Specific Covalent Labeling of Cytochrome P-450\textsubscript{CAM} with 1-(4-Azidophenyl)imidazole, an Inhibitor-derived Photoaffinity Probe for P-450 Heme Proteins*  

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A generally applicable photoaffinity labeling procedure for the active site of P-450 heme proteins has been developed using 1-(4-azidophenyl)imidazole (API), a photolabile analogue of the common inhibitor N-phenylimidazole. The binding of API to P-450\textsubscript{CAM} (\(K_D \approx 1 \mu M\)) elicited a type II spectral shift of the Soret band. Irradiation of the protein-ligand complex at 313 nm caused specific covalent binding. Under similar conditions affinity labeling of pancreatic ribonuclease was negligible. Reconstitution experiments demonstrated that API attachment was accompanied by inhibition of camphor hydroxylation.

At cytochrome P-450\textsubscript{CAM} concentrations up to 10 nM and API concentrations up to a 9-fold molar excess of label over protein, covalent binding increased linearly with label concentration until saturation of a single site was reached at 114% incorporation of tritiated label. More concentrated protein solutions (40 to 50 \(\mu M\)), however, engendered gradual specific labeling at a second discrete site. In these experiments covalent incorporation of API substantially in excess of 100% was observed. The mono- and dilabeled protein derivatives were readily resolved by gel electrofocusing (pl 5.2 and 5.8).

A small heme-bearing fragment was isolated by Sephadex G-75 chromatography (\(M_r \approx 5.0 \times 10^3\)) from API-labeled cytochrome P-450\textsubscript{CAM} after cyanogen bromide degradation. This heme peptide fraction contained \(\sim 80\%\) of the heme of cytochrome P-450\textsubscript{CAM} and \(\sim 70\%\) of the incorporated label; its Soret maximum was at 356 nm in contrast to dissociated heme (390 nm). No significant amount of heme or label was associated with any other BrCN fragment. The resolved mono- and dilabeled heme peptides (pl 4.2 and 4.8) contained all radiolabel associated with the heme peptide fraction.

Thus, photoaffinity labeling of cytochrome P-450\textsubscript{CAM} occurred almost exclusively in the close vicinity of the heme. The binding sites of cytochrome P-450\textsubscript{CAM} for the heme group and both labels are encompassed in a tightly structured domain which can be released in the form of a small heme peptide by selective chemical cleavage.

Attempts to understand the mechanism of monooxygenase reactions have focused on the interaction of the terminal oxidase component, cytochrome P-450, with dioxygen, substrates, or inhibitors. Binding of substrates or inhibitors to P-450 heme proteins has received special attention because it is usually accompanied by a pronounced spectral shift of the Soret maximum suggesting a conformational change in the heme-ligand arrangement (1, 2). Depending on their direction to lower or higher wavelengths, these have been classified into essentially three groups known as type I, associated with a low to high spin conversion of the heme iron, type II, and modified type II (equivalent to reversed type I) spectral changes (3, 4). But compounds eliciting a similar type of spectral response in liver microsomal P-450 heme proteins may have quite unrelated chemical structures. Even with cytochrome P-450\textsubscript{CAM} of the bacterial camphor hydroxylase (5) which shows much higher substrate specificity than the microsomal P-450 heme proteins, it is difficult to rationalize the spectral responses elicited by ligand binding. For instance, binding of the substrate, \(\alpha\)-camphor, causes a strong type I shift while binding of the product, hydroxycamphor, occurs without concomitant spectral change (6). Moreover, chemical modifications of certain hydrophilic amino acid side chains by alkylation can cause shifts reminiscent of substrate binding (7).

Among the more promising current efforts to scrutinize the basic effect leading to the spectral manifestations are kinetic and static studies at subzero temperatures (8), correlations of the spin state of the P-450 heme iron with the electronic configuration of the ligand molecule (9), and studies in model systems (10, 11). In the absence of data on the chemical composition and the exact location of the binding site for a given substrate or inhibitor eliciting a pronounced spectral shift, however, it would appear that our understanding of the nature and size of the effect will remain quite limited because the heme and substrate binding domain may be expected to employ unique structural features for this purpose. At present there is no assurance that a distinct spectral shift upon binding really implies closeness of the ligand to the heme.

The present communication describes synthesis and application of 1-(4-azidophenyl)imidazole a simple, photolabile derivative of the general P-450 inhibitor, N-phenylimidazole, which due to minimal structural alterations still binds with high affinity and specificity to several P-450 heme proteins which have been available for testing (12, 13). This reagent...

1 R. A. Swanson, R. I. Murray, P. J. Hippenmeyer, J. A. Bumpus, and K. M. Dus, manuscript in preparation.
should be applicable to photoaffinity labeling of a large number of functionally different P-450 heme proteins. Thus use of this reagent to determine the location and chemical nature of the inhibitor binding site by means of photoaffinity attachment should open a new avenue for comparative structural studies of P-450 ligand binding sites.

This paper outlines the rationale of the photoaffinity approach to active site studies and gives the details of a simple photoaffinity labeling procedure applied to P-450CAM of the camphor 5-endo-methoxy hydroxylase of Pseudomonas putida (14, 15) which still is the most specific and best characterized P-450 heme protein available today. Partial accounts of certain aspects of this research have been communicated previously (12, 16, 17).

EXPERIMENTAL PROCEDURES

Materials

Homogeneous preparations of cytochrome P-450, putidaredoxin, and putidaredoxin reductase were gifts from Dr. I. C. Gunsalus, University of Illinois at Champaign-Urbana. Bovine pancreatic ribonuclease (3 × crystallized) was purchased from Worthington Biochemical Corp.

Platinum oxide was purchased from Ventron Corp., and p-chloronitrobenzene, imidazole, and cyanogen bromide were from Eastman Organic Chemicals. NADH, grade III, was obtained from Sigma Chemical Co. All other reagents and solvents were purchased from Fisher Scientific and were certified ACS grade except solvents for high performance liquid chromatography which were HPLC grade.

Organic Syntheses

The 3-step synthesis of API is outlined in Fig. 1. 1-(4-Nitrophenylimidazole) (1)—Following the method of Khan and Polya (18), a mixture of p-chloronitrobenzene (23.6 g, 0.15 mol), imidazole (10.2 g, 0.15 mol), anhydrous K$_2$CO$_3$ (15 g), and CuO (0.5 g) were refluxed in anhydrous pyridine (90 ml) for 10.5 h. The cooled reaction mixture was filtered and the residue was extracted with benzene. The combined extracts were dried (Na$_2$SO$_4$), the solvent was removed in vacuo, and the residue was recrystallized from benzene affording 10.7 g (59%) of amber needles, m.p. 203-205°C (literature (19) 204-205°C): ir (nujol mull) 3125, 1605, 1558, 1525, 1350, 1313, 855; pmr [H$_3$]Me$_2$SO 88.40 (s, 1H), 8.25 (m, 2H), 7.90 (m, 2H), 7.83 (s, 1H), 7.12 (s, 1H).

1-(4-Aminophenyl)imidazole (2)—To a suspension of 1 (1.0 g, 5.3 mmol) in 95% ethanol (20 ml) was added platinum oxide (20 mg) and acidified with H$_2$SO$_4$ (1.05 ml) was slowly added dropwise with stirring. Stirring was continued at 0°C for 1 h, then the temperature was allowed to rise to 23°C for 4 h. The reaction mixture was diluted with water (20 ml), filtered, and the residue was extracted with benzene. The combined extracts were dried (Na$_2$CO$_3$) and concentrated in vacuo. The residue was recrystallized from benzene to afford a brown oily residue which was purified by preparative silica gel thin layer chromatography (25:74:1) as eluting solvent.

1-(4-Aminophenylimidazole) (3)—Using a modification of the procedure of Kornblum and Ifland (20) the amine 2 (100 mg, 0.53 mmol) was dissolved in 36% hydrochloric acid (0.26 ml, 3.36 mmol) and water (0.57 ml). The stirred solution was cooled to 0°C and NaNO$_2$ (46 mg, 0.66 mmol) in water (0.16 ml) was added slowly. Stirring was continued at 0°C for 1 h, then the temperature was allowed to rise to 23°C for 4 h. The reaction mixture was diluted with water (20 ml), neutralized with NaOH, and extracted with ethyl acetate. The combined extracts were dried (Na$_2$SO$_4$) and concentrated in vacuo to afford a brown oily residue which was purified by preparative silica gel thin layer chromatography (2 mm × 20 cm × 20 cm) employing ethyl acetate. Yield was 0.204 g (88%) of yellow oil. Pircrate mp 152-153°C (literature (21) 155-156°C).

Methods

Melting points were taken in a Fisher-Johns melting point apparatus and are uncorrected. Microanalyses were performed on a Hewlett-Packard 185B CHN Analyzer at the University of Kansas. Proton magnetic resonance spectra were obtained on a Varian Associates A-60 Spectrometer with tetramethylsilane as the internal standard. Infrared data were recorded using a Perkin-Elmer Model 21 spectrophotometer while UV/VIS data were obtained on an Ammico DW-2 spectrophotometer.

Thin layer chromatography was done on glass plates coated with Silica Gel G (Birckmann). Scintillation counting was carried out with a Packard Tri-Carb Model 3380 counter using 10 ml of ACS solution (Amersham), and thin layer plates were scanned for radioactivity on a Packard Radi chromatogram Scanner Model 7201. High performance liquid chromatography was performed on a Waters Associates Model ALC/GPC-204 instrument equipped with a Model 545 variable wavelength UV detector and a $\mu$Bondapak C$_18$ column (4 mm × 30 cm).

Unless otherwise stated all experiments with cytochrome P-450CAM were carried out in 50 mM potassium phosphate, pH 7.5, which will be referred to as buffer throughout the text.
Binding Assay—The spectral dissociation constant, \( K_d \), for ligand binding to cytochrome P-450\(_{\text{CAM}}\) was determined by difference spectrophotometry at room temperature. In a typical experiment 1.0 ml of a 2.5 \( \mu \)M cytochrome P-450\(_{\text{CAM}}\) solution in buffer was placed in each of two 1-ml (1-cm path length) quartz cuvettes and a base-line of equal light absorbance was recorded. After each consecutive addition of an ethanolic API solution, and an equal volume of ethanol, to the sample and reference cuvettes, respectively, the difference spectrum was recorded. A plot of the reciprocal value of the peak to trough absorbance change (\( \Delta A \)) as a function of the reciprocal of the API concentration yielded a straight line which was extrapolated to the abscissa to determine the \( K_d \).

Photolysis Apparatus—Photolysis experiments were performed at 313 nm employing the apparatus shown in Fig. 2. A polished aluminum reflecting shield was used to direct the emission of a 450 W Hanovia medium pressure quartz-mercury lamp (Ace Glass, Vineland, N. J.) upon an aluminum cuvette housing placed 7 cm from the lamp. The solution to be photolyzed was placed, for preparative experiments, in a 3-ml stirred quartz cuvette (1-cm path length) located in position 3 (Fig. 2) of the cuvette housing; for analytical experiments, a 0.3-ml unstirred quartz cuvette (1-mm path length) was used. The temperature of the photolyzed solution was maintained at 4°C by circulating ice water (through the cuvette housing), and the wavelength of photolyzing radiation was restricted to 313 nm by ensheathing the light source in a tubular Pyrex filter and by placing filter solutions in quartz cuvettes located at positions 1 and 2 in the cuvette housing. Cuvette 1 (4-cm path length) contained a solution 1.38 M in nickel sulfate and 0.37 M in cobalt sulfate and cuvette 2 (1-cm path length) contained a 0.024 M potassium hydrogen phthalate solution. A shutter placed immediately before the cuvette housing allowed timed photolyses to be begun after an initial lamp warm up period (10 to 15 min).

Photodecomposition of API—The rate of photolysis of API at 4°C in the preparative apparatus described above was determined by irradiating a 100 \( \mu \)M API solution in buffer. Photolysis was stopped at time intervals for removal of triplicate aliquots for analysis. The amount of unphotolyzed API in each aliquot was determined by high performance liquid chromatography on a ,aBondapak C\(_18\) column employing methanol/water/acetic acid (25:75:1) as the mobile phase and at least a 100-fold excess of reagent. After completion, the digest was diluted with 20 volumes of distilled water and lyophilized several times in the dark until no more odor was detectable.

Polycrylamide Gel Isoelectric Focusing of Photoaffinity-Labeled Cytochrome P-450\(_{\text{CAM}}\) and Its BrCN-Derived Heme Peptide—After removal of excess API, the mixture of unlabeled, mono-, and dibenzylated P-450\(_{\text{CAM}}\) was electrophoresed in polyacrylamide gel following the procedure outlined by Warner et al. (24) which is, in turn, based on the method of Wrigley (25). Gels of 9 cm in length and 5.6% acrylamide containing 2.0% (v/v) ampholytes of the pH range 3.5 to 10.0 (LKB Produkter) were prepared and 0.5 to 1.0 nmol of heme protein mixture and riboflavin were added before photopolymerization (30 min). PAGIF was carried out at 4°C for 18 to 20 h with 1 mM of initial current/tube. The gels were then washed for 8 h with 5% (w/v) trichloroacetic acid, replaced hourly. Using a modification of the method of Goodman and Matsura (26), the gels were cut into 1.5-mm slices and each slice was allowed to stand in a tightly capped (polyethylene liner) 20-ml glass scintillation vial with 0.5 ml of 30% \( \text{H}_2\text{O}_2/\text{concentrated NH}_3\text{OH} \) (9:1). After the gels had dissolved (1 to 3 days at 23°C in the dark), 10 ml of liquid scintillation mixture was added and counted and counted at 3290 and 4200 channel units.

The conditions applied for PAGIF of the BrCN-derived heme-bearing fragment, resolved by Sephadex G-75 chromatography, were the same as described for the heme protein and its API derivatives.

RESULTS

API was synthesized by a 3-step procedure in 28% overall yield from imidazole and \( p \)-chloronitrobenzene as outlined in Fig. 1. Although this procedure provides only a modest yield, it is simply executed and generally applicable to other derivatives of potential interest.

Difference spectroscopy of the binding of API to cytochrome P-450\(_{\text{CAM}}\) exhibits a type II spectral shift with a characteristic peak at 431 nm and a trough at 411 nm. The total peak to trough change (\( \Delta A \)) was measured as a function of the concentration of API in the sample cuvette from 0.17 to 1.88 \( \mu \)M (Fig. 3, upper inset). The spectral dissociation constant, \( K_d \), obtained from the double reciprocal plot of these data (Fig. 3, lower inset) was found to be 1 \( \mu \)M (1.07 pm). This value is similar in magnitude to that for camphor, 1 to 5 \( \mu \)M (5, 6), and less favorable than that for N-phenylimidazole, 0.1 to 0.6 \( \mu \)M. Since API was added to the protein solution dissolved in ethanol, it was determined that this solvent does not contribute to the spectral changes (data not shown).

Taking advantage of a window of minor absorption of
cytochrome P-450CAM between the aromatic protein bands and the Soret band of the heme group, filters were used to select 313 nm as the compromise wavelength at which API was still readily photolyzed while the heme protein sustained only minor photodamage when maintained at 4°C.

In the photolysis apparatus in Fig. 2, photodecomposition of API to the corresponding nitrene (27) ensued upon irradiation with light of 313 nm (details under "Methods") with a first order rate constant of 0.459 h⁻¹ (τ₁/₂ = 1.51 h). These kinetic parameters were determined in the absence of cytochrome P-450CAM. The intensity of light available for activation of API is somewhat reduced in a photoaffinity labeling experiment since P-450CAM at 4°C and the Soret band of the heme group, filters were used to select 313 nm as the compromise wavelength at which API was still readily photolyzed while the heme protein sustained only minor photodamage when maintained at 4°C.

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The dependence of covalent labeling of P-450cAM with API on label concentration is examined in Fig. 5 using a 10 μM protein solution. Photocovalent labeling increased linearly with label concentration until 114% incorporation had occurred. Since increase of label concentration beyond a 9-fold molar excess over protein concentration did not cause significant increases in total covalent label attachment, this saturation curve is consistent with the interpretation that essentially a single site was labeled. The amount of label incorporated in excess of 100% may be indicative of the extent of labeling which occurred at other sites. In order to determine how much of the bound label resides at a single location, the API derivative it can be expected that labeling with API will produce new protein species clearly separable from unlabeled protein which occurred at other sites. In order to determine how much of the bound label resides at a single location, the API derivative it can be expected that labeling with API will produce new protein species clearly separable from unlabeled protein due to a difference in \( \pi \).

Thus the API derivatives of P-450cAM were resolved by gel electrofocusing. The extent of incorporation of P-450cAM resulting from photolysis at various label concentrations were investigated by gel electrofocusing and by reconstitution experiments. Since the strongly basic group contributed by the label will alter the PI of the protein which occurred at other sites. In order to determine how much of the bound label resides at a single location, the API derivative it can be expected that labeling with API will produce new protein species clearly separable from unlabeled protein due to a difference in \( \pi \).

As shown by the examples given in Fig. 6 (A and B), API incorporations substantially in excess of 100% were, however, observed when e.g. 40 or 100 μM P-450cAM solutions were photolyzed in the presence of 9-fold molar excess of photoaffinity label. Values of \( \pi \) 5.2 and 5.8 were measured for the mono- and dilabeled API-derivative of P-450cAM, respectively. These results suggest that the second molecule of API incorporated per molecule of P-450cAM is at a specific binding site rather than resulting from adventitious labeling of several nonspecific sites. If labeling were occurring at several nonspecific sites of approximately equal affinity for API, one might logically expect to observe in PAGIF, a statistically determined distribution of protein molecules containing 2, 3, and 4, etc. API molecules/molecule of P-450cAM. No derivatives containing more than 2 molecules of API/molecule of P-450cAM have been observed. Only three protein bands are present after PAGIF of photolysis mixtures resulting from preparative labeling experiments. The band of unlabeled protein is getting progressively smaller while increased amounts of dilabeled derivative are found with increases in protein and API concentrations beyond the analytical range.

The amount of unlabeled protein was assessed by measuring only absorption at 600 nm.

Recistitution of camphor hydroxylation with API-labeled P-450cAM was carried out according to the procedure of Gunsalus and Wagner (15). When prephotolyzed P-450cAM (5 h in the absence of API) was employed in the reconstitution experiments, a 40% reduction in the rate of camphor hydroxylation was observed. Although these results indicate a photoinactivation of P-450cAM which is independent of photoaffinity labeling, a further decrease in the rate of hydroxylation, which is attributable to specific covalent labeling, was observed when API-labeled P-450cAM was employed in the reconstitution. During the irradiation of P-450cAM in the presence of API, photoinactivation and photocovalent labeling are occurring.

**Fig. 5.** Dependence of photoaffinity labeling of cytochrome P-450cAM on API concentration. Photoaffinity labeling was performed in the photolysis apparatus as described in detail under "Methods" on cytochrome P-450cAM (3 nmol, 10 μM) and API in 3, 6, 9, or 12-fold molar excess.

**Fig. 6.** Polyacrylamide gel isoelectric focusing of API-labeled cytochrome P-450cAM. Electrofocusing in gels (9 cm, 5.5% acrylamide, 2% ampholines, pH 3.5 to 10, 18 h, 4°C) was carried out with 0.5 to 1.0 nmol of heme protein mixture and riboflavin added before photopolymerization (30 min). The trichloroacetic acid (5%, w/v)-washed gels were cut into 1.5-mm slices and solubilized with 30% H2O2/concentrated NH4OH (90:1, 0.6 ml/slice, 1 to 3 days, 32°C) in the dark. After addition of 10 ml of ACS solution, counting was done at 4°C. A, 40 μM P-450cAM solution, B, 100 μM P-450cAM solution, both photolyzed for 5 h with 9-fold molar excess of API at 313 min.
concomitantly and perhaps in competition with each other. It is therefore difficult to determine the exact contribution of each to the final reduction in rate which is observed. When 70% API-labeled P-450CAM was employed in the reconstitution, an 83.4% reduction in the rate of the enzymatic reaction was seen. But this does not necessarily imply that the reduction in rate due to photoactivation was 13.4% since no linear relationship between the extent of API-labeling and the reduction in hydroxylation rate has been established as yet. Reconstitution with 114% API-labeled P-450CAM showed that no more than 7.5% of the hydroxylase activity remained.

In order to locate the high affinity binding site of API on the linear polypeptide chain, extensively monolabeled cytochrome P-450CAM was subjected to BrCN degradation under conditions which lead to limited degradation with the unlabeled protein (30, 31). Fig. 7 shows the resolution of the BrCN-derived fragments on Sephadex G-75 equilibrated with 20% acetic acid. Although not indicated in the graph, the concentration of peptide material was determined by amino acid analysis of the fractions in parallel to 280 nm absorption to ascertain the concentration of peptides deficient in aromatic residues and to correct for contributions of the photoaffinity label to the absorption in the UV region. While only a rough fractionation of the larger fragments was achieved by this separation step, a low molecular weight fraction containing ~80% of the bound heme (Fig. 7A), and eluting immediately preceding the dissociated heme (~14%), was well resolved from the group of heme-free fragments. This heme-associated peptide fraction also co-eluted with ~70% of the radioactivity incorporated with the label. Since the absence of undigested protein in this digest reflects the advanced state of selective degradation of the protein, it may be assumed that this amount of radioactivity accounts roughly for the amount of specifically bound API while the remaining 20% of covalently bound API migrating with the larger fragments represents the extent of nonspecific labeling. The heme-associated peptide fraction eluted at an effluent volume corresponding to a molecular weight of 5.0 to 5.5 x 10^3. As shown in the upper inset in Fig. 7A, gel electrophoresis of this fraction indicated the presence of one radiolabeled BrCN fragment with a pl of 4.2 corresponding to the monolabeled heme peptide. Thus, the available evidence suggests that this fragment includes the specific binding site of API.

The same BrCN degradation experiment was also carried out with an API-labeled P-450CAM preparation which contained 168% of label distributed over mono- and dilabeled derivatives (Fig. 7B). In this case, much more heme had been dissociated (~31%) and the heme peptide fraction contained >88% of the radioactivity but only 55% of the bound heme. This finding indicates progressive heme dissociation with increased labeling at the second site. Gel electrophoresis of the heme peptide fraction demonstrates the presence of two radiolabeled bands suggesting that the second site is also located on the heme peptide since the total counts are now distributed over two bands of the expected pl values and in proportions corresponding to their distribution in the protein preparation. Fig. 8 compares the Soret band of heme peptide material corresponding to the ascending slope (1), the center of the peak (2), and of the descending slope (3) of the heme peptide fraction obtained from the Sephadex G-75 chromatography shown in Fig. 7, were recorded in 20% acetic acid.
Our concept of the active site structure of cytochrome P-450CAM, where the specific binding of the photoaffinity labels takes place and the subsequent generation of a small heme peptide is schematically summarized in Fig. 9. The scheme proposes probable positions for substrate and label in the microenvironment of the heme and a preferred orientation of the inhibitor-derived label, API, toward the heme group. The position of the putative substrate-binding sulphhydril group is also indicated (32). The amino acid side chains acting as chelating groups to the heme iron in the coordination positions 5 and 6 are indicated as an imidazole group of histidine and a sulphhydril group of cysteine (8, 11, 33, 34).

API was readily synthesized (Fig. 1) and radiolabeled; it proved to be a specific high affinity label for the active site of P-450CAM. Because addition of the azido group in para position of the phenyl ring caused only a minimal change in size and shape of the molecule, it can be assumed that this addition did not change the orientation of the ligand relative to the heme group. This is supported by the typical type II spectral shift (Fig. 3) and the favorable spectral dissociation constant of API. From these data it is apparent that API binds in the same fashion as its parent compound and with an affinity that is at least as high as that of camphor, the natural substrate of P-450 heme proteins regardless of their bacterial, mitochondrial, or microsomal origin. In fact, it has already been shown that it can bind tightly and specifically to the microsomal heme proteins P-450LM-2 and P-450LM-4 (12) of rabbit liver and to P-450 heme proteins of Rhizobium japonicum (13). Thus, the stage is now set for the comparative structural study of the inhibitor binding site of those P-450 heme proteins which bind API with high affinity.

It is important to realize that the photolysis procedure applied in this study evolved as a compromise solution in response to our needs for specific, covalent, active site directed probes tailored to the unique properties and requirements of P-450 heme proteins. Obviously, photoactivation of API could be carried out more efficiently at wavelengths closer to its absorption maximum at 265 nm but the absorption characteristics of P-450 heme proteins necessitated the selection of 313 nm instead (Fig. 2). Although photoactivation of API proceeds only at a modest rate at this wavelength, it was chosen to avoid significant photodamage to the heme protein which shows strong absorption throughout the region between 200 and 600 nm except for a window of minor absorption between 305 and 315 nm.

Since effective photolysis has to proceed for a time period in excess of several half-lives of the label, the need for extended light exposure of the heme protein was clearly established. But this cannot be done completely without penalty, and a small but spectrally recognizable photoinduced alteration of

\[\text{NH} + \text{Cl} \rightarrow \text{NO}_2\]
the P-450 heme protein was unavoidable. Thus, the conditions selected for our photolysis procedure were designed to keep this effect to a minimum while simultaneously adjusting to the modest power output of the mercury lamp at 313 nm.

For proof of specific photocovalent attachment of API to P-450\textsubscript{CAM} the following four conditions were applied: (a) covalent binding of label to protein must be shown to result solely from photolytic activation of API, and (b) it must reach saturation at a level close to stoichiometry; (c) thus only proteins with high specific affinity for this label can show significant covalent attachment, and (d) this attachment must occur at a discrete site, preferably in the vicinity of the heme group.

The extent to which our approach was successful in meeting these conditions is demonstrated in Figs. 4 through 7. From Fig. 4 we conclude that no covalent binding of API occurred in the absence of photolysis and that a protein without specific API binding sites, such as ribonuclease, will not be photoconvalently labeled by the procedure applied in our investigation. From Fig. 5 it becomes apparent that cytochrome P-450\textsubscript{CAM} contains one high affinity binding site for API which can be saturated without significant concomitant incorporation of label elsewhere in the protein. This result is supported by resolution of labeled species of P-450\textsubscript{CAM} from unlabeled protein and from each other in gel electrofocusing shown in Fig. 6. At low protein concentration and 9-fold molar excess of label essentially only one site is labeled while solutions containing protein and label at higher concentrations promote covalent labeling at a second specific site of lower affinity. Since the objectives of this study focus on complete saturation of the high affinity inhibitor binding site, we have chosen stoichiometric labeling of this site as the reference point to which the amount of API incorporated is compared. Thus 114\% API-labeled protein comes very close to having the high affinity site labeled to 100\% while the rest accounts for the level of nonspecific labeling. This interpretation is consistent with the results of our attempts to reconstitute campther hydroxylation with the API derivatives of P-450\textsubscript{CAM}. A preparation containing 70\% label can still show a moderate catalytic activity but after 114\% of API attachment almost all catalytic activity is effectively suppressed. From this finding it is concluded that API is indeed bound at the active site and thus prevents campther binding and hydroxylation. It is of interest that reconstitution of campther hydroxylation with the API-labeled preparations of P-450\textsubscript{CAM} also displays the characteristic rate enhancement with increasing amounts of putidaredoxin known to be obtained for the unlabeled protein (35, 36).

Digestion of the substrate-protected heme protein with BrCN was previously found to cause only limited degradation of P-450\textsubscript{CAM} and to produce a series of heme-bearing fragments the smallest of which was readily accessible by sievo chromatography but accounted for only 10 to 15\% of the heme protein (30). Due to its strong immunological cross-reactivity with antibodies made against the parent protein (31) this heme peptide had gained great significance for comparative studies of P-450 heme proteins (12). Application of the same procedure of BrCN digestion to the photocoylent P-450\textsubscript{CAM}-ligand complex, however, led to complete degradation of the heme protein and afforded the smallest heme peptide exclusively at the expense of the longer heme peptides. The cause of this fortuitous increase in yield to 80\% is not yet clear but it could be due in part to an increase in stability of the heme chelation after covalent binding of the label. Binding of the label may also engender a small conformational change giving better access for BrCN degradation to the 2 methionyl residues surrounding the structural domain which includes both the heme and associated substrate or inhibitor binding sites.

With the introduction of radioactivity a firm balance has been obtained for the amount of label associated with the small heme peptide as shown in Fig. 7 (70\%). Only small amounts of radioactivity were found associated with any of the other BrCN fragments. From this evidence we conclude that monolabeled P-450\textsubscript{CAM} contains roughly a stoichiometric amount of API bound to a single high affinity site in the immediate environment of the heme.

This conclusion is reinforced by the results from gel electrofocusing of the API-derivatized heme peptide (Fig. 7, upper insets) which showed the presence of one and two major peptide bands, respectively, coincident with label.

While it is highly probable that the single residues of histidine and cysteines found in the heme peptide (31) are indeed those involved in heme chelation in the parent protein, no convincing evidence can be provided at this point to show that this assumption is truly justified. Seen from this perspective the spectral characteristics of the heme peptide are at first sight somewhat surprising, especially the blue-shifted maximum of the Soret band which is at 356 nm (Fig. 8) instead of the 390 nm maximum suggested earlier (30, 31) on the basis of BrCN degradation work preceding our photocoylent labeling procedure. But it is now clear that a heme peptide with a Soret maximum at 356 nm can also be obtained from P-450\textsubscript{CAM} in the absence of covalently linked substrate or inhibitor and does not reflect the influence of the label.1 But in this case it represents a very small fraction of the isolated heme peptide while most of the heme peptide preparation contains heme loosely associated and shows a Soret peak at 390 nm which coincides with the maximum exhibited by heme extracted from P-450\textsubscript{CAM}. Thus it appears that under the conditions of BrCN cleavage some of the heme can be dislodged from its original position, and while it is still entrapped in the pouch-like heme peptide domain, it has assumed spectral characteristics similar to those of dissociated heme. Obviously, the heme ligation of the BrCN-derived heme peptide has to be scrutinized by the same physicochemical procedures that were successfully applied to the parent protein, especially electron spin resonance spectroscopy.

The isoelectric points of the API-labeled heme peptides, pl 4.2 and 4.8, contrast with that of the unlabeled heme peptide, pl 4.0, and these values are comparable to pl 4.5 for the unlabeled (33), and pl 5.2 and 5.8, for the mono- and dilabeled hemepeptide, respectively. Due to the basic character of API the mono- and dilabeled derivatives exhibit pl values which are progressively closer to neutrality than that of their acidic parent compounds. In this context it should be emphasized that gel electrofocusing provides significant advantages over sodium dodecyl sulfate-polyacrylamide gel electrophoresis for separating P-450\textsubscript{CAM} from its derivatives and in the study of the API-labeled heme peptides. Since the process of isoelectric focusing imposes and maintains the resolution a supporting matrix of relatively low cross-linkage sufficient which together with the absence of sodium dodecyl sulfate favors the retention of heme. At the very low concentrations needed for electrofocusing in gel the solubilities of the API-derivatives of P-450\textsubscript{CAM} and its heme peptide are adequate while free solution electrofocusing in density gradients presents solubility problems with the protein derivatives.

The second discrete binding site for API on cytochrome P-450\textsubscript{CAM} has significantly lower binding affinity so that a homogenous, monolabeled P-450\textsubscript{CAM}-ligand complex can be readily prepared with API. The binding affinity for API at this second site is, in turn, several orders of magnitude higher 1 K. M. Dus, unpublished results.
than that prevailing for nonspecific binding. The BrCN-derived heme peptides generated from dilabeled P-450CAM were found to contain all of the additional radioactivity of the highly labeled protein preparation. This would be consistent with the suggestion that the second molecule of API binds also at a site in the vicinity of the heme. At present we cannot explain the meaning or the structural basis of the existence of the second discrete binding site for API.

Although the binding of camphor to P-450CAM has been the subject of much concern and repeated investigation\(^6\) (32, 34, 37, 38) the story continues to unfold by added insight and clarification. It is hoped that the data from photoaffinity labeling experiments with P-450CAM presented in this communication will contribute to an improved understanding of this important event in the overall hydroxylation cycle. But the label chosen for this investigation was derived from the inhibitor, N-phenylimidazole, because of its high binding affinity and its general applicability to P-450 heme proteins. Specifically, the observation that API can bind to P-450CAM at two discrete sites, depending on concentration of protein and label, similar to the situation found with camphor (6), may offer a clue concerning the mode of binding of the substrate and the inhibitor. It is consistent with current information to propose that camphor is bound and oriented toward the heme iron (Fig. 9). Studies are now in progress to determine the respective contact residues of both, substrate and inhibitor, at these sites within the sequence of the heme binding domain of cytochrome P-450CAM.

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