Inhibition of Cytochrome P_{450} Reductase by the Diphenyliodonium Cation. Kinetic Analysis and Covalent Modifications

David G. Tew

SmithKline Beecham Research, The Frythe, Welwyn, Hertfordshire AL6 9AR, U.K.

Received October 29, 1992; Revised Manuscript Received July 14, 1993®

ABSTRACT: Diphenyliodonium has been shown to be an irreversible, time-dependent inhibitor of NADPH cytochrome P_{450} oxidoreductase (EC 1.6.2.4) with the K_i for diphenyliodonium chloride being 2.8 mM. Kinetic studies have indicated that diphenyliodonium interacts with the reduced enzyme and NADPH is essential for inactivation to take place. Cytochrome *c* acts as a competitive substrate. The use of radiolabeled diphenyliodonium has enabled two sites of covalent modification to be identified. Isolation of radiolabeled cofactor followed by mass spectrometry has shown that a phenyl group is added to FMN while the FMN is effectively trapped in the reduced state. Trypsin digestion of S-carboxymethylated P_{450} reductase after inhibition with radiolabeled inhibitor shows covalent modification of the protein. Purification of a single radiolabelled peptide followed by automated Edman degradation has enabled identification of the second site of covalent attachment as Trp 419.

Bis(aryliodonium) species have been reported to be potent inhibitors of flavoprotein oxidoreductases acting as part of an electron transport chain. The diphenyleneiodonium cation has been shown to be an inhibitor of NADH ubiquinone oxidoreductase in intact mitochondria (Holland et al., 1973; Gatley & Sherratt, 1974). Further work using purified enzyme confirmed that the diphenyleneiodonium cation was a time-dependent inhibitor showing pseudo-first order kinetics and that covalent modification of a specific polypeptide occurred (Ragan & Bloxham, 1977). More recently, it has been shown that bis(aryliodonium) cations are potent time-dependent inhibitors of neutrophil NADPH oxidase (Cross & Jones, 1986) and macrophage NADPH oxidase (Hancock & Jones, 1987). In both cases inactivation led to a covalent modification of a 45-kDa protein. It has been shown (O'Donnell et al., 1992) that inactivation of NADPH oxidase by the diphenyliodonium cation occurs via a reductive mechanism. Recently, nitric oxide synthetase, which shows significant homology with cytochrome P_{450} reductase, has also been shown to be irreversibly inhibited by diphenyleneiodonium and its analogs (Stuehr et al., 1991).

Many mechanism-based inhibitors have been reported to act via oxidative mechanisms, e.g., the inhibition of horseradish peroxidase and cytochrome P_{450} by alkyl and aryl hydrazines (Ortiz de Montellano, 1987), the inhibition of dopamine β-hydroxylase by *p*-cresol (Goodhart et al., 1987), the inhibition of polyamine oxidase by allylic and allenic amines (Bolkenius & Seiler, 1989), and a diverse array of monoamine oxidase inhibitors including propargylamines, fluoroallylamines, and cyclopropylamines (McDonald et al., 1989). However, there have been few reports to date of mechanism-based inhibitors that act via a reductive mechanism, e.g., inhibition of NADPH 3-oxosteroid oxidoreductase by a 4-diazosteroid (Blohm et al., 1980).

NADPH cytochrome P_{450} oxidoreductase (EC 1.6.2.4) is an essential component of the microsomal mixed function oxidase system (Yasukochi & Masters, 1976). The function of cytochrome P_{450} reductase is to receive two electrons from NADPH and transfer them sequentially to cytochrome P_{450}. The enzyme contains one molecule of both FAD and FMN. The transfer of electrons has been shown to occur in the order NADPH to FAD, then to FMN, and finally to cytochrome P_{450}. However, this sequential transfer of two electrons occurs, rather surprisingly, by redox cycling between the one-electron reduced enzyme and the three-electron-reduced form (Vermilion et al., 1981).

I report here the results of kinetic and structural studies on the inactivation of cytochrome P_{450} reductase by the diphenyliodonium cation. Isolation and analysis of both a single covalently modified flavin and a single, major covalently modified tryptic peptide are consistent with a proposed phenyl radical intermediate in the inactivation process. Kinetic studies described here support this hypothesis.

EXPERIMENTAL PROCEDURES

Materials. Trichloroacetic acid and iodoacetic acid were obtained from Aldrich. NADPH and TPCK-treated trypsin were obtained from Boehringer Mannheim. Cytochrome *c* (Type VI), FAD, and FMN were obtained from Sigma. 2′,5′-ADP-Sepharose and Sephacryl S-200 HR were obtained from Pharmacia. Diphenyliodonium chloride was obtained from Lancaster. Guanidine hydrochloride and TFA[1] were obtained from Pierce. HPLC grade solvents were from Romil.

^{14}C-labeled diphenyliodonium chloride was synthesized from iodosobenzene and ^{14}C-labeled benzene (Amersham International) by a modification of the method of Yamada and Okawara (1972). The product had a specific activity of 38.7 μCi/mg and a radiochemical purity of >99.0%. The isotopic enrichment was 36%.

Preparation of NADPH Cytochrome P_{450} Reductase. Male Wistar rats were fed sodium phenobarbital (1 mg/mL) in their drinking water for 10 days to induce hepatic cytochrome P_{450} reductase. After the rats were sacrificed, their livers were removed and homogenized in ice-cold buffer, pH 7.4, containing 250 mM sucrose and 10 mM Tris-HCl. The homogenate was spun at 10000*g* for 20 min to remove cell

® Abstract published in *Advance ACS Abstracts,* September 1, 1993.

[1] Abbreviations: DCPIP, 2,6-dichlorophenolindophenol; TFA, trifluoroacetic acid; DMSO, dimethyl sulfoxide; TCA, trichloroacetic acid; FAB, fast atom bombardment; PTH, phenylthiohydantoin; HPLC, high-performance liquid chromatography.

0006-2960/93/0432-10209$04.00/0

debris and mitochondria. $CaCl_2$ was added to the supernatant to a final concentration of 10 mM. The microsomes were collected by centrifugation at 25000*g* for 30 min (Schenckman & Cinti, 1978). The microsomal fraction was detergent-solubilized and purified by 2′,5′-ADP-sepharose affinity chromatography according to the method of Shepard et al. (1983). This gave protein which was pure as judged by SDS–PAGE. Purified reductase was stored under liquid nitrogen in 30 mM phosphate buffer, pH 7.7, containing 20% glycerol by volume.

Assay Procedures. NADPH cytochrome P_{450} reductase activity was assayed by measuring the rate of reduction of ferricytochrome *c* (Yasukochi & Masters, 1976). The reduction was monitored at 550 nm relative to 540 nm using a Shimadzu UV 3000 spectrophotometer operating in the dual-wavelength mode. The cuvette temperature was maintained at 25 °C. Data were collected from the Shimadzu UV 3000 using a custom-written software package (Icalis Data) running on an IBM compatible PC. Data were collected every 2 s over a period of 5 min. When the concentration of diphenyliodonium chloride was being varied, each assay was prepared by preincubating ferricytochrome *c* and enzyme at 25 °C for 5 min in 0.3 M potassium phosphate buffer, pH 7.7. Diphenyliodonium chloride (100 mM stock solution in 10% DMSO) was included at this point to an appropriate concentration. The reaction was initiated by the addition of a small amount of a concentrated NADPH solution to give a final concentration of 100 μM. When either cytochrome *c* or NADPH concentrations were varied, the diphenyliodonium chloride concentration was 0.2 or 0.5 mM, respectively.

Measurement of Partition Ratio. NADPH cytochrome P_{450} reductase (10 μL of a 20 μg/mL solution) was mixed with 10 μL of a 0.5 mM NADPH solution in a 1-mL Cuvette. A small volume (up to 5 μL) of diphenyliodonium chloride (10 μM) was added and the solution mixed by repeatedly drawing up and expelling the liquid with a 20-μL pipet. The solution was incubated at 25 °C for 10 min, after which time inactivation was found to have gone to completion. Residual reductase activity was measured by adding a solution of cytochrome *c* (42 μM) and NADPH (100 μM) to a final volume of 1 mL and monitoring cytochrome *c* reduction in the usual way.

Isolation of Modified Flavin and Mass Spectrometric Analysis. NADPH cytochrome P_{450} reductase (1 mg) was exchanged into 0.3 M potassium phosphate buffer, pH 7.7, by size exclusion chromatography (Sepharose S-200 HR, 2.6 × 30 cm). NADPH and ^{14}C-labeled diphenyliodonium chloride were added to a final concentration of 200 μM. The final volume was 5 mL. After 3 h at 25 °C, the enzyme activity had dropped to zero. The inactive reductase was applied to a Sepharose S-200 HR column (2.6 × 30 cm) to remove excess NADPH and diphenyliodonium chloride. All fractions were checked for the presence of flavins by measuring their absorbance spectra between 300 and 600 nm.

The resulting inactivated reductase solution was cooled on ice for 30 min and trichloroacetic acid added to 10%. After standing on ice for a further 30 min, the TCA solution was spun at 8800*g* for 10 min to separate the precipitated polypeptide from soluble cofactor. Scintillation counting confirmed the presence of radiolabel in both phases. The precipitated protein was stored in liquid nitrogen for further use. The supernatant was applied to two preactivated C_{18} cartridges (Bondelut, Analytichem International) by vacuum aspiration. After the cartridges were washed well with water (4 × 1 mL), the bound radioactivity was eluted with methanol (3 × 150 μL). No radioactivity eluted either upon loading or upon washing the cartridges. Methanol was removed from the radioactive fraction using a gyrovap (V. A. Howe).

HPLC of the above radioactive fraction was performed on a Waters C_{18} column (5 × 300 mm) using Waters Model 510 pumps and a Pye Unicam PU4021 diode array detector operating between 190 and 390 nm. Buffer A was 5 mM NH_4OAc in water, and buffer B was 5 mM NH_4OAc in methanol. One radioactive peak was collected yielding 85% of the applied radioactivity. The sample was desalted by HPLC on the above column using a gradient from 100% water to 100% methanol over 10 min. FAB-MS was performed on a VG Analytical 70-250 SEQ instrument using glycerol/thioglycerol (1:1) as the matrix, running in positive ion mode.

S-Carboxymethylation and Tryptic Digestion. Precipitated protein remaining from the flavin isolation was washed well with water by resuspension and centrifugation and then redissolved in Tris base (2 M). Solid guanidine hydrochloride was added to near saturation to effect complete dissolution. This took 4 h at 40 °C.

The redissolved protein was spun at 8800*g* for 20 min to remove any remaining solid and reduced by the addition of β-mercaptoethanol (5% final concentration) under argon at 40 °C. After 4 h, a solution of iodoacetic acid (120 mg) and Tris base (150 mg) in 8 M guanidine hydrochloride (1 mL) was added. After 20 min, β-mercaptoethanol (50 mL) was added to quench the reaction. The protein solution was dialyzed against water (2 × 2 L) and then against 50 mM NH_4HCO_3 (2 L). During the dialysis the volume increased to approximately 5 mL and a small amount of protein precipitated. Ninety-five percent of the radioactivity not released into solution by TCA denaturation of the desalted inactivated enzyme was associated with this solubilized fraction.

To digest the labeled polypeptide, trypsin (50 μg) was added to the protein solution and the temperature was maintained at 25 °C for 24 h. After 2 h, the precipitate had disappeared. After 24 h, TFA (50 μL) was added to stop proteolysis. The tryptic digest was then subjected to reversed phase HPLC without further treatment.

HPLC was performed on the same system as that described for flavin isolation. The initial fractionation took place on a Vydac C_4 column (2 × 150 mm) using a gradient from 100% water containing 0.075% TFA to 70% acetonitrile containing 0.075% TFA over 70 min. Pooled radiolabelled peaks were subjected to further chromatography using this solvent system with a Phenomenex C_{18} column (2 × 150 mm) for peak 1 and using a Vydac phenyl column (2 × 150 mm) for peaks 3–5. Peak 2 was purified further using the Vydac C_4 column with 10 mM NH_4OAc as a buffer followed by a desalt step prior to protein sequencing (peaks 1–5 are indicated on Figure 8).

Sequencing of Tryptic Peptides. Purified tryptic peptides were sequenced on an Applied Biosystems 477A pulsed liquid phase sequenator. Approximately two-thirds of each amino acid PTH derivative was retained in the fraction collector of the 477A and so could be used for scintillation counting.

Data Analysis. Nonlinear regression analysis of data was performed using Grafit (Leatherbarrow, 1992). Progress curves were fitted to the equation

$$[\text{product}] = V_0(1 - \exp(-k_{obs}t))/k_{obs} + at$$

to yield values for k_{obs} (Wilson & Kitz, 1962), where V_0 is the initial rate and *at* represents a linear term added to correct for a small degree of incomplete inhibition.

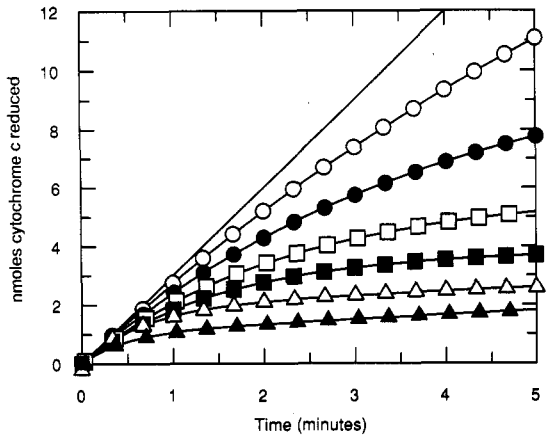

FIGURE 1: Progress curves for the reduction of cytochrome c (42 μM) by cytochrome P_{450} reductase in the presence of diphenyliodonium chloride: (○) 0.2 mM; (●) 0.5 mM; (□) 1.0 mM; (■) 1.5 mM; (△) 2.5 mM; (▲) 5.0 mM. NADPH was 100 μM. Every 10th data point is shown for clarity. The curve with no associated data points is the control rate in the absence of inhibitor. Not all diphenyliodonium chloride concentrations used are shown for clarity.

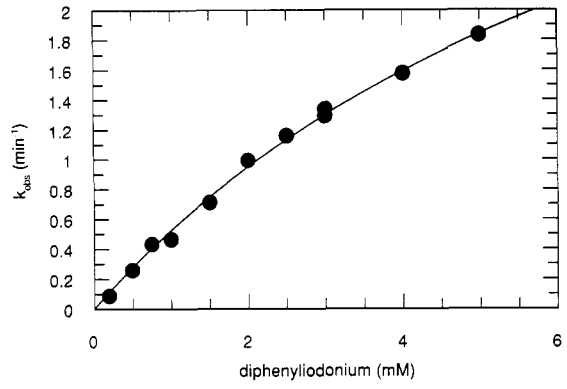

FIGURE 2: Plot of k_{obs} vs diphenyliodonium concentration. k_{obs} values were calculated from the data shown in Figure 1. For diphenyliodonium chloride concentrations not shown in Figure 1, progress curves were similar to those shown in Figure 1.

RESULTS

Inactivation of Cytochrome P_{450} Reductase. Figure 1 shows the reduction of ferricytochrome c with respect to time at various concentrations of diphenyliodonium chloride and a fixed concentration of both NADPH and cytochrome c. Clearly, there are both concentration dependence and time dependence to the inhibition. Each time course has been fitted separately to the curve

$$\text{absorbance} = V_0(1 - \exp(-k_{obs}t))/k + at$$

Figure 2 shows a plot of the rate of enzyme inactivation, k_{obs}, against inhibitor concentration. It is evident from this plot that the rate of inactivation does not vary linearly with inhibitor concentration but rather is approaching a maximum value. This approach to a maximum value indicates that inactivation is a two-step process involving binding of the diphenyliodonium cation to the reductase prior to the inactivation event. However, no effect on V_0 was seen, implying that turnover is a prerequisite for inhibition.

Figure 3 shows the effect on the inactivation rate, k_{obs}, upon varying the NADPH concentration at fixed diphenyliodonium chloride and cytochrome c concentrations. It can be seen that increasing the NADPH concentration results in an increase in the rate of enzyme inactivation.

Finally, Figure 4 shows the effect upon k_{obs} of varying the

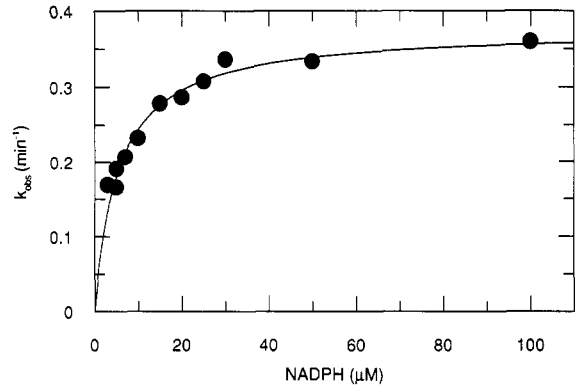

FIGURE 3: Plot of k_{obs} vs NADPH concentration. k_{obs} values were calculated from progress curves obtained using 42 μM cytochrome c and 0.5 mM diphenyliodonium chloride.

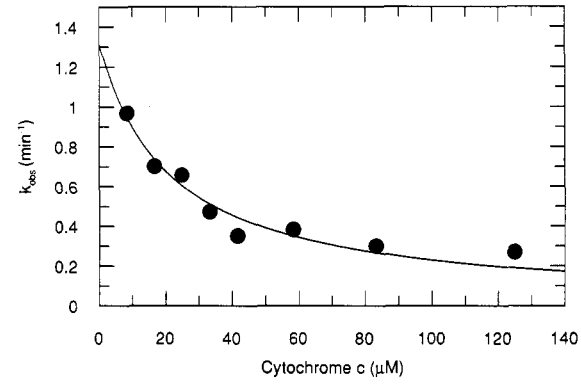

FIGURE 4: Plot of k_{obs} vs cytochrome c concentration. k_{obs} values were calculated from progress curves obtained using 100 μM NADPH and 0.2 mM diphenyliodonium chloride.

cytochrome c concentration at fixed NADPH and diphenyliodonium chloride concentrations. Increasing the cytochrome c concentration results in a decreased rate of inactivation. The data in Figures 2–4 were fitted to the equation

$$k_{obs} = k_{in}[\text{NADPH}][Ph_2I^+]/\{K_{\text{NADPH}}([Ph_2I^+] + K_{Ph_2I^+}[\text{Cyt } c]/K_{\text{Cyt } c}) + [\text{NADPH}]([Ph_2I^+] + K_{Ph_2I^+}(1 + [\text{Cyt } c]/K_{\text{Cyt } c}))\}$$

where k_{in} is the maximal rate of inactivation. This gives a K_m for diphenyliodonium of 2.8 mM and a maximal rate of inactivation of 5.2 min^{-1}.

The relationship between the extent of enzyme inactivation and the amount of diphenyliodonium chloride present was investigated by incubating enzyme, diphenyliodonium chloride, and NADPH together and measuring the residual activity after the reaction had been allowed to go to completion. A plot of residual activity against the number of moles of diphenyliodonium chloride added was found to be linear (data not shown). Extrapolation of these data to zero remaining activity showed that approximately 21 mol of diphenyliodonium chloride was required for complete inhibition of 1 mol of cytochrome P_{450} reductase.

Covalent Modification of Cytochrome P_{450} Reductase. (a) Isolation and Identification of Modified Flavin. When ^{14}C-labeled Ph_2I^+ is used to inactivate cytochrome P_{450} reductase, radiolabel becomes strongly associated with the protein. Approximately 1.4 mol of radiolabel are bound after separation of the inactive enzyme from excess inhibitor by size exclusion chromatography. Denaturation of the inactivated enzyme by cold TCA releases some of the radioactivity into solution. The remaining radioactivity is associated with the protein

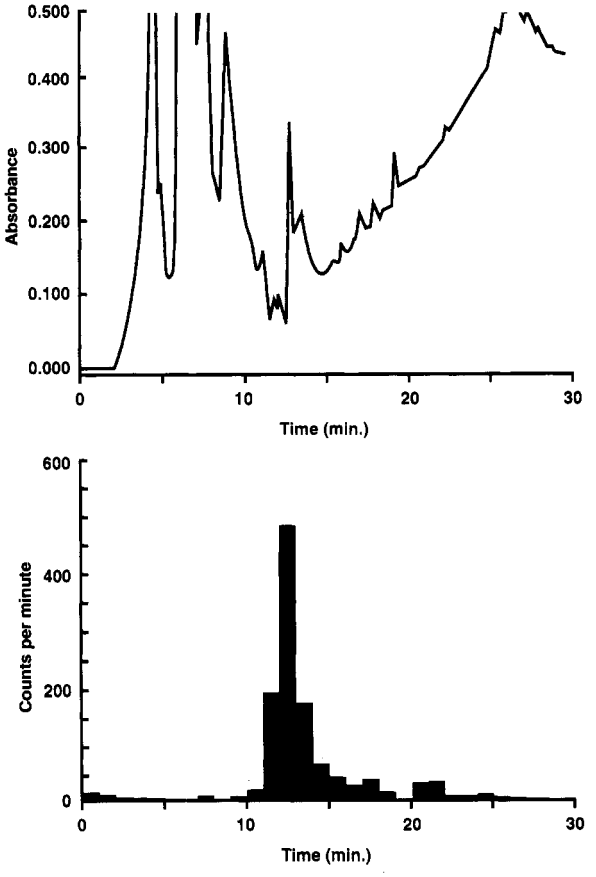

FIGURE 5: Reversed-phase HPLC desalting of radiolabeled covalently modified flavin. The upper trace shows the absorbance at 215 nm, and the lower histogram shows the number of counts per minute for 1-min fractions (1 mL); 10 μL aliquots were counted. A gradient from 100% water to 100% methanol was used.

precipitate. Approximately 0.6 mol of radiolabel is released into solution while 0.8 mol remain bound to the polypeptide. TCA treatment of cytochrome P_{450} reductase has been shown to liberate both FAD and FMN (Iyanagi & Mason, 1973). Control experiments using untreated enzyme confirm that both FAD and FMN are released in approximately equal amounts upon TCA denaturation (data not shown).

The TCA supernatant was concentrated by solid-phase extraction. No significant amounts of radioactivity were lost by this procedure. HPLC of this concentrated radiolabeled fraction shows a number of peaks. However, only one of these peaks has any radioactivity associated with it Figure 5. The yield of radioactivity in this peak is 85%, implying that it is the only significant radiolabeled product. The UV absorption spectrum of this peak is shown in Figure 6. Two broad maxima are visible, one at 280 nm with a shoulder at 300 nm and one at 370 nm. Under the same chromatographic conditions, control HPLC runs indicated that FMN and FAD would both be expected to elute after 5 min. Thus, the radiolabeled product is more hydrophobic than either flavin cofactor. The radiolabeled peak was collected, desalted into aqueous methanol by reversed phase HPLC, and subjected to FAB mass spectrometry. The mass spectrum obtained for the radiolabelled product is shown in Figure 7. A molecular ion (MH^+) with m/e = 535 is clearly visible along with a significant peak at m/e = 537. The large peak at m/e = 369 is a matrix peak.

(b) Identification of the Site of Protein Modification. The radiolabeled precipitated protein was dissolved in Tris (1 M) and saturated guanidinium hydrochloride. The solubilized protein was S-carboxymethylated with iodoacetic acid. Ex-

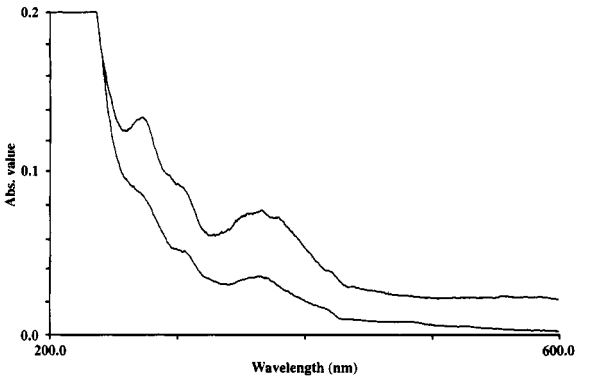

FIGURE 6: (Lower trace) UV absorbance spectrum of the radiolabeled peak isolated by HPLC from the TCA-soluble fraction after inactivation of cytochrome P_{450} reductase. (Upper trace) UV absorbance spectrum for the flavin adduct prepared according to the method of O'Donnell et al. (1993). Both spectra were recorded in a 100-μL microcell.

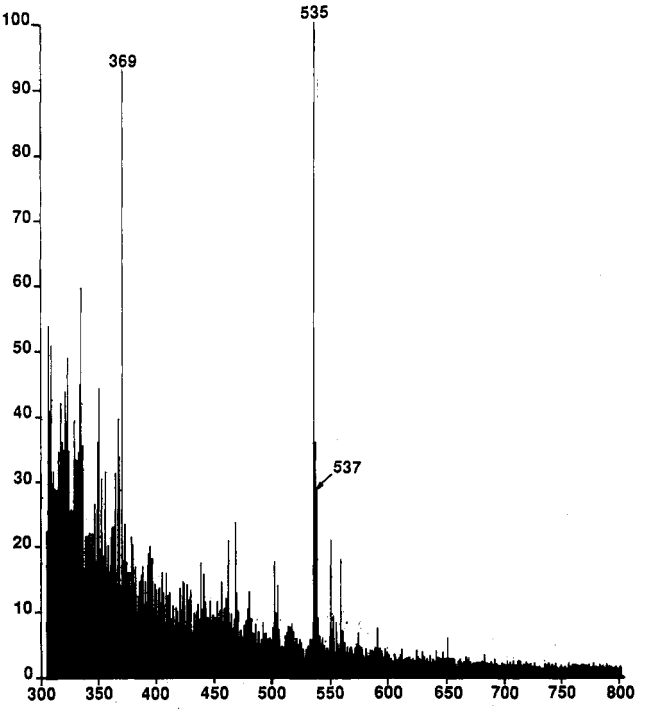

FIGURE 7: Positive ion FAB mass spectrum of the radiolabeled flavin adduct. This spectrum is not background corrected. The large peak at m/e = 369 is a matrix peak. In addition to M + H at m/e = 535 and 537, an M + Na peak is visible at m/e = 557 along with a number of unassigned low-abundance ions at m/e > 550.

tensive dialysis of the S-carboxymethylated protein to remove guanidinium hydrochloride resulted in no significant loss of radiolabel from the protein, thus demonstrating the covalent nature of the modification. Treatment of the radiolabeled protein with trypsin followed by reversed-phase HPLC gave the chromatogram shown in Figure 8. The most obvious feature of this chromatogram is that the radioactivity seems to be distributed over many of the peptides. However, superimposed upon this apparent nonspecific labeling are a number of small sharp peaks and one large sharp peak. Five peaks were collected as indicated and submitted to rechromatography. Rechromatography of peaks 1 and 3–5 indicated that the radioactivity was not significantly associated with any single peptide but was related to the total amount of peptide in a given fraction. These peaks were considered to be nonspecifically labelled and not analyzed further. Peak 2 contained 30% of the peptide bound radiolabel. This corre-

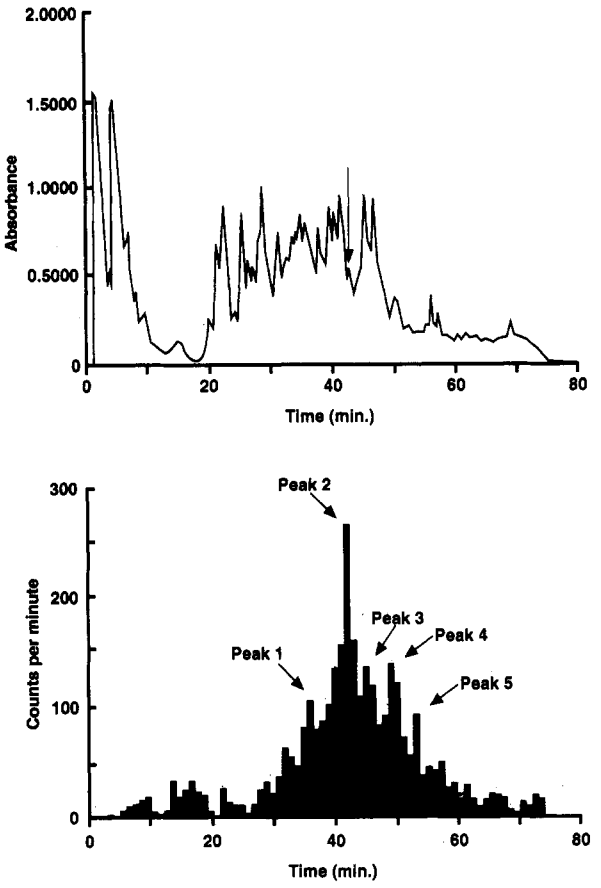

FIGURE 8: Reversed-phase HPLC profile of a tryptic digest of S-carboxymethylated cytochrome P_{450} reductase which had been inactivated with ^{14}C-labeled diphenyliodonium. The upper trace shows the absorbance at 214 nm, and the lower histogram shows the number of counts per minute for 1-min fractions (0.2 mL); 20-μL aliquots were counted. A gradient from 100% water to 70% acetonitrile/30% water over 70 min was used. Both the acetonitrile and the water contained 0.075% TFA.

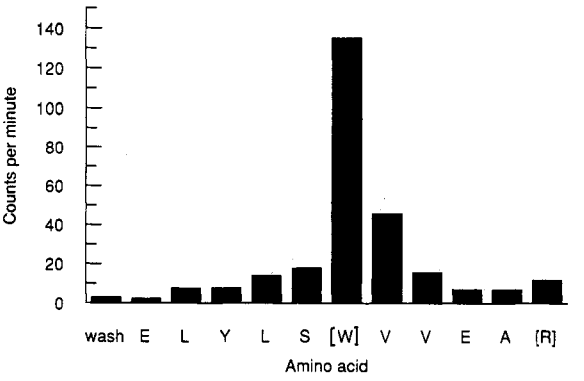

FIGURE 9: Bar chart showing the result of scintillation counting approximately two-thirds of each amino acid PTH derivative released by automated Edman degradation of the peptide isolated from peak 2. The amino acid corresponding to each cycle is indicated. Arg 424 was not identified but is added as the C-terminal amino acid by inference from the known sequence.

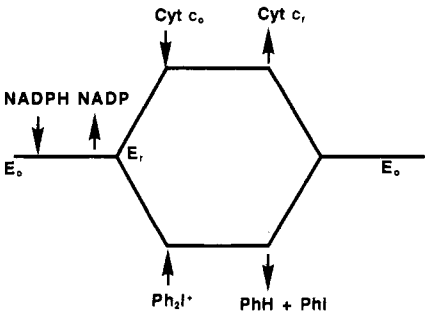

FIGURE 10: Simplified kinetic scheme in which diphenyliodonium and cytochrome *c* are considered to be alternative substrates acting on reduced cytochrome P_{450} reductase. Subscripts "o" and "r" indicate oxidized and reduced forms of enzyme, respectively.

sponds to approximately 0.25 mol of radiolabel. Rechromatography of peak 2 showed that the radioactivity appeared to be associated with one peptide. This peptide is indicated in the HPLC trace. The radiolabelled peptide was collected, desalted, and subjected to automated Edman degradation. Figure 9 shows the number of counts retrieved from the sequencer and the corresponding amino acid found in the peptide. More than 90% of the radiolabel is released during the sixth cycle of Edman degradation. No amino acid is seen at this point. The sequence obtained from the rest of the peptide allows unequivocal identification of the site of modification as Trp 419.

DISCUSSION

The results presented here indicate that the diphenyliodonium cation is a time-dependent inhibitor of cytochrome P_{450} reductase. Despite the obvious dissimilarity between the endogenous substrate for cytochrome P_{450} reductase, cytochrome P_{450}, and the diphenyliodonium cation, it appears that this inhibitor fulfills all of the usual criteria associated with mechanism-based inactivators: loss of enzyme activity is pseudo-first-order, exhibits saturation kinetics, and is directly proportional to low inhibitor concentrations. Irreversibility of the inhibition is confirmed both by size exclusion chromatography of the inactivated enzyme, which does not result in any significant reactivation, and by dilution experiments, where inactivation of small concentrated samples of enzyme (as used for the determination of partition ratio) is not reversed upon dilution.

The rate of enzyme inactivation, k_{obs}, at a given inhibitor concentration was obtained from progress curves according to the method of Kitz and Wilson (1962). However, to eliminate any effects due to incomplete inactivation, a linear term, *at*, was added to this equation. This was found to be necessary as a residual slow reduction rate was often apparent even after long incubations at high inhibitor concentrations. Without this linear correction, the fitted curve showed small but significant deviations from the data. Thus, the final equation used was

$$[\text{product}] = V_0(1 - \exp(-k_{obs}t))/k_{obs} + at$$

The kinetic mechanism for the P_{450} reductase catalyzed reduction of cytochrome *c* by NADPH has been shown to follow a Hexa Uni Ping Pong scheme (Kominami et al., 1982). Three different substrates are involved in this general mechanistic scheme giving rise to three different products. All three substrates and all three products are kinetically distinguishable. Cytochrome P_{450} reductase is a special example of this mechanistic class because two of the substrates (the first and the second molecule of cytochrome *c*) and their corresponding products are identical. We cannot, therefore, distinguish between the molecule of cytochrome *c* which binds first to the reduced enzyme and that which binds second. If a full kinetic scheme is drawn up for the inhibition of cytochrome P_{450} reductase by the diphenyliodonium cation and the site of inhibition is assumed to be only one of the reduced enzyme intermediates, then it is not possible to arrive at a simplified rate equation that allows determination of K_i

for the inhibitor. Consequently, we are forced to consider a simplified kinetic scheme in which the two cytochrome *c* requiring steps are treated as one and the different reduced forms of the enzyme are equivalent. This effectively reduces the kinetic model to a Ping Pong Bi Bi system (Segel, 1975). Cytochrome *c* and diphenyliodonium chloride may then be considered to be alternative substrates for the reduced enzyme Figure 10. The rate of inactivation of the enzyme, k_{in}, is directly proportional to the rate of turnover of diphenyliodonium and so can then be described by the equation (Segel, 1975)

$$k_{obs} = k_{in}[\text{NADPH}][Ph_2I^+]/\{K_{\text{NADPH}}([Ph_2I^+] + K_{Ph_2I^+}[\text{Cyt } c]/K_{\text{Cyt } c}) + [\text{NADPH}]([Ph_2I^+] + K_{Ph_2I^+}(1 + [\text{Cyt } c]/K_{\text{Cyt } c}))\}$$

K_m values for NADPH and cytochrome *c* are found to be unchanged in the presence of diphenyliodonium chloride.

As is shown by Figure 4, cytochrome *c* protects the enzyme against inactivation by acting as a competitive inhibitor. This is as predicted by the kinetic scheme in Figure 10. However, this tells us nothing concerning the site of action of diphenyliodonium.

Figure 3 shows that NADPH is qualitatively uncompetitive with respect to inactivation by the diphenyliodonium cation. NADPH will only be purely uncompetitive when the partition ratio becomes unity, and so the diphenyliodonium cation acts as a dead-end inhibitor. Strictly, while turnover of inhibitor regenerates active enzyme, NADPH will always show mixed kinetics with respect to the diphenyliodonium cation (Segel, 1975). If the diphenyliodonium cation inhibits by interacting with reduced enzyme, then we would indeed expect a qualitatively uncompetitive effect upon varying NADPH.

When cytochrome P_{450} reductase is inactivated by diphenyliodonium chloride, the characteristic yellow chromophore due to the flavin cofactors is irreversibly lost. Removal of NADPH and diphenyliodonium by size exclusion chromatography does not result in regeneration of oxidized flavin either associated with the protein or in the low molecular weight fractions as judged by the absorbance spectrum between 300 and 600 nm. The loss of this chromophore implies either that the isoalloxazine moiety is destroyed or that it is trapped in a reduced form.

Inhibition of the enzyme with radiolabeled diphenyliodonium chloride has allowed isolation of both radiolabelled protein and a radiolabeled TCA-soluble fraction. HPLC of the TCA-soluble fraction indicates that only one significant radiolabeled product is present. Mass spectrometry of this product (Figure 7) results in an ion with m/e = 535 being observed. FMN (empirical formula $C_{17}H_{21}N_4O_9P$) has a mass of 456 for the oxidized free acid form. Under the conditions employed for the mass spectrum, we would therefore expect to see an ion with m/e = 457 for FMN corresponding to the protonated molecular ion. This is 78 less than the ion observed. If inhibition by diphenyliodonium chloride were to result in the addition of a phenyl group (C_6H_5, mass = 77) and a proton to FMN, then this would result in a product of the required molecular weight, 534 (although the product is isolated from NH_4OAc containing buffer and is presumably an ammonium salt, the mass spectrum corresponds to the free acid as ammonium salts are not seen as such under the conditions used here). The ^{14}C label has an abundance of 18% in a single phenyl group. Thus, if the mass spectrum is truly that of a phenyl adduct, then a molecular ion of corresponding increased mass should be seen. A peak at m/e = 537, 2 mass units higher than the unlabeled molecular ion, is clearly visible in Figure 7. The ratio of the 535 peak to the 537 peak is approximately 4 to 1, which agrees well with the degree of ^{14}C enrichment.

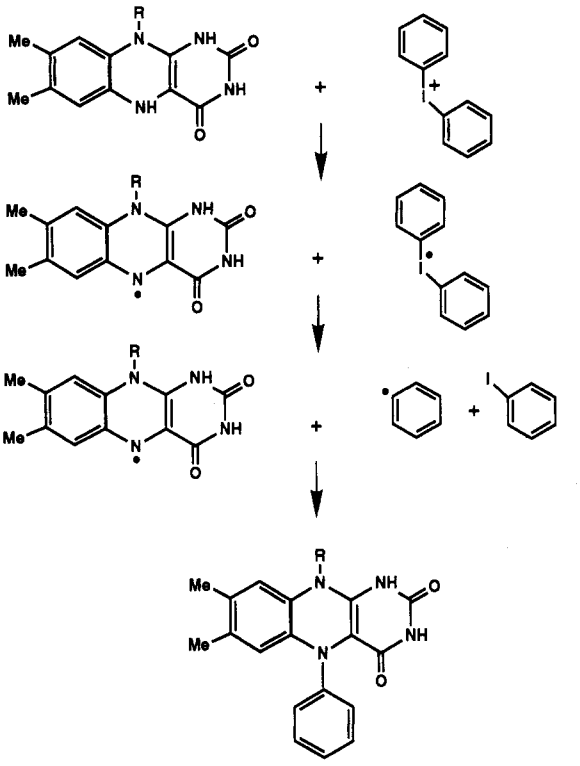

FIGURE 11: Proposed mechanism of inactivation and covalent modification of FMN. R = ribityl 5′-phosphate. Only an N_5 adduct is chown for simplicity.

The UV spectrum shown in Figure 6 shows only a weak absorption maximum at ca. 370 nm. This indicates that the isolated FMN adduct is effectively trapped in a reduced form and is consistent with the loss of the yellow flavin chromophore upon inactivation. Given the change in the UV spectrum of the FMN adduct and the requirement to add both a phenyl group and a proton to the parent FMN molecule, it is only reasonable to suggest that the phenyl group adds to one of the following four positions: N_1, N_5, C_{4a}, or C_{10a}. Addition at other positions would not eliminate the flavin chromophore and only give rise to a mass increase of 76 by substituting a proton with a phenyl group. The C_{4a} and the N_5 positions are the most likely sites for modification (Silverman & Zieske, 1984). The UV maxima for C_{4a} and N_5 adducts of flavins have been reported to be in the ranges 360–370 and 296–355 nm, respectively (Maycock et al., 1976). A phenyl adduct of FMN has been isolated from the reaction of photoreduced FMN with diphenyliodonium chloride and assigned the stucture of either a C_{4a} or N_5 adduct on the basis of UV, NMR, and mass spectrometry data (O'Donnell et al., 1993). The UV spectrum observed for this flavin adduct is also shown in Figure 6 for comparison. The two UV spectra are cleary very similar indeed. Thus, although unequivocal identification of the flavin adduct is not possible, the UV data support either a C_{4a} or a N_5 adduct which is identical to that formed by the reaction of reduced FMN with diphenyliodonium chloride (O'Donnell et al., 1993).

The kinetic experiments indicate that the diphenyliodonium cation interacts only with the reduced enzyme. Therefore, starting with a fully reduced FMN and diphenyliodonium, I propose the mechanism shown in Figure 11. Reduced flavin transfers one electron to diphenyliodonium. This gives a flavin

semiquinone and a neutral diphenyliodyl radical species. This diphenyliodyl radical fragments rapidly to give iodobenzene and a phenyl radical. It is known that aryl iodonium cations react via free-radical mechanisms (Banks, 1966; Hampton et al., 1964). The phenyl radical can now partake in a radical recombination with the flavin semiquinone, the net result of which is to have added C_6H_6 across one of the isoalloxazine double bonds. The observed partition ratio of 21 is consistent with this mechanism.

Only 0.6 mol of FMN adduct is recovered. This does not, by itself, account for the complete inhibition seen. It is possible that the modification of the protein at Trp 419 results in inactivation. This would account for a further 0.25 mol of inhibited enzyme. However, it is not known whether Trp 419 has a functional role in catalysis so this is, at best, speculative. Another possibility is that the remaining 0.4 mol of inhibited enzyme is due to FAD modification or destruction. No FAD is recovered despite the fact that the conditions used allow recovery of both FAD and FMN from native protein. Examination of the UV spectrum of all fractions obtained after size exclusion chromatography of the inactivated enzyme indicates that native FAD has not simply dissociated. Thus, the only conclusion I can come to concerning the FAD is that either it has been destroyed or it is trapped in a reduced form in such a way that it is not obviously detectable in the experiments performed here. Further work is required to determine the fate of the FAD cofactor.

Recovery of radiolabeled polypeptide yields the tryptic map shown in Figure 8. A high degree of nonspecific labeling is evident. This is in accord with the proposed generation of phenyl radicals. Sequencing of the labeled peptide with the highest specific radioactivity unequivocally identified Trp 419 as the covalently modified amino acid by comparison of the deduced sequence with the published sequence for rat cytochrome P_{450} reductase (Porter & Kasper, 1985). This indicates that the diphenyliodonium cation binds at, or very close to, Trp 419.

In conclusion, I have shown that the diphenyliodonium cation is a time-dependent irreversible inhibitor of cytochrome P_{450} reductase. Inhibition requires reduction of the enzyme. The electron acceptor, cytochrome *c*, acts as a competitor to the inhibition reaction in accord with the proposed mechanism. I have demonstrated covalent modification of both the flavin and the polypeptide. Isolation of the radiolabeled flavin adduct has allowed unequivocal identification of this as a phenylated FMN moiety. Trypsin cleavage of radiolabeled S-carboxymethylated polypeptide has allowed a single covalently modified peptide to be isolated. Automated Edman degradation has shown the site of attachment to be Trp 419.

ACKNOWLEDGMENT

I thank Dr. K. Lawrie for synthesis of ^{14}C-labeled diphenyliodonium chloride, Dr. N. Haskins for performing FAB mass spectrometry, and Dr. C. Southan for assistance with the peptide sequencing.

REFERENCES

Banks, D. F. (1966) *Chem. Rev. 66*, 243–266.
Blohm, T. R., Metcalf, B. W., Laughlin, M. E., Sjoerdsma, A., & Schatzman, G. L. (1980) *Biochem. Biophys. Res. Commun. 95*, 273–280.
Bolkenius, F. N., & Seiler, N. (1989) in *The Design of Enzyme Inhibitors as Drugs* (Sandler, M., & Smith, H. J., Eds.) pp 245–256, Oxford University Press, Oxford, U.K.
Cross, A. R., & Jones, O. T. G. (1986) *Biochem. J. 237*, 111–116.
Gatley, S. J., & Sherratt, H. S. A. (1974) *Biochem. Soc. Trans. 2*, 517–519.
Goodhart, P. J., DeWolf, W. E., Jr., & Kruse, L. I. (1987) *Biochemistry 26*, 2576–2583.
Hampton, K. G., Harris, T. M., & Hauser, C. H. (1964) *J. Org. Chem. 29*, 3511–3514.
Hancock, J. T., & Jones, O. T. G. (1987) *Biochem. J. 242*, 103–107.
Holland, P. C., Clark, M. G., Bloxham, D. P., & Lardy, H. A. (1973) *J. Biol. Chem. 248*, 6050–6056.
Iyanagi, T., & Mason, H. S. (1973) *Biochemistry 12*, 2297–2308.
Kitz, R., & Wilson, I. B. (1962) *J. Biol. Chem. 237*, 3245–3249.
Kominami, S., Ogishima, T., & Takemori, S. (1982) in *Flavins and Flavoproteins* (Massey, V., & Williams, C. H., Eds.), pp 715–718, Elsevier, New York.
Leatherbarrow, R. J. (1992) *Grafit*, version 3.0, Erithacus Software Ltd., Staines, U.K.
Maycock, A., Abeles, R., Salach, J., & Singer, T. (1976) *Biochemistry 15*, 114–125.
McDonald, I. A., Bey, P., & Palfreyman, M. G. (1989) in *The Design of Enzyme Inhibitors as Drugs* (Sandler, M., & Smith, H. J., Eds.) pp 227–244, Oxford University Press, Oxford, U.K.
O'Donnell, V. B., Tew, D. G., Jones, O. T. G., & England, P. J. M. (1993) *Biochem. J. 290*, 41–49.
Ortiz de Montellano, P. R. (1987) *Acc. Chem. Res. 20*, 289–294.
Porter, T. D., & Kasper, C. B. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 973–977.
Porter, T. D., & Kasper, C. B. (1986) *Biochemistry 25*, 1682–1687.
Ragan, C. I. & Bloxham, D. P. (1977) *Biochem. J. 163*, 605–615.
Schenckman, J. B., & Cinti, D. L. (1972) *Methods Enzymol. 52*, 83–89.
Segel, I. H. (1975) *Enzyme Kinetics*, Wiley, New York.
Shepard, E. A., Pike, S. P., Rabin, B. R., & Philips, I. R. (1983) *Anal. Biochem. 129*, 430–433.
Silverman, R. B., & Zeiske, P. A. (1984) *Biochemistry 23*, 2128–2138.
Stuehr, D. J., Olufunmilayo, A. F., Kwon, N. S., Gross, S. S., Gonzalez, J. A., Levi, R., & Nathan, C. F. (1991) *FASEB J. 5*, 98–103.
Vermilion, J. L., Ballou, D. P., Massey, V., & Coon, M. J. (1981) *J. Biol. Chem. 256*, 266–277.
Yamada, Y., & Okawara, M. (1972) *Bull. Chem. Soc. Jpn. 45*, 2515–2519.
Yasukochi, Y., & Masters, B. S. S. (1976) *J. Biol. Chem. 251*, 5337–5344.