , the oxidized, semiquinone, and reduced forms of flavodoxin.

For the isolation of reaction products, for practical reasons we chose to use FMN for reaction of reduced flavin with Ph_2I^+ , because it is much more soluble than riboflavin and simpler to deal with in subsequent analytical procedures than FAD. Commercial FMN is only 85% pure, whereas commercial FAD is >95% pure, with the impurity being authentic FMN. Hence we used FAD as starting material and converted it to FMN using the phosphodiesterase of snake venom. In a typical experiment, ~500 mg of FAD was dissolved in ~50–60 ml of 0.01 M potassium P_i , pH 7.0, and converted to FMN by incubating overnight at 25 °C with ~50 mg of *Naja naja* venom, a potent source of phosphodiesterase. The course of the conversion was monitored by the spectral change associated with the conversion ($\epsilon_{450} \text{ FAD} = 11,300 \text{ M}^{-1} \text{ cm}^{-1}$, $\epsilon_{445} \text{ FMN} = 12,500 \text{ M}^{-1} \text{ cm}^{-1}$), using suitably diluted aliquots for the spectral measurements. On complete conversion, the reaction mixture was boiled for 1–2 min to precipitate the venom and the solution was centrifuged to get rid of the precipitate. A sample was tested by HPLC to make sure that no unconverted FAD remained. The FMN solution was further purified by Sep-Pak C-18 reversed phase cartridges, eluting with 10% CH_3CN in water, and concentrated to ~250 ml using a rotary evaporator at 38–40 °C.

The pure FMN solution thus obtained was brought up to 0.1 M potassium P_i , pH 7.0; placed in a 500-ml three-necked round-bottomed flask; 20 ml of 0.3 M EDTA was added in the dark and made anaerobic by bubbling argon, at 0–5 °C for 45 min. The argon-equilibrated solution was then irradiated with a sun-gun for 10 min until all of the green fluorescence had disappeared, indicating full reduction. Diphenyliodonium chloride, 50 ml of 0.025 M, previously made anaerobic by bubbling with argon for 30 min was added to the reduced FMN solution with argon gas still flowing. The solution immediately turned orange with some precipitate, which, however, disappeared on incubating at 25 °C for ~1 h. The reaction mixture was opened to air and left overnight on ice.

The solution was then loaded on four 35-ml Sep-Pak prepacked columns, prewashed with methanol and equilibrated with water. After washing with water to remove salts, the flavin reaction products were eluted with acetonitrile-water mixtures. The columns were protected from light throughout. The solutions were concentrated as quickly as possible using a rotary evaporator.

Spectroscopic Studies—UV-visible spectra were recorded with a Hewlett-Packard diode array spectrophotometer, or a Cary (Varian) model 219 double-beam spectrophotometer. NMR spectra were recorded in D_2O with a Bruker 500-MHz instrument, and chemical shift values are reported in ppm relative to tetramethylsilane.

HPLC Analysis—Reversed phase HPLC analyses were carried out with a Beckman System Gold instrument (model 126), using a UV detector and a C-18 250 × 10-mm column. The column was equilibrated and washed with 20% acetonitrile in 20 mM ammonium acetate, pH 5.7, for 5 min, and then the compounds were eluted by applying a gradient to 50% acetonitrile in 20 mM ammonium acetate over a period of 30 min. The eluate was monitored at 350 nm.

Stopped Flow Kinetics—Stopped flow experiments were carried out using a Hi-Tech SF-61 instrument in absorbance mode. For anaerobic reactions, the system was flushed and incubated overnight with a solution containing 3,4-dihydroxybenzoate (protocatechuic acid) and protocatechuate 3,4-dioxygenase to scavenge any oxygen (21). All buffers and Ph_2I^+ solutions were made anaerobic by bubbling with highly pure argon for 15 min in a syringe before use. Experiments were done at 25 °C. Analyses were conducted by fitting data to exponential equations using the Marquardt algorithm (22) incorporated into Program A and developed by C. J. Chiu, R. Chang, J. Diverno, and D. P. Ballou at the University of Michigan (Ann Arbor, MI). Simulations were carried out by using numerical integrations of the differential equations for the reaction sequences using a fourth order Runge-Kutta algorithm (22), also in Program A.

For studies with free flavin, usually FAD was used. Typically, an ~50 μM solution was placed in a glass tonometer in the presence of 0.05 M potassium P_i , pH 7.0, 15 mM EDTA, and 500 μM protocatechuic acid. A catalytic amount of protocatechuate dioxygenase was placed in the side arm of the tonometer. The contents of the tonometer were made anaerobic by repeated cycles of evacuation and subsequent equilibration with high quality argon, and finally left under positive pressure of argon for transfer to the stopped flow apparatus. The flavin was reduced by shining visible light from a commercial sun-gun, mixed with the protocatechuate dioxygenase, and allowed to equilibrate for 30 min (to remove any residual oxygen) before loading into the stopped flow spectrophotometer at 25 °C and mixed with various concentrations of Ph_2I^+ or other solutions, as described in the text.

For experiments with flavoproteins, the same protocol was followed,

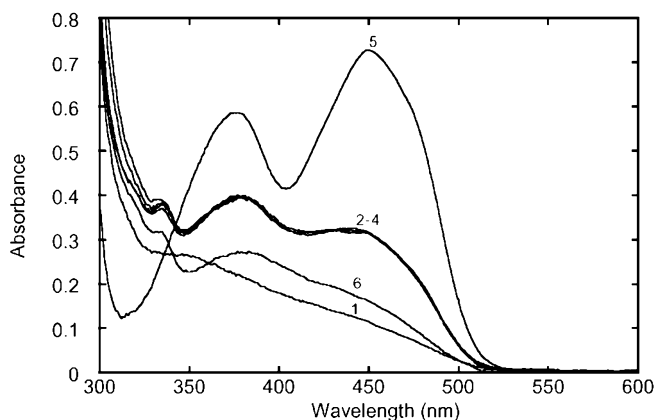


FIG. 1. Spectra obtained on reaction of diphenyliodonium chloride with reduced FAD. The experiments were done with a stopped flow spectrophotometer at 25 °C in 0.05 M potassium P_i , pH 7.0, as described under "Materials and Methods." Spectrum 1 was that obtained on mixing the photoreduced flavin with anaerobic buffer; spectra 2–4 were obtained at the end of the reactions with 0.5, 1.0 and 2.0 mM Ph_2I^+ . Spectrum 5 was obtained by reaction of the reduced flavin with air-equilibrated buffer in absence of Ph_2I^+ ; spectrum 6 shows the corrected spectrum for the flavin-phenyl adduct mixture, obtained by subtracting 20% of spectrum 5 from the average of spectra 2–4.

except that reduction of the enzyme was accomplished in the tonometer by introducing the reductant from a side arm.

RESULTS AND DISCUSSION

Stopped Flow Studies

The reaction of Ph_2I^+ with reduced FAD at pH 7.0 results in a relatively rapid spectral change, as shown in Fig. 1. The extent of spectral change was independent of the concentration of Ph_2I^+ , a finding of considerable mechanistic importance, as will be developed later. The progress curves at all concentrations of Ph_2I^+ and at all wavelengths from 320 to 500 nm were fit satisfactorily to a single exponential, yielding k_{obs} values that were linearly dependent on Ph_2I^+ concentration, and that in each case did not show any systematic variation with the wavelength of observation. Such experiments were repeated over the pH range of 6.0–8.5, with similar results in terms of the extent of spectral changes involved and the linear dependence of reaction rate constant on Ph_2I^+ concentration. The observed second order rate constants were, however, dependent on pH (Fig. 2), indicating a pK_a for the reaction of 6.7. This pH dependence strongly suggests that the primary reaction of Ph_2I^+ is with the reduced flavin anion, because reduced flavins are known to have an almost identical pK_a value (23).

In separate experiments carried out under identical conditions, but in anaerobic cuvettes, similar spectral changes to those shown in Fig. 1 were observed. In those experiments it could be further shown that on exposure to air there was no further spectral change, indicating that there was no remaining reduced flavin, and that most of the reduced flavin had been consumed in formation of adducts. Some, however, had been converted to oxidized flavin in the anaerobic reaction. As will be described below, this was estimated to be ~20% of the total flavin. Fig. 1 shows the calculated spectrum of the reaction mixture with this percentage of oxidized flavin subtracted from the observed final spectrum. The spectrum of the adduct mixture indicates species with remaining absorbance in the 400–500-nm region, as well as absorbance maxima at 370 and 330 nm. The nature of this mixture will be considered below.

The results described are not fully consistent with the model of Tew and require a substantial portion of the reaction to occur through what appears to be direct reaction of reduced flavin

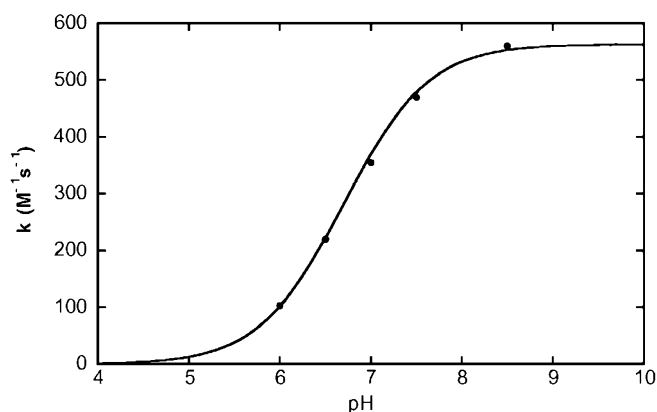
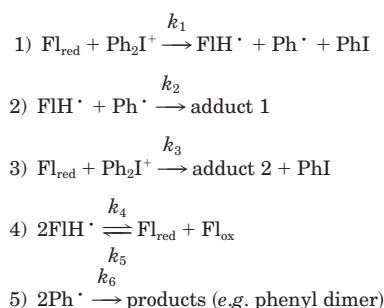


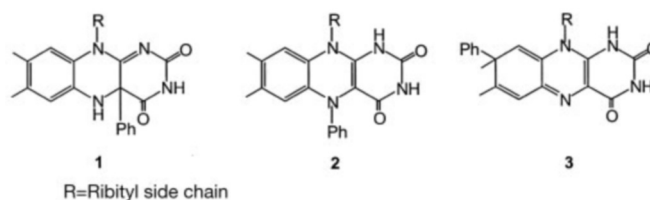
FIG. 2. The pH dependence of the rate constant for reaction of reduced FAD with diphenyliodonium chloride. The rate constants were obtained from stopped flow experiments at 25 °C, as described under "Materials and Methods."

with Ph_2I^+ , without any radical intermediates. This conclusion is based on comparison with simulations to Scheme 2.



SCHEME 2

In this scheme, reaction steps 1 and 2 represent the mechanism proposed by Tew (7), simplified to take into account that the primary iodonidiphenyl radical fragments on a picosecond time scale to the more stable phenyl radical and iodobenzene (24). However, this mechanism must be expanded to include reaction 4, the well known rapid dismutation of flavin radical to a mixture of oxidized and reduced flavin (25). The rate constants k_4 and k_5 are known, at pH 7.0, being 6×10^8 and $2 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$, respectively (25, 26). Thus, for an appreciable yield of adducts and finite formation of oxidized flavin, k_2 in the above scheme has to be very large, $\sim 10^7 \text{ M}^{-1} \text{ s}^{-1}$ or greater. However, in the absence of direct reaction between reduced flavin and Ph_2I^+ (reaction 3 in Scheme 2, with rate constant k_3), simulations show that the percentage of oxidized flavin increases markedly with the concentration of Ph_2I^+ , the reaction time profile is frequently not fit with a single exponential, and the observed rate constants are not linear with Ph_2I^+ concentration, appearing to reach a saturation value at high concentrations. It is also necessary to include a step involving the non-productive removal of phenyl radicals (exemplified by reaction 5 and rate constant k_6 in Scheme 2). In the absence of such a step, simulations show frequently the transitory accumulation of oxidized flavin in excess of the final, a situation that we have never encountered experimentally. The only situation that approximates to the observed results is inclusion of k_3 that can compete effectively with the radical formation step, k_1 . The best fits to the pH 7.0 data are in fact determined with a ratio of $k_1/k_3 \sim 0.5$, with numerical values of ~ 250 and $\sim 500 \text{ M}^{-1} \text{ s}^{-1}$, respectively, and with negligible contribution from the radical recombination step k_2 . The extent of total reaction proceeding through step 2 is of course quite dependent on the initial



STRUCTURES 1–3

concentration of reduced flavin. Within experimental error we have not observed dependence of the composition of the final products on the initial reduced flavin concentration, provided that the latter is no greater than one-tenth of the lowest Ph_2I^+ concentration used, *i.e.* that the observed reaction is pseudo first order.

Adduct Characterization

Addition of a phenyl residue to the reduced flavin ring could give rise to different products, with the phenyl substitution at the C4a, N5, or C8a positions, as shown in Structures 1–3. Reaction of reduced FMN with Ph_2I^+ , described under "Materials and Methods," resulted in a mixture of products, as shown by HPLC (Fig. 3). A similar result was reported by Covès *et al.* (10) for reaction of reduced FAD with Ph_2I^+ .

Unfortunately, some of the products were not very stable, making identification difficult. To obtain enough material for characterization, the bulk reaction mixture obtained from reaction of reduced FMN and Ph_2I^+ was chromatographed on C-18-based Sep-Pak columns. After loading on the column, buffer salts and excess Ph_2I^+ were removed by thorough washing with water before fractionation with acetonitrile-water mixtures. The first compound, which was eluted with water and 1% CH_3CN , was underivatized FMN ($\sim 20\%$ of the starting material).

The next compound isolated from the reaction mixture was eluted with 2% CH_3CN . Evaporation of the solvent yielded a compound with a blue fluorescence, characterized by an excitation maximum at 360 nm and an emission maximum at 430 nm. This compound was found to be unstable; hence, we could not obtain good NMR and mass spectral data. However, its visible spectrum showed an absorption band at 364 nm with tailing into the 400 nm region, and the absorption maximum was shifted to 342 nm in 6 N HCl. This hypsochromic shift in acid is reported to be a general characteristic of flavin N5 adducts (27). Based on this property, it is concluded that this adduct probably has the structure shown as Structure 2. This adduct is presumably responsible for the peak at 18 min in the HPLC separation (Fig. 3). The spectroscopic properties of this and the other isolated adducts are summarized in Table I.

Elution of the Sep-Pak column with 2–2.5% CH_3CN gave a fraction whose UV-visible spectrum changed slowly over the course of days, corresponding to the peak at 15 min in the HPLC profile of Fig. 3. Immediate evaporation of the solvent by Speed-Vac resulted in a yellow solid with absorption maxima at 320, 332 (sh), and a broad band centered around 400 nm. The fluorescence spectrum showed excitation peaks at 320 and 460 nm with emission maximal at 530 nm. The nonagreement of the absorbance and fluorescence excitation spectra indicated that this fraction was a mixture of at least two compounds. An apoflavodoxin column (28) was employed for further purification. Adducts were bound to the column as a pink band. After washing with 10 mM potassium P_i , 0.3 mM EDTA, pH 7.0, what proved to be still a mixture of adducts was eluted with 5% TCA in 0.3 mM EDTA. After removal of the TCA by extraction with ether, the solution was rechromatographed on a small Sep-Pak column with $\text{H}_2\text{O}:\text{CH}_3\text{CN}$ mixtures. Several minor fractions

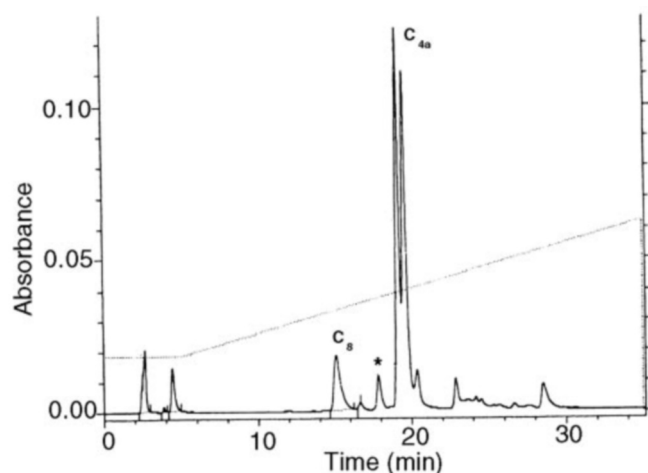


FIG. 3. HPLC separation of the reaction mixture from reduced FMN and diphenyliodonium chloride. The gradient used was 20% CH₃CN in 20 mM ammonium acetate, pH 5.7 (5 min), followed by 20–50% CH₃CN in 20 mM ammonium acetate, pH 5.7 (30 min), and products were monitored at 350 nm. The peak (marked with an asterisk) eluting at 18 min was unstable and disappeared within 1 h. It probably represents the N5-phenyl adduct.

TABLE I
Spectroscopic properties of isolated adducts
ND, not determined.

Compound	UV-Vis	NMR δ (ppm)	Mass Spec (m/z for M ⁺ -1)	Fluorescence
1 	λ_{max} =364 nm and shifts to 388 nm in 6N HCl	CH ₃ protons at 2.14 and 2.17, aromatic protons at 7.0–7.8.	533	Non fluorescent
2 	λ_{max} =364 nm with tailing into 400 nm region. Shifts to 342 nm in 6N HCl	ND	ND	λ_{ex} =360nm, λ_{em} =430 nm
3 	ND	CH ₃ protons at 1.57, 1.63; aromatic protons at 7.2–7.4	533	ND
4 	λ_{max} =320, 332 (sh), broad band at 400 nm	CH ₃ protons at 1.61; CH ₂ protons at 4.37; aromatic protons at 7.2–7.4	549	λ_{ex} = 320, 460nm, λ_{em} = 530 nm

were eluted with water that had 320- and 332-nm maxima, but with variable secondary bands between 360 and 500 nm. The main fraction, eluted with 5% CH₃CN, and had absorbance maxima at 320, 332, and 432 nm (Fig. 4). This fraction was evaporated to dryness at room temperature to prevent any further decomposition and dissolved only when ready to use.

Fig. 4 shows the titration of this adduct with apoflavodoxin in 0.05 M potassium P₁, pH 7.0, resulting in a dramatic shift in the absorption spectrum. From this titration, it can be estimated that the K_d for binding to apoflavodoxin is <1 μM , and the extinction coefficients of the free flavin can be calculated to

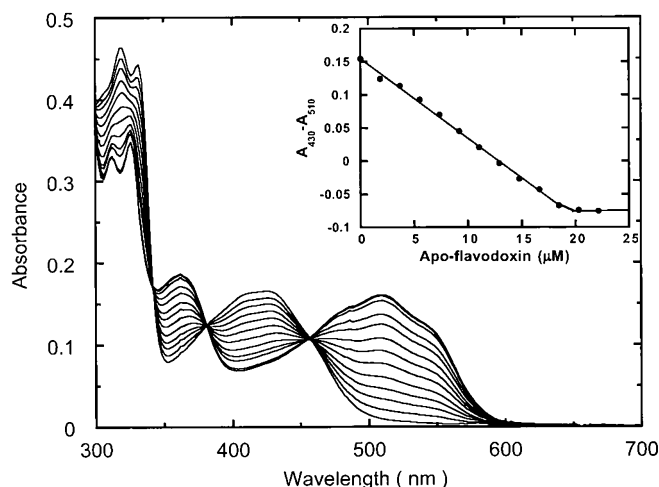


FIG. 4. Titration of C8-phenyl FMN (see text for details) with *C. beijerinckii* apoflavodoxin. The starting spectrum before addition of apoprotein had an absorbance maximum at 430 nm; successive spectra, all corrected for dilution, were obtained on addition of apoflavodoxin at the concentrations shown in the inset. Inset, plot of the absorbance difference ($A_{430} - A_{510}$) as a function of apoflavodoxin concentration. Titrations were carried out in 0.05 M potassium P₁, pH 7.0, 25 °C.

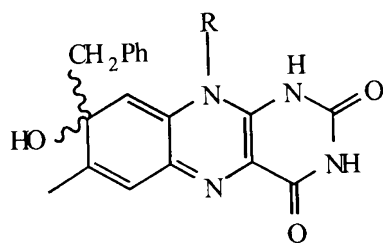
be $\epsilon_{318} = 24,000 \text{ M}^{-1} \text{ cm}^{-1}$, $\epsilon_{332} = 23,000 \text{ M}^{-1} \text{ cm}^{-1}$, and $\epsilon_{430} = 8,500 \text{ M}^{-1} \text{ cm}^{-1}$.

Despite the impressive isosbestic points of the spectra of Fig. 4, the ¹H NMR spectrum of the new flavin was quite complex and appears to be the result of a mixture of two compounds. The addition of a phenyl ring to the flavin was evidenced by the appearance of new complex signals in the aromatic region between 7.2 and 7.4 ppm, and there were three methyl signals that were shifted upfield compared with normal FMN (observed at δ 1.57, 1.61, and 1.63 ppm). An additional two-proton signal was observed at δ 4.37 ppm (Table I). From the UV-visible spectra, it was predicted that C8 of flavin is involved in the formation of this adduct and detailed analysis of the ¹H NMR spectrum is consistent with its being a mixture of two C8-phenyl adducts having Structures 3 and 4 in the ratio of ~65:35, Structure 3 comprising CH₃ signals at δ 1.57 and 1.63 ppm, and the signal at δ 1.61 ppm resulting from Structure 4. Further support for Structure 4 comes from the mass spectral data, which showed a molecular ion peak at 549 mass units; the observed increase of 94 units suggests the addition of a phenyl and a hydroxyl group to the FMN (Structure 4). The addition of one phenyl group to FMN accounts for an additional 77 mass units, giving rise to the observed peak at m/e 533 (Structure 3).

It is known that flavodoxin forms a red protein-bound chromophore on photoirradiation in presence of certain alcohols or acids (29). A 1,8-dihydroflavin structure with a substituent at the C8 carbon was assigned to this chromophore on the basis of detailed NMR studies (30). A flavin benzoquinoid structure with similar spectral properties was also assigned to the primary photoproduct of flavins and phenylacetate (31).

Structures 3 and 4 are also consistent with spectral changes associated with a pK_a of 6.5 (λ_{max} of the protonated species 320, 470 nm), probably because of ionization of the N1 position (data not shown). Structures 3 and 4, with extended conjugation through the benzoquinoid structure of the flavin, would also be consistent with the unusual spectral properties of the components of this fraction. An adduct with a similar spectrum has been reported by Covès *et al.* (10) from reaction of Ph₂I⁺ with FAD.

Further elution of the Sep-Pak cartridges with 2.5–3% CH₃CN afforded a pale yellow nonfluorescent solution with absorption maximum at 364 nm. This fraction corresponds to



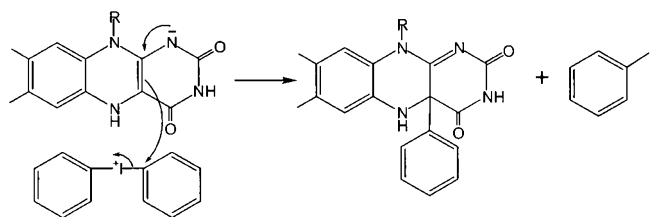
STRUCTURE 4

the double peak around 19 min in the HPLC of Fig. 3. The negative ion FAB Mass spectroscopic data showed an $M^+ - 1$ at 533 mass units, consistent with the addition of a single phenyl group to FMN. The ^1H NMR data showed two clear singlets for the two aromatic methyls and the complex multiplets observed in the aromatic region account for the extra phenyl protons. The possible sites for the adduct formation are either N5 or C4a. Distinction between these two structures was made by making use of ^{13}C NMR, as well as absorption spectral changes in 6 N HCl. An N5 adduct corresponds structurally to reduced flavin, and the ^{13}C signal for the C4a carbon in an N5 adduct is expected to be around the same region as that of the reduced flavin, which is 103 ppm (32). The ^{13}C NMR spectrum of the adduct has no signal around the region ~ 103 ppm, consistent with the adduct involving the C4a position. It is reported that flavin-C4a adducts show a bathochromic shift in 6 N HCl, which is observed as a general characteristic property irrespective of the substituent (27). The adduct obtained in the present reaction showed a bathochromic shift of 22 nm in 6 N HCl from 364 to 388 nm, further supporting Structure 1.

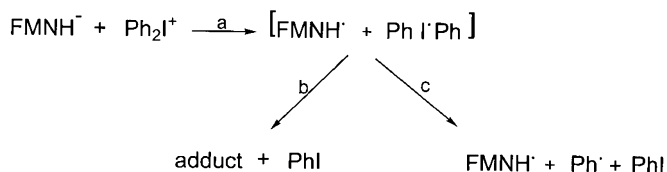
Addition of a phenyl group to the C4a could occur from either side of the tetrahedral carbon to give rise to two diastereomers, evidenced by the double peak at ~ 19 min in Fig. 3. These two isomers could be resolved by HPLC using an isocratic run with 20% CH_3CN in 20 mM NH_4OAc , pH 5.7, over a period of 20 min. The two isolated peaks were identical in their UV-visible and mass spectra and showed the same bathochromic shift in 6 N HCl, confirming them to be isomeric compounds. The NMR spectra were almost identical with the exception of one aromatic proton. In one isomer the peak was at 7.37 ppm, and in the other one it was at 7.32 ppm. The diastereomeric nature of the adducts was also confirmed by their specific binding to different apoproteins. For example, the first eluting isomer binds tightly to apoflavodoxin and apoOYE, but there is no binding of these apoproteins with the second isomer. These results and those with other proteins will be published separately.

Accurate quantification of the yields of the various adducts was difficult, owing to the instability of C8 and N5 adducts, and because of incomplete recovery of UV-visible absorbing material from HPLC and preparative Sep-Pak columns. Oxidized FMN was easy to quantify, because it is stable and elutes first. With Ph_2I^+ in excess, the yield of FMN was routinely found to be $\sim 20\%$ of the initial flavin taken. Spectral deconvolution of the visible spectrum of the remaining reaction products (*cf.* Fig. 1) indicate $\geq 50\%$ formation of the C4a adduct, 10–20% C8 adducts, and $< 10\%$ N5 adduct.

As detailed in a previous section, simulations require that approximately two-thirds of the total reduced flavin react directly with Ph_2I^+ in a fashion not involving detectable radical intermediates, and that this is the major route of flavin adduct formation. Radical reactions do indeed occur, as detected previously by EPR (8), and are required in our own experiments to account for the formation of oxidized flavin (reaction 4 in Scheme 2). However, adduct formation from free flavin radicals does not appear to be a viable pathway. Although direct nu-



SCHEME 3



SCHEME 4

cleophilic attack of the reduced flavin anion on Ph_2I^+ is feasible for formation of C4a-phenyl flavin (see Scheme 3), it is not feasible for formation of N5 and C8 adducts.

In these cases radical reaction pathways seem mandatory. This seeming dilemma may be explained if the apparent direct reaction involves a caged radical pair of neutral flavin semiquinone and diphenyliodoxy radical (or its breakdown product, phenyl radical). In that case, the first three steps of Scheme 2 may be replaced by steps *a–c* of Scheme 4.

The 2:1 ratio of the rate constants for steps *b* and *c*, derived from simulations, was confirmed by experiments in which reduced flavin was reacted anaerobically with an equimolar concentration of Ph_2I^+ . Under these conditions the final reaction mixture contained 16% unreacted reduced flavin, easily quantified by the spectral changes on admission of air (results not shown). This result is consistent with 32% of the total reduced flavin being converted to the free radical species (step *c* of Scheme 4), which, instead of reaction to form adducts, rapidly disproportionates to an equimolar mixture of oxidized and reduced flavin.

Studies with Enzymes

Kinetics of Inactivation under Steady State Turnover Conditions—With most enzymes studied, where inactivation by Ph_2I^+ was observed under steady state conditions, the apparent first order rate constant for inactivation, $k_{\text{inact}}^{\text{app}}$, was obtained by analysis of the time course of decrease in catalytic velocity. For example, in assays involving the oxidation of NADH or NADPH, a continuous trace of the absorbance at 340 nm was recorded until complete inactivation was obtained, evidenced by the trace having approximately the same slope as that of a control reaction without enzyme. Semi-log plots *versus* time of the observed absorbance minus that obtained from the extrapolated slope of the inhibited reaction were linear for at least 90% of the total change, yielding a value of $k_{\text{inact}}^{\text{app}}$. With all of the enzymes studied where inactivation occurred, within the error of measurement, the $k_{\text{inact}}^{\text{app}}$ values were directly proportional to the Ph_2I^+ concentration, consistent with lack of specific binding of Ph_2I^+ , and yielding a second order rate constant for the inactivation. These results differ from those reported previously with xanthine oxidase and other enzymes, where apparent specific binding of the inhibitor was deduced from inhibition progress curves (6, 7, 8, 10). Table II summarizes the data obtained with the flavoprotein enzymes studied.

TABLE II
Steady state inactivation of selected flavoprotein enzymes

Enzymes	Substrates	Conditions	k_{inact}
Old Yellow Enzyme	NADPH, O ₂	pH 7.0, 25 °C	None
	NADPH, cyclohexenone	pH 7.0, 25 °C	None
Glucose oxidase	Glucose, O ₂	pH 5.6, 25 °C	None
D-Amino acid oxidase	D-Proline, O ₂	pH 8.5, 25 °C	None
Xanthine oxidase	Xanthine, O ₂	pH 8.5, 25 °C	$\sim 800 \text{ M}^{-1} \text{ s}^{-1}$
Lipoamide dehydrogenase	NADH, lipoate	pH 6.5, 25 °C	$\sim 17 \text{ M}^{-1} \text{ s}^{-1}$
	NADH, lipoate (NAD ⁺) ^a	pH 6.5, 25 °C	None
Glutathione reductase	NADPH, GSSG	pH 7.6, 25 °C	None
Thioredoxin reductase (<i>Escherichia coli</i>)	NADPH, DTNB, ^b thioredoxin	pH 7.6, 25 °C	$\sim 250 \text{ M}^{-1} \text{ s}^{-1}$
Melilotate hydroxylase	NADH, O ₂ , 2-hydroxyphenylpropionate	pH 7.6, 25 °C	None
Anthranilate hydroxylase	NADPH, O ₂ , anthranilate	pH 7.6, 25 °C	None
	NADPH, O ₂ , (salicylate)	pH 7.6, 25 °C	None
MHPC oxygenase	NADH, O ₂ , 2-methyl-3-hydroxypyridine-5-carboxylate	pH 7.6, 25 °C	None
	NADH, O ₂ , 5-hydroxynicotinate	pH 7.6, 25 °C	None
Phenol hydroxylase	NADPH, O ₂ , phenol	pH 7.6, 25 °C	$\sim 15 \text{ M}^{-1} \text{ s}^{-1}$
	NADPH, O ₂ , resorcinol	pH 7.6, 25 °C	$\sim 23 \text{ M}^{-1} \text{ s}^{-1}$
<i>p</i> -Hydroxybenzoate hydroxylase	NADPH, O ₂ , 4-hydroxybenzoate	pH 7.6, 25 °C	$30 \text{ M}^{-1} \text{ s}^{-1}$
	NADPH, O ₂ , 2,4-dihydroxybenzoate	pH 7.6, 25 °C	None
	NADPH, O ₂ , tetrafluoro-4-hydroxybenzoate	pH 7.6, 25 °C	$16 \text{ M}^{-1} \text{ s}^{-1}$
	NADPH, O ₂ , 2-fluoro-4-hydroxybenzoate	pH 7.6, 25 °C	$6 \text{ M}^{-1} \text{ s}^{-1}$
	NADPH, O ₂ , 3-fluoro-4-hydroxybenzoate	pH 7.6, 25 °C	$60 \text{ M}^{-1} \text{ s}^{-1}$
	NADPH, O ₂ , 4-aminobenzoate	pH 7.6, 25 °C	None
	NADPH, O ₂ , (6-hydroxynicotinate)	pH 7.6, 25 °C	None

^a Parentheses indicate a non-substrate effector.

^b DTNB, 5,5'-dithiobis(nitrobenzoic acid).

Reaction of Reduced Flavoproteins with Diphenyliodonium Chloride

Old Yellow Enzyme—Although OYE did not show any inhibition by Ph₂I⁺ in the standard enzymatic assays with oxygen or cyclohexenone as electron acceptors (Table II), the reduced enzyme did react very slowly with Ph₂I⁺, as shown in Fig. 5. In this experiment the enzyme was maintained in the reduced state under anaerobic conditions by a catalytic amount of NADP and an NADPH-regenerating system. On tipping Ph₂I⁺ from the side arm of the anaerobic cuvette, very slow spectral changes were observed, with a $t_{1/2}$ of 77 min⁻¹ (Fig. 5, inset), corresponding to a second order rate constant of $0.6 \text{ M}^{-1} \text{ s}^{-1}$. Flavin adduct formation was complete, because on exposure to air there was no sign of return of the spectral characteristics of oxidized enzyme. The modified enzyme was completely inactive in the standard catalytic assays. It was strongly fluorescent, with an emission maximum at 530 nm and excitation spectrum similar to that of the absorption spectrum, with maxima at 320 and 440 nm, characteristics of a flavin-C8-phenyl adduct. On denaturation of the protein with 50% ethanol or acetone, the fluorescence of the liberated flavin increased ~ 5 -fold, with slight shifts of the excitation maxima to 325 and 445 nm. HPLC analysis of the liberated product also revealed the presence of a small amount of flavin-C4a adduct, as evidenced by its retention time and by the characteristic shift in absorbance maximum in strong acid. Inactivation of the enzyme is clearly the result of modification of the flavin, because the modified flavin could be removed by dialysis versus 2 M KBr as with native enzyme (48) and the resulting apoprotein could be quantitatively reconstituted with FMN with full return of catalytic activity (results not shown). Although this point has not been checked with the other enzymes studied, it is reasonable to assume that it may be a general phenomenon, i.e. the inactivation of flavoproteins by Ph₂I⁺ is caused by the chemical modification of the flavin prosthetic group.

***p*-Hydroxybenzoate Hydroxylase**—In catalytic assays, the inactivation of *p*-hydroxybenzoate hydroxylase by Ph₂I⁺ was very dependent on the substrate (hydroxylation reaction) or effector (stimulation of oxidase reaction) that was employed (Table II). Striking differences are apparent between *p*-hy-

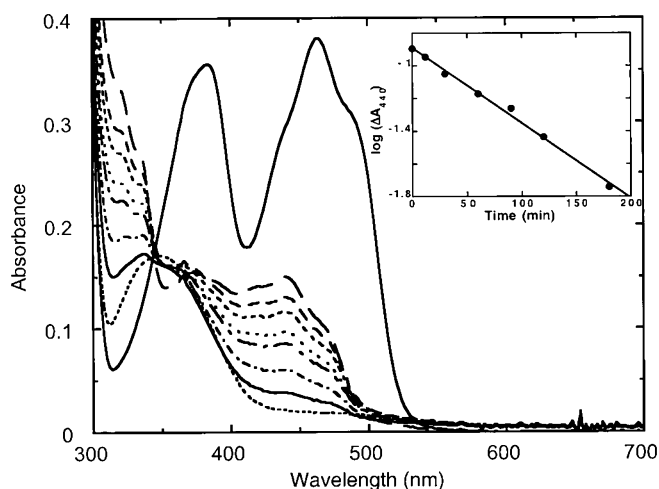


FIG. 5. Reaction of reduced Old Yellow Enzyme from brewers' bottom yeast with diphenyliodonium chloride. 36 μM enzyme was reacted with Ph₂I⁺ in 0.1 M potassium P_i, pH 7.0, 25 °C. Solid top line, enzyme under anaerobic conditions, before reduction. Bottom dashed line, after reduction with an NADPH-regenerating system comprising 2 μM NADPH, 2 mM glucose 6-phosphate, and 2 units of *Leuconostoc mesenteroides* glucose-6-phosphate dehydrogenase. Spectra with increasing absorbance at 440 nm were recorded at 12, 30, 60, 90, 120, 180 min and 18 h after addition of 250 μM Ph₂I⁺. Inset, $\log A_{440}(t_{\infty} - t)$ as a function of reaction time.

droxybenzoate as substrate, where rapid inactivation was found, and 2,4-dihydroxybenzoate, where no inactivation was observed. Nevertheless, when reduced enzyme was incubated with Ph₂I⁺, with either substrate, phenyl adduct formation was found, at approximately the same rate, with essentially complete inactivation of the enzyme when subsequently assayed with either substrate. Results of a typical experiment, in the presence of *p*-hydroxybenzoate, are shown in Fig. 6. At the concentration of Ph₂I⁺ used (500 μM), the spectral changes shown are complete within 15 min, with a half time of $\sim 70 \text{ s}$, corresponding to a second order rate constant of $\sim 20 \text{ M}^{-1} \text{ s}^{-1}$. A similar experiment, carried out in the presence of 2,4-dihydroxybenzoate, gave the same spectral changes, with a second

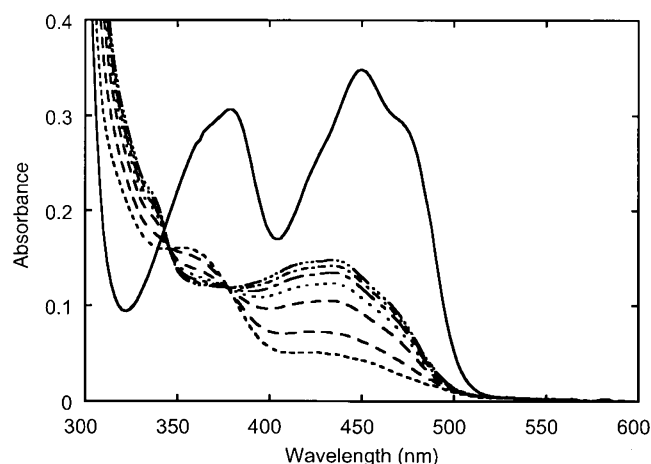


FIG. 6. Reaction of *p*-hydroxybenzoate hydroxylase with diphenyliodonium chloride. Conditions were identical to those of Fig. 5, except for pH 7.6. Solid line, spectrum of oxidized enzyme ($\sim 35 \mu\text{M}$), in the presence of $230 \mu\text{M}$ *p*-hydroxybenzoate. Bottom dashed line, enzyme after reduction with NADPH-regenerating system. Subsequent curves with increasing absorbance at 440 nm were obtained at 20, 90, 150, 210, 300, and 480 s after addition of $500 \mu\text{M}$ Ph_2I^+ .

order rate constant of $23 \text{ M}^{-1} \text{ s}^{-1}$. In both the cases, the product had an intense green fluorescence, emission maximum at 530 nm and with excitation maxima at 330 nm and 420 nm, characteristics similar to those assigned to a flavin-C8-phenyl adduct. The reaction was also followed in more detail by stopped flow techniques. Enzyme, reduced with a catalytic concentration of NADPH maintained by glucose 6-phosphate and glucose-6-phosphate dehydrogenase in the presence of *p*-hydroxybenzoate, was reacted with concentrations of Ph_2I^+ of 0.125, 0.25, 0.5, and 1.0 mM. The reaction curves at all wavelengths studied were fit by single exponential functions, with observed pseudo first order rate constants directly dependent on the Ph_2I^+ concentration, yielding a second order rate constant for adduct formation of $26 \text{ M}^{-1} \text{ s}^{-1}$ (results not shown).

Glucose Oxidase—Diphenyliodonium chloride was without effect on the catalytic activity of glucose oxidase, assayed at its pH optimum of 5.6, and employing the coupled assay for H_2O_2 with horseradish peroxidase and *o*-dianisidine (33). Not only was it without effect in steady state assays, but it was also without effect on incubation of the glucose-reduced enzyme for prolonged periods (24 h) with 1 mM Ph_2I^+ , at pH 5.6, 7.0, or 8.5. In these experiments, on opening to air and allowing turnover of the enzyme, complete return to the starting spectrum of oxidized enzyme was obtained. This enzyme appears, therefore, to be remarkably resistant to reaction with Ph_2I^+ .

Xanthine Oxidase—Reaction of dithionite-reduced enzyme with Ph_2I^+ appears only to involve the FAD center of the enzyme. Evidence for this is illustrated in Fig. 7. On mixing Ph_2I^+ from the side arm of the anaerobic cuvette containing reduced enzyme, there was an immediate increase in absorbance mainly in the wavelength region below 500 nm. The difference spectrum associated with this change is shown in Fig. 7 (inset). The small increase in the long wavelength region is likely caused by some reoxidation of the reduced iron-sulfur centers by Ph_2I^+ , or associated radical species. On opening to air, there is a slow return of absorbance over the whole spectral region, notably complete return to the original in the long wavelength region, but with missing absorbance at wavelengths below 500 nm. The difference spectrum between the original oxidized enzyme and the reoxidized Ph_2I^+ -treated enzyme is shown in Fig. 7 (inset), is clearly flavin-like in character, and indicates complete retention of the spectrum of the

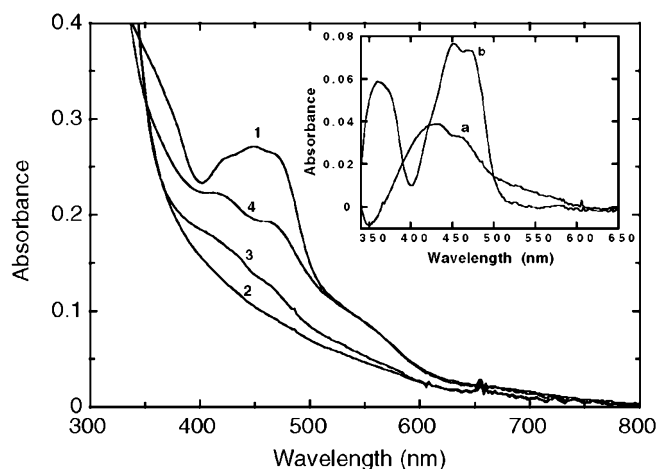


FIG. 7. Reaction of milk xanthine oxidase with diphenyliodonium chloride. The enzyme concentration was $\sim 7.6 \mu\text{M}$ in 0.02 M sodium pyrophosphate, pH 8.5, 25°C . Curve 1, oxidized enzyme; curve 2, after addition of an ~ 2 -fold excess of sodium dithionite; curve 3, immediately after addition of $250 \mu\text{M}$ Ph_2I^+ . This spectrum remained unchanged under anaerobic conditions over a period of 2 h. Curve 4, final stable spectrum 18 h after air reoxidation. Inset, difference spectra; a, curve 3 - curve 2; b, curve 1 - curve 4.

iron-sulfur chromophores in the inactivated enzyme. The Ph_2I^+ -treated enzyme had less than 0.2% the activity of the starting enzyme in conventional xanthine-oxygen reductase assays (34).

Confirmation that it is the flavin site in xanthine oxidase that reacts with Ph_2I^+ comes from a study with the deflavo enzyme. This form, which retains the two iron sulfur centers and the molybdopterine center, and is readily reduced by xanthine (34), was reduced with dithionite under anaerobic conditions and then mixed with 0.5 mM Ph_2I^+ . No spectral changes occurred over a period of 2 h, and on admitting air the spectrum of the original deflavo enzyme was slowly regained. Subsequent addition of xanthine resulted in rapid reduction of the deflavo enzyme, as with the untreated enzyme (results not shown).

Flavodoxin—Flavodoxins are low molecular weight, FMN-containing flavoproteins of plant and microbial origin that are involved in numerous single electron transfer reactions (see Ref. 35 for a review). They are characterized by the thermodynamic stability of the flavin semiquinone oxidation state, and hence provide an ideal opportunity to test for the involvement of radical intermediates in the inactivation of flavoproteins by Ph_2I^+ . In the following experiments we have used the flavodoxin of *C. beijerinckii*, which is known to yield almost quantitative stabilization of the blue neutral semiquinone on photoirradiation in the presence of EDTA (35), and which can also be converted to the fully reduced state photochemically with 5-deaza flavin as photocatalyst. Fig. 8 shows the results of an experiment in which the protein was reduced to the semiquinone level under anaerobic conditions and then reacted with Ph_2I^+ . The reaction was extremely slow, extending over a period of 4 days before completion. The final spectrum clearly showed the absence of semiquinone, with its characteristic absorbance between 600 and 700 nm, and was dominated by a major return of the absorbance of the oxidized protein. There was evidence of some modified flavin, from the absorbance in the 500–600 nm region and in the 300–400 nm region.

By contrast, when fully reduced flavodoxin was mixed with Ph_2I^+ , there was a very rapid reaction, resulting in an initial spectrum with wavelength maxima at 510, 352, and 324 nm (Fig. 9). This reaction is very rapid, requiring stopped flow

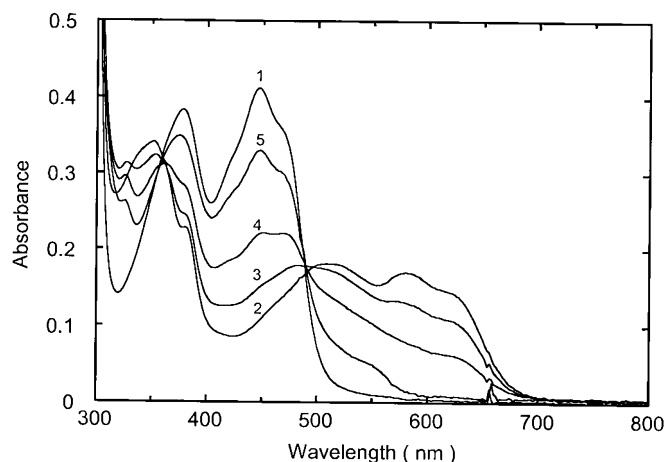


FIG. 8. **Reaction of flavodoxin semiquinone with diphenyliodonium chloride.** 40 μM enzyme in 0.05 M potassium P_i , pH 7.0, 25 $^\circ\text{C}$ was used. Curve 1, system under anaerobiosis before reduction. Curve 2, after careful photoreduction to the maximum semiquinone level in presence of 15 mM EDTA and 1 μM 5-deazaflavin. Curves 3–5, spectra 2.5, 25, and 91 h after addition of 500 μM Ph_2I^+ . All spectra have been corrected for dilution.

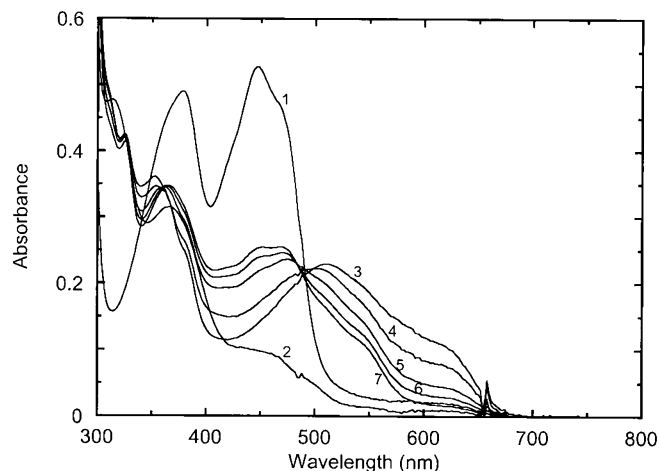


FIG. 9. **Reaction of fully reduced flavodoxin with diphenyliodonium chloride.** Conditions are the same as those detailed in Fig. 8. Curve 1, anaerobic oxidized protein ($\sim 50 \mu\text{M}$). Curve 2, after full photochemical reduction. Curves 3–7, 3 min, 134 min, 18 h, 48 h, and 96 h after addition of 500 μM Ph_2I^+ .

measurement. The observed rate was directly proportional to Ph_2I^+ concentration, yielding a second order rate constant at pH 7.0, 25 $^\circ\text{C}$ of $2.2 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$, independent of wavelength (results not shown). The spectrum of the rapidly formed species appears to be the composite of an approximately equal mixture of semiquinone and adduct, with semiquinone again disappearing over a period of 4 days (loss of absorbance in the 600–700 nm region), and clearly with much less return of the absorbance of oxidized flavoprotein. That the initial absorbance in the 600–700 nm region was indeed caused by flavin semiquinone was shown by anaerobic addition of $\text{K}_3\text{Fe}(\text{CN})_6$, which is known to oxidize very rapidly the semiquinone form (we measured this independently to have a rate constant of $1.8 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ at pH 8.5, 25 $^\circ\text{C}$ (results not shown)). Assuming that the flavin-phenyl adduct formed has no absorbance in the 600–700 nm region, it can be calculated that 48–50% of the initial reaction results in formation of the semiquinone. These results suggest that the reaction of reduced flavodoxin with Ph_2I^+ proceeds by two parallel pathways of equal rate, one yielding directly the

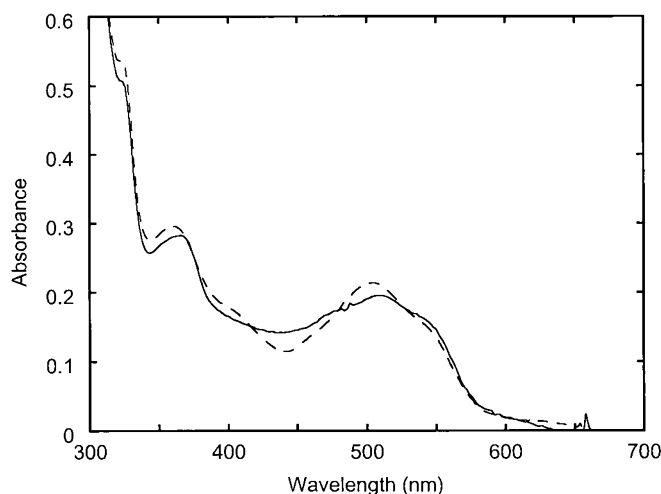
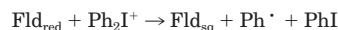
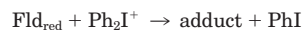


FIG. 10. **Calculated spectra of the phenyl adduct(s) obtained on reaction of reduced flavodoxin with diphenyliodonium chloride (spectra numbers are from experiment shown in Fig. 9).** Solid line, difference spectrum obtained by subtracting 33% of spectrum 1 from that of spectrum 7, and multiplying by 100/67 to estimate the spectrum if 100% adduct had been formed. Dashed line, difference spectrum obtained by subtracting 50% of the spectrum of full semiquinone (data obtained during the photoreduction, but not shown) from that of spectrum 3, and multiplying by 2, to account for the presumed equimolar mixture of semiquinone and adduct formed immediately on reaction of Ph_2I^+ with fully reduced flavodoxin.

adduct, the other flavin semiquinone, in the same way as concluded with free flavins (see Schemes 2–4).



REACTIONS 1 AND 2

In the case of flavodoxin, the semiquinone appears to be very unreactive, so that the fate of the unstable phenyl radical product is probably dimerization rather than adduct formation. The slow formation of phenyl adduct from the semiquinone is probably the result of a slow dismutation of the semiquinone to an equimolar mixture of oxidized and reduced flavodoxin, with the reduced flavin form reacting with Ph_2I^+ as it is formed.



REACTION 3

Thus, several cycles of reaction of Fld_{red} with Ph_2I^+ , dismutation, and reaction of the re-formed Fld_{red} would result in a final distribution of 67% adduct and 33% Fld_{ox} . By contrast, when the reaction is started with Fld_{sq} and Ph_2I^+ , the final distribution should be 33% adduct and 67% Fld_{ox} , close to that actually observed (cf. Fig. 8).

With the above assumption, the spectrum of the adduct was calculated in two ways from the data of Fig. 9. First, from the final spectrum after 4 days of reaction, 33% of the initial oxidized spectrum was subtracted and then multiplied by 100/67 to give an estimate of the spectrum if 100% adduct had been formed. Second, from the spectrum of the reaction mixture immediately after addition of Ph_2I^+ , 50% of the spectrum of the semiquinone form (obtained before Ph_2I^+ addition) was subtracted, then multiplied by 2. The results of these calculations are gratifyingly similar, and shown in Fig. 10. It should be noted that the calculated spectrum is very similar to that obtained on titration of the isolated C8-phenyl-FMN with apoflavodoxin described in Fig. 4, confirming the identification of the reaction product of Ph_2I^+ with flavodoxin as a flavin-C8-phenyl adduct.

Because iodonium compounds such as Ph_2I^+ are well established as essentially irreversible 1-electron oxidants, because of the instability of the primary diphenyliodo radical, which decays on a picosecond time scale to phenyl radical and iodobenzene, it is likely that formation of flavin-phenyl adducts would involve flavin radical intermediates, as proposed by earlier workers (5–10). The present study, both with free flavins and flavoproteins, shows that this concept has to be modified to admit the possibility of direct nucleophilic attack of reduced flavin on Ph_2I^+ , or that phenyl adducts are formed only from a caged pair of flavin radical and phenyl radical, rather than from the dissociated free radicals. The results with free flavins indicate that the reactive species is the reduced flavin anion, FlH^- , rather than neutral flavin, FlH_2 . This situation probably applies also with flavoproteins, because NMR studies have indicated that most reduced flavoproteins are in the anion form (36). There are several factors that would be expected to influence the reactivity of a flavoprotein with Ph_2I^+ . Given the unlikelihood of specific binding of Ph_2I^+ at the enzyme active center, a concept supported by the second order character of the interaction, accessibility of Ph_2I^+ to the flavin is an obvious candidate for determining whether any reaction will occur, and if it does, its contribution to rate. A second obvious factor would be expected to be the redox potential of the enzyme flavin, particularly that of the $\text{Fl}_{\text{sq}}/\text{Fl}_{\text{red}}$ couple, because ideally this should be lower than that of the $\text{Ph}_2\text{I}^+/\text{Ph}_2\text{I}$ couple. Because the flavin species that is reactive with Ph_2I^+ is the fully reduced form, as shown previously (6, 7), and confirmed in the present study, the steady state level of reduced flavin in catalytic turnover would also be expected to be an important factor in whether an enzyme is inactivated by Ph_2I^+ .

The concept that inhibition of catalytic activity by diphenyliodonium and similar compounds is an indicator of a catalytic mechanism involving radical intermediates is clearly not correct, because several enzymes listed in Table II that are inactivated by Ph_2I^+ do not involve flavin radicals in their catalytic mechanism, *e.g.* lipoamide dehydrogenase, thioredoxin reductase, phenol hydroxylase, and *p*-hydroxybenzoate hydroxylase. The latter enzyme is particularly informative, because inactivation in steady state assays is very dependent on the substrate or non-substrate effector. Thus inactivation is readily observed with *p*-hydroxybenzoate as substrate, but no inactivation was observed with 2,4-dihydroxybenzoate as substrate. A series of fluorine-containing substrates lead to inactivation, but at quite different rates, and no inactivation is observed with *p*-aminobenzoate as substrate or 6-hydroxynicotinate as a non-substrate “effector,” which causes the enzyme to function as an NADPH oxidase rather than as a hydroxylase. The different results with *p*-hydroxybenzoate hydroxylase do not appear to be explicable in terms of redox potential, because in general the midpoint potential of the enzyme flavin is only marginally different in the presence or absence of substrate (37). It should be noted also that, in general, the enzyme does not stabilize a flavin radical form on 1-electron reduction, indicating that the potential for the $\text{EFl}_{\text{sq}}/\text{EFl}_{\text{red}}$ couple must be of the order of 100 mV more positive than that of the midpoint potential, $\text{EFl}_{\text{ox}}/\text{EFl}_{\text{red}}$, *i.e.* ~ -60 mV (38). With the potential of the $\text{Ph}_2\text{I}^+/\text{Ph}_2\text{I}$ couple at -332 mV (39), this difference makes the primary 1-electron reduction of Ph_2I^+ thermodynamically very unfavorable, perhaps accounting at least in part for the slowness of the reaction. However, clearly this is not the reason for the big differences in reactivity in the presence of different substrates. In the case of *p*-hydroxybenzoate hydroxylase, the chief factor influencing the reactivity with Ph_2I^+ would appear to be level of reduced enzyme in steady state catalysis. Under the assay conditions of Table II, it can be estimated that $\sim 80\%$

of the enzyme should be in the reduced state in steady state turnover with 4-hydroxy benzoate present (40), whereas with 2,4-dihydroxybenzoate less than 1% should be present in the reduced state (41). The same situation is expected in the presence of the effector, 6-hydroxynicotinate, where reduction of the enzyme flavin by NADPH is much slower than its reoxidation by molecular oxygen (42).

Phenol hydroxylase is also readily inactivated during catalytic turnover (Table II). Again, it can be estimated both with phenol and resorcinol as substrates that appreciable concentrations (30–60%) of the enzyme should be in the reduced form in the steady state of catalysis (43, 44).

Although with these two enzymes the steady state level of the reduced flavin would appear to provide a good rationale for the results, this explanation is not necessarily valid in general. For example, with another flavoprotein monooxygenase, MHPC oxygenase, it can be calculated with MHPC as substrate, $\sim 80\%$ of the enzyme should be in the reduced state in turnover, and with 5-hydroxynicotinate, $\sim 60\%$ should be in the reduced form (45, 46). However, no inactivation with Ph_2I^+ was found with either substrate. With this enzyme the flavin radical state is stabilized on 1-electron reduction, with the potential for the $\text{EFl}_{\text{sq}}/\text{EFl}_{\text{red}}$ couple estimated at -48 mV in the presence of MHPC and -82 mV in the presence of 5-hydroxynicotinate. Although these values indicate that the reaction of the reduced enzyme with Ph_2I^+ is thermodynamically unfavorable, the situation is no worse than with *p*-hydroxybenzoate hydroxylase, where the potential of the $\text{EFl}_{\text{sq}}/\text{EFl}_{\text{red}}$ couple is equally high (see discussion above). The fact that the redox potential is not the only factor determining reactivity is clear from Table II, *e.g.* both D-amino acid oxidase and Old Yellow Enzyme have redox potentials for the $\text{EFl}_{\text{sq}}/\text{EFl}_{\text{red}}$ couple of -204 and -215 mV, respectively (47, 48), values more thermodynamically favorable for the reaction, yet no inactivation with Ph_2I^+ was found in steady state assays. In such cases, presumably lack of accessibility of Ph_2I^+ to the flavin, or electrostatic repulsion by suitably positioned positively charged residue(s) in the vicinity of the flavin, must be dominant in preventing reaction. In the case of glucose oxidase, which is remarkably resistant to reaction with Ph_2I^+ , the flavin is well buried in the protein structure, accessible through a funnel-shaped pocket, which can accommodate only a single substrate molecule (49). With this protein, difficulty of access would therefore appear to be responsible for the nonreactivity with Ph_2I^+ .

The reaction of reduced flavodoxin with Ph_2I^+ is quite remarkable, because it results in the very rapid ($k = 2.2 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$ at pH 7, 25°C) equimolar mixture of flavin-C8-phenyl adduct and flavodoxin semiquinone. The very slow further reaction of the semiquinone appears to be a result of the slow disproportionation of the semiquinone to an equimolar mixture of oxidized and reduced flavoprotein, with the latter reacting with Ph_2I^+ as it is formed. With flavodoxin the only part of the flavin that is readily accessible to solvent is the benzene sub-nucleus (35), providing a good rationale for the reaction product being a flavin-C8 adduct. Similar accessibility of the flavin-C8 position in *p*-hydroxybenzoate hydroxylase (50) and the Old Yellow Enzyme (51) could explain why flavin-C8-phenyl adducts are also formed with these enzymes. What remains as an intriguing unanswered problem is the large difference in reaction rates, $0.6 \text{ M}^{-1} \text{ s}^{-1}$ for Old Yellow Enzyme, $26 \text{ M}^{-1} \text{ s}^{-1}$ for *p*-hydroxybenzoate hydroxylase, and $2.2 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$ for flavodoxin, especially because the latter value is 50–100 times greater than for reaction with free flavin.

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