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Involvement of Phenyl Radicals in Iodonium Compound Inhibition of Flavoenzymes

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SUMMARY

Iodonium inhibition of the flavoenzymes neutrophil NADPH oxidase and cytochrome P450 reductase has been suggested to require reductive metabolism of the inhibitor to a phenyl radical. Inhibition would ultimately result from covalent attachment of phenyl radicals to either the flavin cofactor or adjacent amino acid side chains important in catalysis. In this paper we provide evidence, using EPR techniques, that phenyl radicals are formed during reaction of iodonium diphenyl with reduced free flavin (FMN) and protein-bound (cytochrome P450 reductase or xanthine oxidase) flavin. Kinetic analysis indicated iodonium diphenyl to be an uncompetitive inhibitor of xanthine oxidase, suggesting the need for reduced enzyme for inhibition. A study of the catalytic and structural properties of different flavoenzymes suggested that only enzymes containing flavins that function in one-electron transfer are targets for iodonium inhibition.

Reactive oxygen species such as superoxide, hydrogen peroxide, and hydroxyl radicals are known to be continuously produced *in vivo* from a variety of sources, ranging from phagocyte defense against disease to production as a reaction intermediate during mitochondrial oxidation. Recently, however, they have become increasingly implicated as second messengers in a number of biological signaling processes (1), including neurotransmission (2), regulation of proliferation and proto-oncogene expression in fibroblasts (3), and signal transduction by oxygen-sensing carotid body cells (4).

As the diversity of effects of reactive oxygen species becomes apparent, reliable tools for elucidation of oxygen radical-dependent events become essential. DPI and its analogue IDP are potent inhibitors of the flavoenzymes neutrophil NADPH oxidase (5), mitochondrial NADH dehydrogenase (6, 7), xanthine oxidase (8), both endothelial and macrophage nitric oxide synthase (9), and cytochrome P450 reductase (10). All of these are capable of generating reactive oxygen species either directly or indirectly, via reduction of redox cycling agents (cytochrome P450 reductase). Iodonium inhibition of these enzymes is time dependent and irreversible and exhibits saturation kinetics. For all but one enzyme (nitric oxide synthase), inhibition is uncompetitive with respect to reducing substrate, suggesting that catalytic turnover/enzyme reduction is necessary for inhibition. In addition to flavoenzyme inhibition, IDP is capable of reductant-dependent arylation of cytochrome P450 heme (11) and may interact with reduced cytochrome b_{-245} of bovine neutrophil NADPH oxidase (8).

Diaryliodonium salts are potent arylating agents, mediating the addition of phenyl groups to a wide variety of nucleophiles and bases, for example, halide ions (12), sulfydryl groups (13), aromatic amines (14), and pyridine (15). It has been suggested by analysis of reaction intermediates and use of radical scavengers that these reactions occur via a radical mechanism whereby an electron is abstracted from a nucleophile to form a phenyl radical, which then adds back to the nucleophile to form phenylated adducts (16). Electrochemical reduction and redox potentiometry have indicated the formation of phenyl radicals upon one-electron reduction of both DPI ($E_{m,7.0}$ = −422 mV) and IDP ($E_{m,7.0}$ = −332 mV) (17). As shown in Fig. 1, the formation of a phenyl radical from IDP is associated with splitting of the molecule and loss of iodobenzene into solution. In contrast, due to the presence of an extra cross-bridge, DPI forms an intact radical (18).

Reduced FAD and FMN react with DPI or IDP to form phenylated flavin adducts (5). Inhibition of both neutrophil NADPH oxidase and cytochrome P450 reductase may occur by similar mechanisms (5, 10). A redox center in the enzyme,

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ABBREVIATIONS: DPI, diphenylene iodonium; IDP, iodonium diphenyl; PBN, *N*-*t*-butyl-α-phenylnitrone; DMPO, 5,5-dimethyl-1-pyrroline *N*-oxide; MNP, 2-methyl-2-nitrosopropane; DTPA, diethylenetriaminepentaacetic acid; PBS, phosphate-buffered saline.

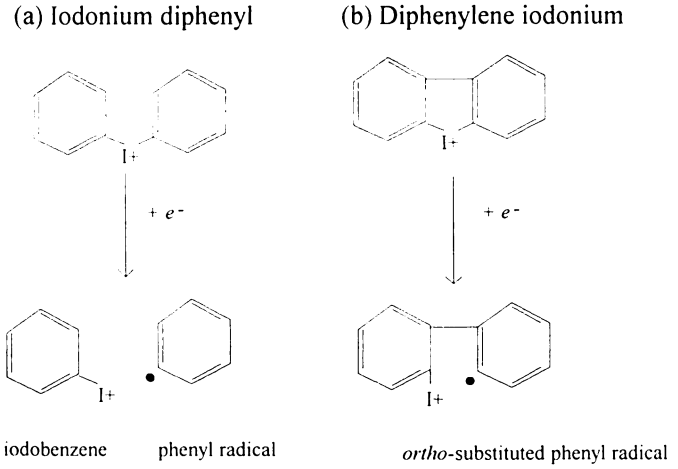


Fig. 1. Phenyl radical formation by the iodonium compounds IDP and DPI. Both DPI and IDP react readily with nucleophiles by electron abstraction, leading to reactive radical formation.

reduced during enzyme turnover, could act as an electron donor to the inhibitor, allowing generation of phenyl radicals that would then inhibit by direct attack on either the redox center or amino acid side chains important in enzyme catalysis.

We have used EPR spectroscopy to provide evidence that phenyl radicals are formed during IDP interaction with reduced FMN in solution. In addition, we describe substrate-dependent radical generation during inhibition of both xanthine oxidase and cytochrome P450 reductase. This supports the inhibitory mechanism proposed previously (5). In addition, a number of flavoenzymes have been examined for iodonium inhibition. The catalytic and structural characteristics of iodonium-sensitive and -insensitive enzymes have been compared in an effort to explain the specificity of iodonium compounds as flavoenzyme inhibitors.

Experimental Procedures

Materials

DPI sulfate was a gift from Mr. T. Reid (Department of Biochemistry, University of Bristol). It was dissolved in dimethylsulfoxide to give a 10 mM stock solution. IDP was obtained from Fluka Chemica A.G. (Buchs, Switzerland). FMN, FAD, PBN, DMPO, MNP, xanthine oxidase (grade 1, EC 1.1.3.22), glutathione reductase (bovine intestinal mucosa, EC 1.6.4.2), D-amino acid oxidase (porcine kidney, EC 1.4.3.3), L-amino acid oxidase (*Crotalus adamanteus*, EC 1.4.3.2), glucose oxidase (*Aspergillus*, EC 1.1.3.4), EDTA, and DTPA were obtained from Sigma Chemical Co. (St. Louis, MO).

Isolation of Rabbit Liver NADPH Cytochrome P450 Reductase (EC 1.6.2.4)

NADPH-cytochrome P450 reductase was purified from hepatic microsomes of phenobarbital-treated rabbits by the method of Yasukochi and Masters (19). The purified enzyme was solubilized in 10 mM phosphate buffer, pH 7.7, containing 20% glycerol, 0.1 mM dithiothreitol, 0.1 mM EDTA, and 0.1% Emulgen 911. Specific activity was calculated as NADPH-cytochrome *c* reductase activity (cytochrome *c* reduction monitored at 550–540 nm, using $\Delta\epsilon = 19.1\ mM^{-1}\ cm^{-1}$) (20). Protein concentration was determined by the method of Bradford (21).

EPR Spin Trapping of Phenyl Radicals

Free flavin assays. FMN was anaerobically photoreduced at room temperature in the presence of either DMPO (80 mM) or PBN (40 mM), as follows. A solution of FMN (0.75–1 mM), spin trap, and EDTA (1 mM) in Chelex-treated phosphate buffer (100 mM, pH 7) was placed in a quartz EPR flat cell (final volume, 500 μl). A stream of nitrogen was bubbled through from below to remove oxygen and the cell was illuminated, using a 250-W tungsten filament lamp, for 20 min to facilitate flavin reduction. Degassed IDP (final concentration, 900 μM) in phosphate buffer was then added and the sample was placed immediately in the cavity of the ESR spectrometer (Varian E-104) and scanned with the following instrument settings: sweep width, 100 G; modulation frequency, 100 kHz; modulation amplitude, 0.8–1.25 G; sweep time, 4 min; power, 20 mW; time constant, 0.25 sec (see figure legends).

Cytochrome P450 reductase assays. Enzyme protein (1.5 mg; NADPH-cytochrome *c* reductase activity, 1.8×10^{-5} mol of cytochrome *c* reduced/min/mg) was mixed with IDP (2.2 mM), PBN (33 mM), and DTPA (1 mM) at 20°. The sample was incubated for 5 min at 25° before the addition of NADPH (2 mM) (final volume, 1 ml). After an additional incubation of 10 min the sample was placed in a quartz EPR flat cell and scanned (Bruker E-106) with the following instrument settings: sweep width, 60 G; modulation frequency, 100 kHz; modulation amplitude, 1 G; sweep time, 168 sec; power, 20 mW; time constant, 41 msec. For some experiments, NADPH, IDP, or cytochrome P450 reductase was replaced with phosphate buffer, as indicated in the figure legends.

Xanthine oxidase assays with DMPO. Enzyme protein (0.4 mg) was mixed in the EPR flat cell with DMPO (80 mM) in phosphate buffer, pH 7.2. Deoxygenation was started by bubbling with nitrogen from below. Degassed xanthine (1.5 mM) was then added and deoxygenation was continued for 10 min. After the addition of deoxygenated IDP (1 mM), the sample was placed in the EPR spectrometer (Varian E-104) and scanned with the following settings: sweep width, 100 G; modulation frequency, 100 kHz; modulation amplitude, 2.5 G; sweep time, 16 min; power, 20 mW; time constant, 1 sec.

Xanthine oxidase assays with PBN. Enzyme protein (0.8 mg) was mixed with IDP (1 mM), PBN (50 mM), and DTPA (0.7 mM) in Chelex-treated phosphate buffer, pH 7.2. The sample was deoxygenated by bubbling with nitrogen for 10 min. Degassed xanthine (2 mM) was added and the mixture was degassed for an additional 2 min (final volume, 1 ml). The sample was placed in the EPR flat cell and scanned with settings identical to those described above for cytochrome P450 reductase. For some experiments, xanthine, xanthine oxidase, or IDP was replaced with phosphate buffer, as indicated in the figure legends.

Chemical generation of PBN/·C_6H_5. Phenyl radicals were generated by the oxidation of phenylhydrazine, by a modification of the method of Augusto *et al.* (22). Phenylhydrazine (500 μM) in Chelex-treated phosphate buffer (60 mM, pH 7.2) was mixed with PBN (40 mM) and $CuCl_2$ (10 μM), immediately placed in the EPR flat cell, and scanned with settings identical to those described above for cytochrome P450 reductase.

Assays of Enzyme Activity

Glutathione reductase. Enzyme activity was measured spectrophotometrically by monitoring the absorbance of NADPH at 340 nm (23). Enzyme (1 μl, 100 units/ml) was added to 1 ml of assay buffer (50 mM phosphate, 200 mM KCl, 1 mM EDTA, 1 mM oxidized glutathione, pH 7.6) at 25°. NADPH (0.1 mM) was added to start the reaction. For inhibition assays, DPI (to 200 μM) was added before NADPH.

Glucose oxidase. Activity was measured by oxygen uptake using a Clark-type oxygen electrode. Enzyme (0.25 unit) was placed in 2.4 ml of buffer [0.2 M D-glucose, 10 μg (24 units) of catalase, 0.1 M acetate, pH 5.6] at 35° (24). The dissolved oxygen concentration at 35° was calculated by the method of Truesdale and Downing (25). For inhibition

assays, DPI (to 50 μM) was added before enzyme.

D-Amino acid oxidase. Enzyme activity was measured using an oxygen electrode, as described above. Enzyme (0.15 unit) was added to 2.4 ml of buffer (0.1 M Tris-methylamine, 0.1 M D-alanine, pH 8.3, 25°) containing 20 μg (48 units) of catalase (26). DPI (to 400 μM) was added before enzyme.

L-Amino acid oxidase. Activity was measured using an oxygen electrode at 37°. Enzyme (0.12 unit) was added to 2.4 ml of PBS, pH 7.2, containing 0.1 M L-methionine and 20 μg (48 units) of catalase, with or without DPI (to 400 μM).

Xanthine oxidase. Enzyme activity was assayed as the superoxide dismutase-sensitive rate of reduction of cytochrome *c* (27). Enzyme (0.05 unit) was added to a cuvette containing 1 ml of assay buffer (PBS, pH 7.7) with 100 μM cytochrome *c*, 500 μM xanthine, and 0–50 μM DPI, at 20°. Cytochrome *c* reduction was monitored at 550–540 nm, using $\Delta\epsilon = 19.1\ mM^{-1}\ cm^{-1}$ (20). Superoxide dismutase (100 μg) was added at the end of the assay to ensure that all reduction resulted from superoxide generation.

Results

EPR trapping of phenyl radicals. After anaerobic photoreduction (in the presence of EDTA) of a solution of FMN with the spin trap PBN, the addition of degassed IDP resulted in the formation of a PBN radical adduct with hyperfine coupling constants as follows: $a_N = 15.9$ G and $a_H^\beta = 4.2$ G (Fig. 2a). No radical adducts were formed if either FMN or IDP was omitted from the reaction mixture. Traces of the adduct were observed if the sample was not illuminated before IDP addition (Fig. 2c). However, it was not possible to exclude all light during the incubation and a small amount of natural light-catalyzed flavin reduction was unavoidable. The need for illumination for formation of the strong signal in Fig. 2a strongly suggests that flavin reduction is essential for formation.

Formation of a PBN radical adduct with identical hyperfine coupling constants ($a_N = 15.9$ G, $a_H^\beta = 4.2$ G) was also observed during inhibition of cytochrome P450 reductase by IDP, but only in the presence of NADPH as reductant (Fig. 3a). If NADPH or IDP was omitted from the reaction mixture, no signal from this adduct was seen (Fig. 3, b and c). A very small amount of this signal was formed in the absence of enzyme (i.e., NADPH, IDP, and PBN only) (Fig. 3d). This may result from iodonium reduction by NADPH when both are present at very high concentrations. Another carbon-centered PBN radical adduct was also observed ($a_N = 16$ G, $a_H^\beta = 3.2$ G) (Fig. 3b); however, the formation of this adduct required only enzyme and NADPH, and not IDP, and may arise from some nonspecific reductase activity of the enzyme preparation. Omission of the metal chelator DTPA, included to guard against potential metal-dependent effects, had no effect on this latter signal (data not shown). The need for NADPH together with IDP and enzyme indicates that reduced enzyme is required for generation of the strong radical adduct signal shown in Fig. 3a.

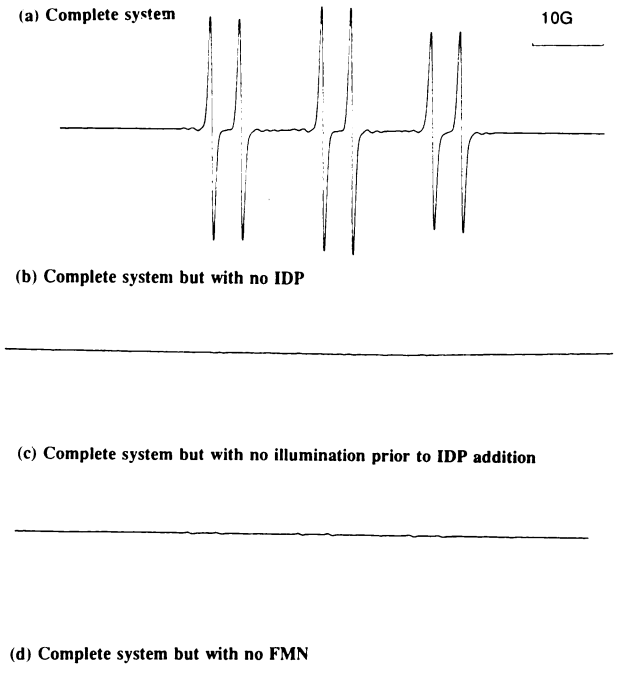


Fig. 2. EPR spin trapping of radicals produced after addition of IDP to EDTA/*hν*-reduced FMN. a, A solution of FMN (1 mM), PBN (40 mM), and EDTA (1 mM) in Chelex-treated phosphate buffer, pH 7, was degassed and photoreduced in the EPR flat cell as described in Experimental Procedures. Degassed IDP (900 μM) was then added and spectra were recorded immediately. b, IDP was replaced with phosphate buffer. c, The sample was not illuminated before IDP addition. d, FMN was replaced with phosphate buffer. Instrument settings were as described, with modulation amplitude of 0.8 G.

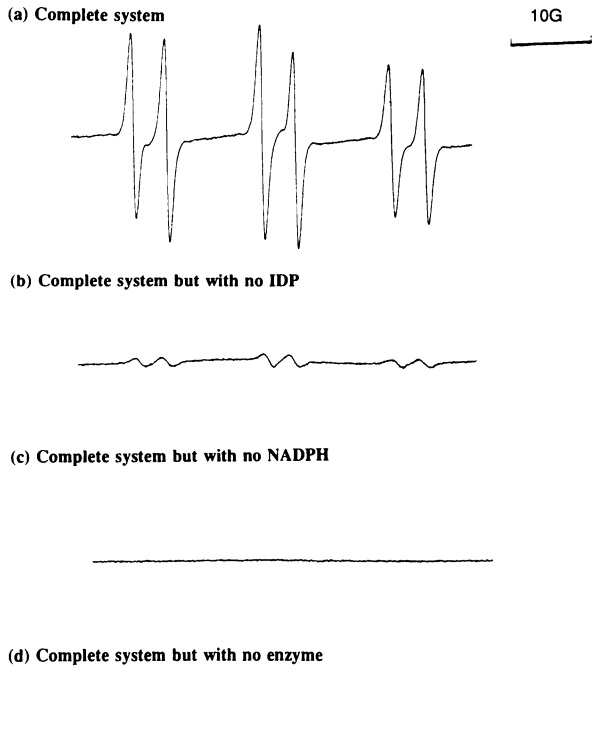


Fig. 3. EPR spin trapping of radicals produced during IDP inhibition of cytochrome P450 reductase. a, Enzyme (1.5 mg) was incubated at 25° for 5 min with IDP (2.2 mM), PBN (33 mM), and DTPA (1 mM). After the addition of NADPH (2 mM), the sample was incubated for an additional 10 min before EPR spectra were recorded. b, IDP was replaced with phosphate buffer. c, NADPH was replaced with phosphate buffer. d, Enzyme was replaced with phosphate buffer.

Formation of an adduct with hyperfine coupling constants identical to those described above (a_N = 15.9 G, a_H^β = 4.2 G) was also seen after inhibition of xanthine-reduced xanthine oxidase by IDP (Fig. 4a). No signals were observed if IDP, enzyme, or substrate was omitted from the reaction mixture (Fig. 4, b-d). In these experiments it was necessary to include the metal chelator DTPA to guard against artifacts arising from Fenton chemistry, because some oxygen contamination may occur while the inhibited sample is being transferred to the ESR cell. To identify the IDP-dependent PBN radical adduct, phenyl radicals were generated chemically by the one-electron oxidation of phenylhydrazine by Cu^{2+} in the presence of PBN. The resulting PBN/$\cdot C_6H_5$ adduct had hyperfine coupling constants of a_N = 15.9 G and a_H^β = 4.2 G (Fig. 5), which are identical to those observed in the flavin and enzyme systems.

Two other spin traps, the nitroso MNP and the nitrone DMPO, were used in place of PBN in the xanthine oxidase and free FMN experiments to obtain more structural information about the trapped radicals. MNP failed to trap radicals in either experimental system. In addition, in experiments involving photoreduction of free FMN it was necessary to change the order of reaction to avoid illumination of the spin trap, because this itself led to formation of photodegradation products. Finally, when used with the chemical phenyl radical-generating system (phenylhydrazine and Cu^{2+}) (22), a very low yield of poorly resolved radical adduct was obtained under conditions identical to those which produced a very large amount of PBN/$\cdot C_6H_5$ (data not shown). Using DMPO, an adduct with coupling constants of a_N = 16.0 G and a_H^β = 24.6 G was observed in both the free FMN and xanthine oxidase experimental systems (Fig. 6). This is consistent with previously reported coupling constants for DMPO/$\cdot C_6H_5$ (22). Unfortunately, it appeared that under certain conditions DMPO could interact with free flavin itself, leading to the formation of artifactual radical adducts (data not shown). However, formation of the strong carbon-centered adduct shown in both experimental systems was always dependent on the presence of both flavin and inhibitor.

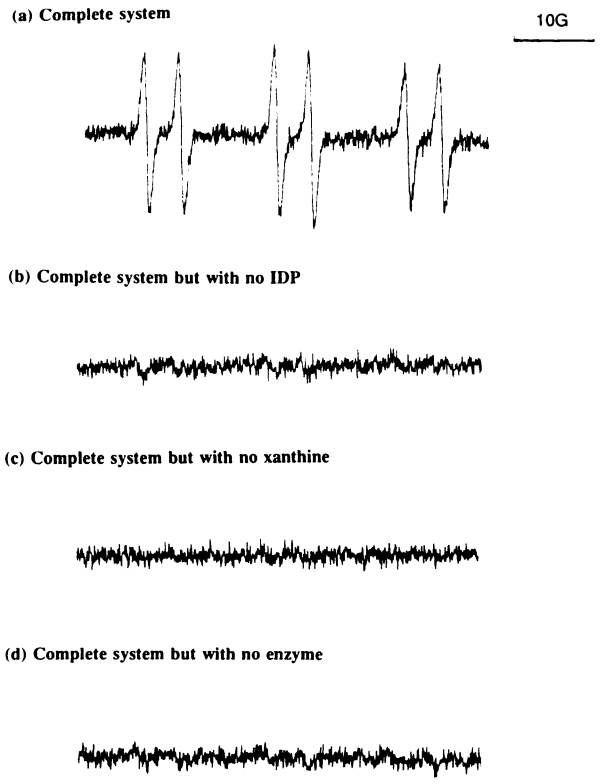


Fig. 4. EPR spin trapping of radicals produced during IDP inhibition of xanthine oxidase. a, Enzyme (0.8 mg), IDP (1 mM), PBN (50 mM), and DTPA (0.7 mM) were degassed for 10 min before the addition of degassed xanthine (2 mM). The sample was degassed for an additional 2 min before being placed in the EPR flat cell, and spectra were then recorded. b, IDP was replaced with phosphate buffer. c, Xanthine was replaced with phosphate buffer. d, Enzyme was replaced with phosphate buffer.

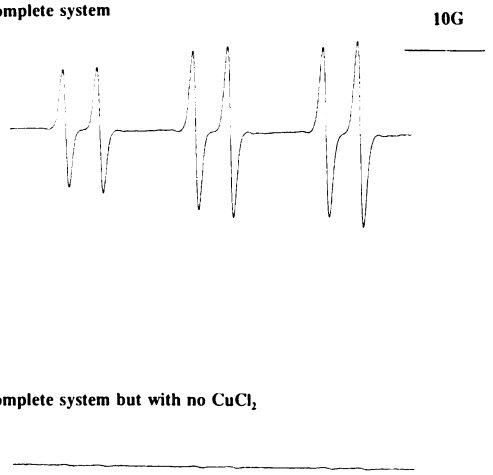


Fig. 5. Chemical generation of phenyl radicals. a, Phenylhydrazine (500 μM) in Chelex-treated phosphate buffer (60 mM) was mixed with PBN (40 mM) and $CuCl_2$ (10 μM) and scanned immediately. b, $CuCl_2$ was omitted.

Effects of DPI or IDP on flavoenzyme activity. Of five flavoenzymes examined for iodonium inhibition, only xanthine oxidase was inhibited by DPI (Fig. 7). There was no inhibition of the activities of glutathione reductase, glucose oxidase, D-amino acid oxidase, or L-amino acid oxidase. Inhibition of xanthine oxidase was both time dependent and concentration dependent at saturating xanthine concentrations. This closely resembles the inhibition by DPI or IDP of neutrophil NADPH oxidase (5), mitochondrial NADH dehydrogenase (6), and cytochrome P450 reductase (10). Kinetic analyses of the inhibition of xanthine oxidase were performed as described previously (5). The anticipated reaction scheme for irreversible enzyme inhibition involving binding of inhibitor before inhibition can be given by

$$E + I \overset{K_i}{\rightleftarrows} E \cdot I \overset{k_3}{\rightarrow} E^*$$

where E is enzyme, I is inhibitor, E·I is reversible complex formed between enzyme and inhibitor, and E^* is fully inhibited enzyme. K_i is the dissociation constant for the initial reversible reaction, and k_3 is the first-order rate constant for the conversion of reversible complex to irreversibly inhibited enzyme. For an irreversible enzyme inactivation, the rate of enzyme inacti-

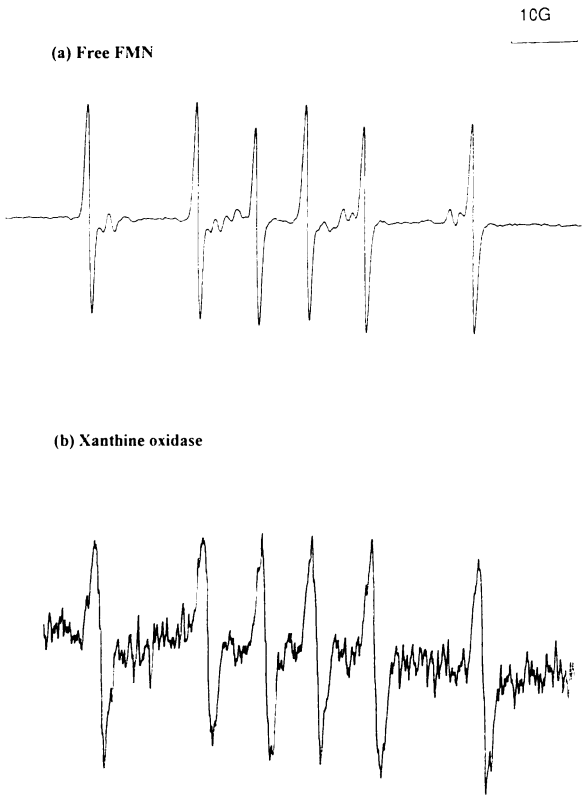


Fig. 6. EPR spin trapping of radicals produced during IDP interaction with either free FMN or xanthine oxidase. a, FMN (0.75 mM), DMPO (80 mM), and EDTA (1 mM) were degassed and photoreduced in the EPR flat cell as described in Experimental Procedures. Degassed IDP (900 μM) was then added and the spectrum was recorded immediately. Instrument settings were as described, with modulation amplitude of 1.25 G. b, Xanthine oxidase (0.4 mg) was degassed in the EPR cell with DMPO (80 mM) and xanthine (1.5 mM) as described in Experimental Procedures. Deoxygenated IDP (1 mM) was then added and the spectrum was recorded immediately. Instrument settings were as described in Experimental Procedures.

vation (k) can be given by

$$k = -dE/dt + e^{-kt}$$

where E is active enzyme concentration. Therefore, for any reaction proceeding with an initial rate A, the rate (v) at any time (t) is given by

$$v = Ae^{-kt}$$

Integrating with respect to t, between $t = 0$ and $t = \infty$, gives total substrate turnover (V) as

$$V = A(1 - e^{-kt})/k$$

Fitting the observed absorbance-time traces (Fig. 7) to this equation allows calculation of values of k. Plotting k versus the DPI concentration (Fig. 8) showed that, at high inhibitor concentrations, the rate of inactivation (k) approached a maximum ($k_3 = 2.46 \times 10^{-2}$ sec^{-1}). This plot was also used to give a value for the apparent K_i for DPI, using the equation (28)

$$k = k_3 \cdot I/(K_i + I)$$

This gave a K_i value of 6.6 μM. In another experiment, the

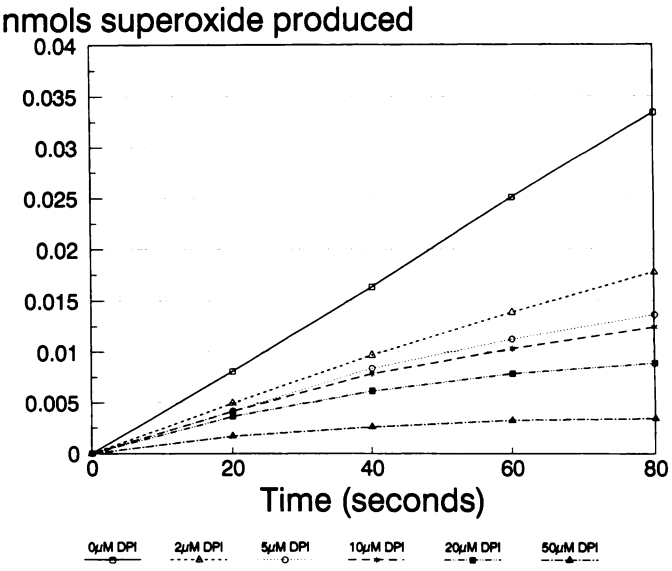
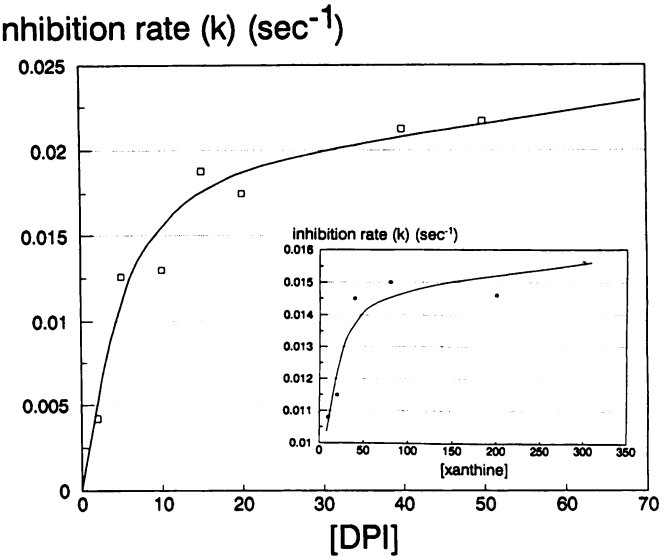


Fig. 7. Superoxide generation by xanthine oxidase and inhibition by DPI. Superoxide generation was measured as described in Experimental Procedures. For assay, enzyme (0.05 unit) was added to 1 ml of assay buffer containing cytochrome c (100 μM), xanthine (500 μM), and DPI (0–50 μM) in PBS, pH 7.7, and absorbance was monitored at 540–550 nm.



$K_i = 6.6\mu M$

$k_3 = 2.46 \times 10^{-2} sec^{-1}$

Fig. 8. Inhibition of xanthine oxidase by DPI and calculation of K_i. The inhibition rate (k) was calculated as the rate of decrease of superoxide generation, using the data shown in Fig. 6 and the equation $k = k_3 \cdot [I]/(K_i + [I])$. *Inset*, effect of changing xanthine concentrations on inhibition of xanthine oxidase by DPI. Enzyme activity was measured as described for Fig. 6. The xanthine concentration was varied around the K_m value (50 μM), whereas the DPI concentration was kept constant at the K_i value (6.6 μM).

inhibition rate was calculated at a constant DPI concentration (6.6 μM) with varying xanthine concentrations around the K_m value (50 μM) (29). Under these conditions, the inactivation rate was found to increase to a maximum with increasing

substrate concentrations (Fig. 8, *inset*), indicating that DPI is an uncompetitive inhibitor with respect to xanthine. This suggests that DPI reacts with reduced enzyme only.

Discussion

There is indirect evidence that inhibition of flavoenzymes (e.g., neutrophil NADPH oxidase, NADH dehydrogenase, and cytochrome P450 reductase) by iodonium compounds involves reductive metabolism of the inhibitor, by the enzyme, to reactive phenyl radicals that inhibit by attaching directly to the flavin ring or adjacent amino acid groups important in catalysis. Inhibition of xanthine oxidase by DPI has been reported previously (8) and was suggested to require redox turnover of the enzyme. Here, kinetic analysis demonstrated that inhibition of this enzyme by IDP was uncompetitive with respect to xanthine, supporting the need for reduced enzyme for reaction with this analogue also.

Although phenyl radicals are known to form during electrochemical reduction of both DPI and IDP, direct evidence of their involvement in iodonium inhibition of flavoenzymes has previously been lacking. Here, using spin-trapping techniques, we have examined the interaction of IDP with photoreduced free FMN in buffered aqueous solution, using conditions under which we previously demonstrated reductant-dependent phenylation of flavins (FMN or FAD) by iodonium compounds (IDP or DPI) (5). Using the spin trap PBN, a strong signal with hyperfine coupling constants identical to those of the chemically generated PBN/$\cdot C_6H_5$ was observed. This adduct was only formed if flavin reduction took place before iodonium addition. The formation of this radical under conditions identical to those that allow phenylation of flavins (i.e., the presence of reduced flavin) strongly suggests the involvement of this radical in adduct formation.

The inhibition of the flavoenzymes cytochrome P450 reductase and xanthine oxidase by IDP was shown by similar techniques to involve radical generation. As for free flavins, formation of the radical adduct was dependent on the presence of reductant (enzyme substrate), indicating the need for reduced enzyme. Very small amounts of this radical were formed from a sample containing IDP, NADPH, and PBN. This was only a fraction of that formed when enzyme (cytochrome P450 reductase) was present, indicating that radical formation in the latter case was an enzymatic process. The demonstration of radical adduct formation during xanthine oxidase inhibition by IDP was complicated by the need to exclude oxygen as an electron acceptor. Due to the viscosity of the enzyme preparation, it was necessary to dilute samples to allow gassing with nitrogen to take place. This unfortunately resulted in low signal intensity of the radical adduct observed (Fig. 4a).

Initially, PBN was chosen as the spin trap for these experiments due to the stability of its carbon-centered adducts. Oxygen radicals, which are readily generated by both free reduced flavin and xanthine oxidase, are also trapped by this spin trap, but these adducts are less stable. However, these advantages are offset by the limited structural information that PBN may afford. Therefore, additional experiments were carried out with two other spin traps, the nitroso MNP and the nitrone DMPO, to examine whether the radical trapped by PBN was indeed a phenyl radical. MNP did not prove to be a useful spin trap for these experiments, because either it was not capable of efficiently trapping the phenyl radical in aqueous solution or the MNP/$\cdot C_6H_5$ formed was not sufficiently stable for spectroscopic analysis. In general, nitroso-derived radical adducts are less stable than those formed from nitrones, such as PBN (30). Chignell *et al.* (31) have previously reported on the failure of MNP to trap several substituted phenolic compounds in aqueous solution. Using DMPO as the spin trap, in both the free FMN and xanthine oxidase experimental systems we observed a signal with coupling constants consistent with those previously reported for DMPO/$\cdot C_6H_5$ (a_N = 16.0 G, a_H^β = 24.6 G) (22), the formation of which always required the presence of both flavin and inhibitor.

Cytochrome P450 reductase is a flavoenzyme containing 1 mol each of FMN and FAD per 1 mol of enzyme. Because no other redox centers are present to participate in catalysis, demonstration of radical formation from iodonium interaction with this enzyme confirms the reactivity of protein-bound flavins towards iodonium compounds. Kinetic analyses and covalent modification of cytochrome P450 reductase by IDP have recently been reported by Tew (10). In that work, uncompetitive inhibition with respect to NADPH, competitive inhibition with respect to cytochrome *c*, covalent modification of an amino acid residue (Trp-419), and formation of phenylated FMN adducts were reported after iodonium inhibition of reduced enzyme. Taken together with our EPR data, these results support a mechanism whereby inhibition of cytochrome P450 reductase ultimately results from covalent modification of cofactor/amino acid side chains by reactive phenyl radicals generated by reductive metabolism of the inhibitor.

Xanthine oxidase contains at least two other redox-active centers (Fe-S and Mo^{2+}) (32) in addition to flavin, either of which may be capable of electron donation to IDP. It was not possible to distinguish the site of electron donation, but similarities between inhibition of this enzyme and inhibition of other flavoenzymes by iodonium compounds might suggest involvement of the flavin component. Interaction of iodonium compounds with Fe-S centers or molybdenum have not been reported in other enzyme systems.

To examine the specificity of iodonium compounds as flavoenzyme inhibitors, we compared the structural and catalytic properties of a number of flavoenzymes. Flavoproteins previously reported to be inhibited by iodonium compounds are NADH dehydrogenase (6, 7), cytochrome P450 reductase (10), nitric oxide synthase (9), neutrophil NADPH oxidase (5), and xanthine oxidase (8). All inhibition reactions share features suggestive of a common mode of action, particularly the irreversible nature and time dependence. For cytochrome P450 reductase, NADH dehydrogenase, neutrophil NADPH oxidase, and xanthine oxidase, inhibition has also been demonstrated to be dependent on catalytic turnover.

With the exception of xanthine oxidase, all inhibited flavoenzymes possess relatively low midpoint redox potentials (Fl_{ox}/Fl_{red}) (*Fl*, flavin; see Table 1). This may facilitate electron donation to the more electronegative DPI ($E_{m,7.0}$ = −422 mV) (17) or IDP ($E_{m,7.0}$ = −332 mV) (17). Redox potentials of iodonium-insensitive flavoenzymes, where known, are close to

TABLE 1
Characteristics of flavoenzymes that have been examined for iodonium inhibition

System	Inhibited?	Donor	Redox center	Acceptor	Semiquinone involved in catalysis?	Redox potential ($E_{m,7.0}$)
						mV
NADPH oxidase	Yes	NADPH	FAD	Cytochrome *b*	Yes	−280
NADH dehydrogenase	Yes	NADH	FMN	Ubiquinone	Yes	−300
Cytochrome P450 reductase	Yes	NADPH	FAD/FMN	Cytochrome P450	Yes	−270
Xanthine oxidase	Yes	Xanthine	FAD	Oxygen	Yes	0
Nitric oxide synthase	Yes	L-Arginine	FAD	Oxygen	Yes	?
D-Amino acid oxidase	No	D-Amino acids	FAD	Oxygen	No	−4
L-Amino acid oxidase	No	L-Amino acids	FAD	Oxygen	No	?
Glutathione reductase	No	NADPH	FAD	Oxidized glutathione	No	?
Glucose oxidase	No	β-D-Glucose	FAD × 2	Oxygen	No	?

0 mV. In addition, all known flavoenzymes inhibited by iodonium compounds function as one-electron donors, accepting two electrons but passing them on singly to the terminal acceptor during catalysis. All known insensitive flavoenzymes transfer two electrons during catalysis. It is possible that flavins oriented within the active site in such a way that they are rendered obligate two-electron donors are incapable of one-electron donation to iodonium compounds. Alternatively, flavins functioning in one-electron transfer must exist as semiquinones during enzyme turnover. It is possible that this radical may be the inhibitor-reactive form, rather than the fully reduced flavin. This is unlikely, however, because mass spectra and UV absorbance spectra of adducts formed after reaction of IDP with either free FMN (5) or cytochrome P450 reductase (10) suggest that these are substituted dihydroflavins. Tew (10) has proposed that formation of phenylated FMN from IDP during inhibition of cytochrome P450 reductase occurs after one-electron oxidation of the fully reduced dihydroflavin by IDP. Adduct formation would then occur via radical recombination of the phenyl radical with the flavin semiquinone. A comparison of structural and catalytic characteristics of sensitive and resistant systems is shown in Table 1.

The activities of iodonium compounds as flavoenzyme inhibitors share features suggestive of a common mode of action. We have proposed a mechanism of action, and understanding of this mechanism may lead to the design of analogues specific for certain flavoprotein-active sites.

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