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Affinity Labeling of the Active Site and the Reactive Sulfhydryl Associated with Activation of Rat Liver Phenylalanine Hydroxylase†

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ABSTRACT: A pterin analogue, 5-[(3-azido-6-nitrobenzylidene)amino]-2,6-diamino-4-pyrimidinone (ANBADP), was synthesized as a probe of the pterin binding site of phenylalanine hydroxylase. The photoaffinity label has been found to be a competitive inhibitor of the enzyme with respect to 6,7-dimethyltetrahydropterin, having a K_i of 8.8 ± 1.1 μM. The irreversible labeling of phenylalanine hydroxylase by the photoaffinity label upon irradiation is both concentration and time dependent. Phenylalanine hydroxylase is covalently labeled with a stoichiometry of 0.87 ± 0.08 mol of label/enzyme subunit. 5-Deaza-6-methyltetrahydropterin protects against inactivation and both 5-deaza-6-methyltetrahydropterin and 6-methyltetrahydropterin protect against covalent labeling, indicating that labeling occurs at the pterin binding site. Three tryptic peptides were isolated from [^{3}H]ANBADP-photolabeled enzyme and sequenced. All peptides indicated the sequence Thr-Leu-Lys-Ala-Leu-Tyr-Lys (residues 192–198). The residues labeled with [^{3}H]ANBADP were Lys198 and Lys194, with the majority of the radioactivity being associated with Lys198. The reactive sulfhydryl of phenylalanine hydroxylase associated with activation of the enzyme was also identified by labeling with the chromophoric label 5-(iodoacetamido)fluorescein [Parniak, M. A., & Kaufman, S. (1981) J. Biol. Chem. 256, 6876]. Labeling of the enzyme resulted in 1 mol of fluorescein bound per phenylalanine hydroxylase subunit and a concomitant activation of phenylalanine hydroxylase to 82% of the activity found with phenylalanine-activated enzyme. Tryptic and chymotryptic peptides were isolated from fluorescein-labeled enzyme and sequenced. The modified residue was identified as Cys236.

Phenylalanine hydroxylase (EC 1.14.16.1) (PAH)[1] catalyzes the hydroxylation of phenylalanine to tyrosine in the presence of molecular oxygen. PAH also shows an absolute requirement for a tetrahydropterin (Kaufman, 1959). Although tetrahydrobiopterin is the natural substrate (Kaufman, 1963) a number of synthetic pterins such as 6-MPH$_4$, 6,7-DMPH$_4$, and 6-phenyltetrahydropterin can be used as cofactors for PAH (Bailey & Ayling, 1978; Kaufman & Levenberg, 1959). A pyrimidine analogue of 6-phenyltetrahydropterin, 5-(benzylamino)-2,6-diamino-4-pyrimidinone is also active as a cofactor for PAH (Bailey & Ayling, 1978, 1980). It binds with the same affinity as 6-phenyltetrahydropterin but has a maximum velocity only 1.5% that of the analogous pterin.

Although much is known about the pterin cofactor requirements, nothing is known about the pterin binding site. There is little structural information available regarding PAH or any of its binding sites. For this reason an azido analogue of 6-phenyltetrahydropterin has been synthesized in this laboratory to probe the pterin binding site of PAH. The photoaffinity label 5-[(3-azido-6-nitrobenzylidene)amino]-2,6-diamino-4-pyrimidinone (ANBADP) is an analogue of 6-phenyldihydropterin lacking the 7-carbon of the pteridine ring and containing azido and nitro substituents on the phenyl ring. We report here the interaction of this photoaffinity label with PAH, its covalent binding to the enzyme upon photolysis, and the isolation and sequence of a labeled peptide.

Rat liver PAH is also under allosteric control and is reversibly activated by its substrate, phenylalanine (Shiman et al., 1979, 1990; Shiman & Gray, 1980). This allosteric binding site is a unique site and separate from the catalytic site (Shiman et al., 1990; Shiman, 1980). In rat liver PAH there is one free exposed sulfhydryl in the native enzyme (Fisher et al., 1972), and modification of this sulfhydryl with *N*-ethylmaleimide results in activation of the enzyme (Parniak & Kaufman, 1981). A concomitant apparent decrease in total phenylalanine binding suggests that this sulfhydryl is at or adjacent to the allosteric activation site. In order to understand more about this site, we have labeled the free sulfhydryl with the chromophore 5-(iodoacetamido)fluorescein (IAF). We report here the isolation and sequence of fluorescein-labeled peptides isolated from proteolytic digestion of labeled native PAH.

Experimental Procedures

Materials. Manganese dioxide, 3-fluorobenzaldehyde, sodium borodeuteride, and 2,5,6-triamino-4-pyrimidinone

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[1] Abbreviations: PAH, phenylalanine hydroxylase; ANBADP, 5-[(3-azido-6-nitrobenzylidene)amino]-2,6-diamino-4-pyrimidinone; FNBADP, 5-[(3-fluoro-6-nitrobenzylidene)amino]-2,6-diamino-4-pyrimidinone; 5-IAF, 5-(iodoacetamido)fluorescein; 6-MPH$_4$, 6-methyl-5,6,7,8-tetrahydropterin; 6,7-DMPH$_4$, 6,7-dimethyl-5,6,7,8-tetrahydropterin; DTT, dithiothreitol; HPLC, high-pressure liquid chromatography; TFA, trifluoroacetic acid.

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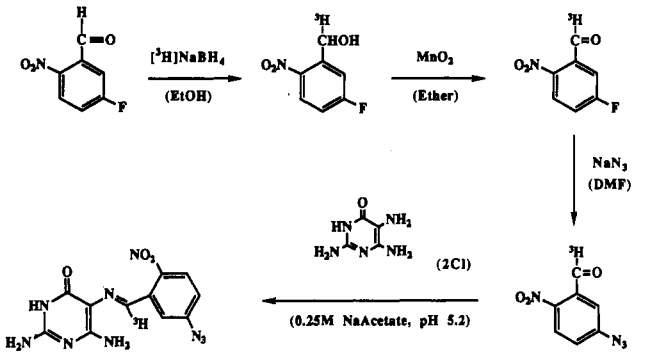
(sulfate salt) were purchased from Aldrich. 5-(Iodoacetamido)fluorescein was purchased from Molecular Probes. [^{3}H]Sodium borohydride was purchased from New England Nuclear. 6-MPH$_4$ and 6,7-DMPH$_4$ were prepared as previously described (Storm et al., 1971). Rat liver PAH was purified by the procedure of Wallick et al. (1984). PAH from *Chromobacterium violaceum* was the kind gift of Dr. Akiko Onishi. All other materials were of the highest quality commercially available and were used without further purification.

Synthesis of ANBADP. 3-Fluoro-6-nitrobenzaldehyde was synthesized from 3-fluorobenzaldehyde as described by Pelchowicz et al. (1961). The purified product was reacted with sodium azide [1:1 (mol/mol)] in DMF to produce 3-azido-6-nitrobenzaldehyde (Goldman, 1969; Miller & Parker, 1961). The product was isolated by adding ice to the reaction mixture and collecting the resulting precipitate. Proton and ^{13}C NMR confirmed that all the starting material had been converted to product. This reaction and all subsequent reactions were carried out in the absence of light. The final step in the synthesis of ANBADP was the condensation of 3-azido-6-nitrobenzaldehyde with 2,5,6-triamino-4-pyrimidinone according to the procedure of Rogers and Ulbricht (1968). 2,5,6-Triamino-4-pyrimidinone (sulfate salt) was reacted with barium acetate at 100 °C for 30 min to form the acetate salt of the pyrimidinone. The precipitated barium sulfate was removed by filtration under an argon atmosphere, and the filtrate was collected in a flask containing a slight excess of 3-azido-6-nitrobenzaldehyde dissolved in a minimal volume of ethanol. The reaction mixture was kept at 4 °C overnight. The product, a dark orange precipitate, was isolated by filtration, washed extensively with anhydrous ether, and dried under vacuum. The sample was not recrystallized to avoid degrading the azide at high temperature. The overall yield was usually greater than 70%. Anal. Calcd: C, 42.04; H, 2.86; N, 40.13. Found: C, 41.61; H, 2.60; N, 40.50. ^{1}H NMR (360 MHz) (DMSO-d_6) δ 6.4–7.0 (5 H, amide protons, br m), 7.15 (1 H, dd, J = 8.72, 2.5 Hz), 7.95 (1 H, d, J = 8.7 Hz), 8.0 (1 H, d, J = 2.5 Hz), 9.95 (1 H, Schiff base proton, s); ^{13}C NMR (90 MHz) (DMSO-d_6) δ 101.9, 117.6, 118.8, 126.4, 135.0, 140.2, 144.1, 144.5, 154.2, 157.3, 163.6. Fast atom bombardment mass spectroscopy gave a parent ion peak (MH^+) at 316. Ultraviolet and visible molar extinction coefficients were λ_{max} 285 nm (ϵ 18 290 M^{-1} cm^{-1}) and λ_{max} 353 nm (ϵ 16 380 M^{-1} cm^{-1}).

The fluoro analogue, FNBADP, was also synthesized as a control. The procedure was the same as described above except that 3-fluoro-6-nitrobenzaldehyde was used in the condensation step and no care was taken to exclude light.

The stability of ANBADP under experimental conditions was determined. The double bond of the Schiff base is subject to hydrolysis at acidic pH. Tritium-labeled ANBADP was hydrolyzed in 0.1 M HCl for 10 min, and the products were separated by TLC developed in ethanol containing 1% triethylamine. The spots were isolated and the tritium was found to be associated with the spot corresponding to the aldehyde. In 0.1 M phosphate buffer, pH 6.8, less than 3% of ANBADP was hydrolyzed over a 5-min time span as determined by the disappearance of the absorbance at 365 nm due to the Schiff base. Under HPLC conditions, 0.08% TFA in a water–acetonitrile gradient, approximately 50% of the label was hydrolyzed by the time it eluted 39 min into the gradient. The products were identified as 2,5,6-triamino-4-pyrimidinone and 3-azido-6-nitrobenzaldehyde by comparing their elution times (24 and 40 min, respectively) and UV–vis spectra with standards.

Scheme I



ANBADP

The apparent K_i values of both the azido and fluoro analogues with respect to 6,7-DMPH$_4$ were determined by using the PAH assay described by Ayling et al. (1973). Kinetic data were fit to the inhibition programs of Cleland (1979) by using programs adapted for use on a Macintosh microcomputer by Dr. James Robertson of the Pennsylvania State University.

Molecular modeling and energy minimization studies were carried out by using the Macromodel program developed by Clark and Still to compare the structure of ANBADP with that of 6-phenyltetrahydropterin and 6-phenyldihydropterin. MM2 energy minimization was accomplished by a Monte Carlo global conformational search in dihedral angle space about rotational bonds.

Synthesis of [^{2}H]- and [^{3}H]ANBADP. The deuterated and tritiated forms of the photoaffinity label, ANBADP, were synthesized as shown in Scheme I. 3-Fluoro-6-nitrobenzaldehyde was converted to the alcohol by reduction in the presence of a 1:1 (mol/mol) ratio of either [^{2}H]- or [^{3}H]-labeled sodium borohydride in ethanol. The reaction vessel was kept on ice, and the reaction was stopped after 30 min by pouring the mixture over ice. The precipitated product was collected by filtration and dried under vacuum. The resulting alcohol was then oxidized back to the aldehyde in the presence of manganese dioxide (5-fold excess by weight) in anhydrous ether (Dollimore & Tonge, 1967; Gritter & Wallace, 1959). The reaction was monitored by TLC developed in a solvent system of 5% ethyl acetate in chloroform. After 2 h the reaction was ~50% complete. An additional 2-fold excess (by weight) of manganese dioxide was added and the reaction proceeded for an additional 24 h until it was essentially complete. The mixture was filtered through Celite, and the filtrate was dried over magnesium sulfate, filtered again, and dried under a stream of argon. Both proton and ^{13}C NMR indicated that the deuterium was incorporated at the α-position and that the isotope incorporation was greater than 90% (Goldman 1969). The labeled benzaldehyde was then used to synthesize ANBADP as described above. For the deuterated product the proton NMR singlet at δ 9.95 (Schiff base proton) was not observed. In the ^{13}C spectra, the singlet at δ 163.6 was replaced by a triplet at δ 163.6 ($J \approx 36$ Hz). Both deuterated and tritiated products yielded one spot (R_f 0.84) by TLC developed in ethanol containing 1% triethylamine. The specific radioactivity of [^{3}H]ANBADP was 1.8 mCi/μmol.

Modification of Phenylalanine Hydroxylase with 5-IAF. The reaction mixture for modification contained 20 μM PAH and 40 μM 5-IAF in 0.1 M potassium phosphate buffer, pH 6.8, at 25 °C (Parniak & Kaufman, 1981). Aliquots were removed at various time points and assayed for hydroxylase activity spectrophotometrically at 275 nm (Shiman & Gray,

1980) without preincubation with phenylalanine (Parniak & Kaufman, 1981) and monitored for at least 2 min. The 1-mL reaction mixture contained 0.1 M potassium phosphate buffer, pH 6.8, 0.25 mM phenylalanine, 30 μg of crystalline beef catalase, 60 μM 6-MPH_4, and 6 mM dithiothreitol. Controls were also run to determine the initial velocity of the enzyme activated with phenylalanine. These assays contained 1 mM phenylalanine. Aliquots were also removed, and the protein was isolated from excess reagent by 1-mL spun gel-filtration columns containing Sephadex G-50 equilibrated with 0.1 M potassium phosphate buffer, pH 6.8, according to the method of Penefsky (1977, 1979). The amount of fluorescein bound was determined spectrophotometrically at 495 nm by using a molar extinction coefficient of 85 000 M^{-1} cm^{-1} (Franzen et al., 1980). PAH concentration was determined by the method of Lowry as modified by Peterson (1977). When modification was completed, the hydroxylase was separated from excess reagent by spun gel-filtration columns, consisting of 5-mL disposable syringes filled with Sephadex G-50 equilibrated in double-distilled water. The columns were spun for 4 min before and after sample application. Protein recovery was typically 90–95%.

Photoaffinity Labeling with ANBADP. The reaction mixture for the labeling of PAH with nonradiolabeled ANBADP consisted of 0.1 M potassium phosphate, pH 6.8, 4 μM PAH, 30% DMF (v/v, final concentration) and 0–50 μM ANBADP in a final volume of 0.1 mL. The samples were photolyzed (Spectroline Model XX-15B UV lamp, 302-nm output maximum, no filters) at a distance of 5 cm for 2 min (16.2 mW/cm^2). Immediately after photolysis, samples were placed on ice and aliquots were removed to assay for activity (Shiman et al., 1979).

Labeling with [^{3}H]ANBADP was carried out in a reaction mixture containing 0.1 M potassium phosphate, 4 μM PAH, and 10–30% (v/v) DMF in a 10 × 75 mm borosilicate glass tube. [^{3}H]ANBADP in DMF, specific radioactivity of 400 μCi/μmol, was added to give a final concentration of 0–200 μM photoaffinity label and 30% DMF in a final volume of 100 μL. The samples were photolyzed as described above. Unbound label was removed by immediately applying the sample to a 1-mL spun gel-filtration column containing Sephadex G-50 equilibrated with double-distilled water. The amount of label bound was calculated from the specific radioactivity, and the protein concentration was determined as previously described.

For preparative photolabeling of PAH, the radioactive label was diluted with nonradioactive label to give a final specific radioactivity of 30 μCi/μmol. The reaction mixture and procedure were the same as for analytical labeling, and 30 100-μL samples were photolyzed. Unreacted label was removed by using 5-mL spun gel-filtration columns with 0.5 mL of sample applied to each column.

Predicted Values for Stoichiometry. The predicted values for the stoichiometry of labeling were calculated by using

$$[\text{E–L}] = \frac{(E_0 + K_L + L_0) - [(E_0 + K_L + L_0)^2 - 4E_0L_0]^{1/2}}{2}$$

where [E–L] is the concentration of the PAH–ANBADP complex, E_0 is the initial active-site concentration of PAH, L_0 is the total concentration of ANBADP, and K_L is the K_i determined for ANBADP. The calculated values for the stoichiometry assume that once the ANBADP is bound there will be 100% efficiency in labeling.

The predicted values for the substrate protection studies were calculated by using

$$\frac{[EL]}{[ES]} = \frac{K_m[L_0]}{K_i[S_0]}$$

where [EL] is the concentration of ANBADP-labeled PAH, [ES] is the concentration of the enzyme–substrate species, K_m is the Michaelis constant for the competing substrate ligand (35 μM for 6-MPH_4 or 25 μM for 5-deaza-6-MPH_4), and K_i is the inhibition constant for ANBADP (8.8 μM). $[L_0]$ and $[S_0]$ are the total concentrations of ANBADP and the pterin ligands, respectively. The calculated values assume that once the ANBADP is bound there will be 100% efficiency in labeling.

Proteolytic Digestion of Labeled PAH. Samples containing 1 mg/mL of PAH labeled with either ANBADP or fluorescein were adjusted to pH 8 by the addition of 1 M Tris, pH 8, to a final concentration of 0.1 M. Trypsin or chymotrypsin was added to a final concentration of 50:1 (w/w) PAH to protease. Digestion was carried out at 37 °C for 24 h for fluorescein-labeled PAH and 12–14 h for ANBADP-labeled PAH.

HPLC Separations. All HPLC chromatographic separations were performed on a Waters 990+ chromatography work station interfaced to a NEC Powermate II computer for data capture. Reverse-phase HPLC was performed on a Waters Delta Pak C_{18} 100-Å column (3.9 × 300 mm) equilibrated with HPLC-grade water containing 0.1% trifluoroacetic acid (solvent A). Peptide fractionation and purification were accomplished by using a linear gradient of 0.07% trifluoroacetic acid in acetonitrile (solvent B) at 1–2%/min for peak isolation and at 0.5–0.2%/min for peak purification. All separations were done at a flow rate of 1 mL/min.

Isolation and Identification of Fluorescein-Labeled Peptides. Labeled peptides resulting from proteolytic cleavage with trypsin or chymotrypsin were isolated by HPLC after digestion for 24 h at 37 °C. The chromatographs were monitored at both 220 and 440 nm, and those peaks absorbing at 440 nm were collected. These samples were taken to dryness in vacuo (Savant Speed Vac), resuspended in a minimal volume of solvent A, and rechromatographed. Labeled peaks were collected manually and submitted for N-terminal sequence analysis. Sequence analysis was performed with an Applied Biosystems 447A gas–liquid amino acid sequenator.

Isolation and Identification of [^{3}H]ANBADP-Labeled Peptides. Radioactive labeled peptides resulting from tryptic cleavage were isolated after digestion at 12–14 h at 37 °C. Samples were adjusted to pH 4–5 by using trifluoroacetic acid, and 200–500-μL samples were injected onto the HPLC column. Fractions (1 mL) were collected and 20-μL aliquots were removed to be counted. Fractions containing radioactivity were pooled for each peak and the volume was reduced in vacuo. Each sample was then rechromatographed until a single radioactive peak was obtained. These peaks were then submitted to N-terminal amino acid sequence analysis.

Results

Biological Activity of the Photoaffinity Label ANBADP. Kinetic experiments were performed to determine whether ANBADP was an inhibitor of PAH with respect to tetrahydropterin substrates. ANBDAP was found to be a competitive inhibitor of the enzyme with respect to 6,7-$DMPH_4$ with a K_i of 8.8 ± 1.1 μM. The large standard error may be due to the absence of DTT or quinonoid dihydropteridine reductase in the assay. ANBADP was found to be an inhibitor of quinonoid dihydropteridine reductase. DTT was also unacceptable in the assay as it reduced the azide to the amine, giving spurious results at low inhibitor concentrations. To reduce the large autooxidation rate of 6,7-$DMPH_4$ in the

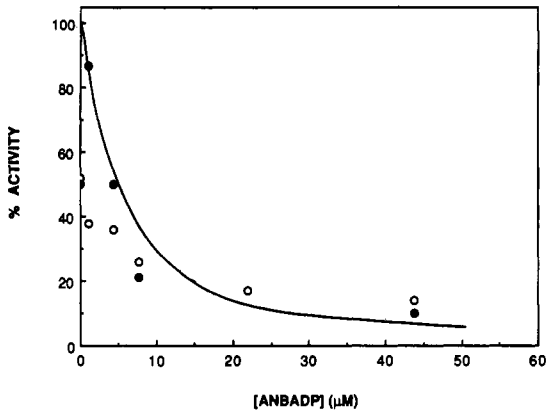


FIGURE 1: Photoinactivation of PAH in the presence of ANBADP. The reaction mixtures contained 4 μM PAH (active-site concentration) and increasing concentrations of ANBADP in 0.1 M potassium phosphate buffer, pH 6.8. Photolysis was for 1 min at 302 nm as described under Experimental Procedures. PAH was either preactivated with 1 mM phenylalanine (○) or was not preactivated (●). The solid line represents the expected decrease in activity upon photolysis (see Experimental Procedures).

absence of these reductants, the catalase was increased 2-fold from 30 to 60 μg in a 1-mL assay mixture (Ayling et al., 1973).

Effect of Photolabeling on PAH Activity. The effect of ANBADP on PAH activity upon photolysis is shown in Figure 1. Inactivation is concentration dependent with a decrease in activity with increasing inhibitor concentration. Preactivation with 1 mM phenylalanine (preincubated for 3 min at 25 °C) affects inactivation at lower ANBADP concentrations but not at higher concentrations. The solid line in Figure 1 represents the calculated percentage of ANBADP bound at various inhibitor concentrations (see Experimental Procedures), while the open and closed circles are the measured values with and without phenylalanine activation, respectively. It should be noted that the calculated values are for the percentage of ANBADP reversibly bound to the enzyme. Upon photolysis, not every ANBADP molecule associated with the enzyme will be covalently bound. Less than 100% efficiency in labeling would account for the approximately 15% residual activity observed in Figure 1.

Photolysis of PAH at 302 nm in the absence of ANBADP results in a 50% loss of activity as compared to the unphotolyzed control. The presence of three tryptophan and 22 tyrosine residues may be responsible for this loss of activity. ANBADP offers some protection against the photoinactivation of PAH at low concentrations. The presence of 1 μM ANBADP increases the activity recovered after photolysis to 87%, compared to 51% in the absence of ANBADP when PAH is not preactivated by phenylalanine. The same effect is observed with the fluoro analogue, FNBADP. The activity recovered increased from 50% to 76% in the presence of 5 μM FNBADP. Inactivation of PAH with increasing concentrations of ANBADP in the absence of phenylalanine activation is then assumed to be due to irreversible binding of the photoaffinity label to the enzyme and not due to photoinactivation of the enzyme alone. The effectiveness of ANBADP labeling and therefore inactivation at lower ANBADP concentrations is greater with PAH that is preactivated (Figure 1).

If ANBADP is inactivating PAH by modifying the pterin binding site, the presence of a pterin substrate should protect the enzyme against inactivation. In the presence of $6MPH_4$, however, PAH is inactivated upon photolysis whether or not ANBADP is present. Removing any oxygen from the system by evacuating the sample and flushing with argon did not significantly decrease the amount of inactivation. Oxidized

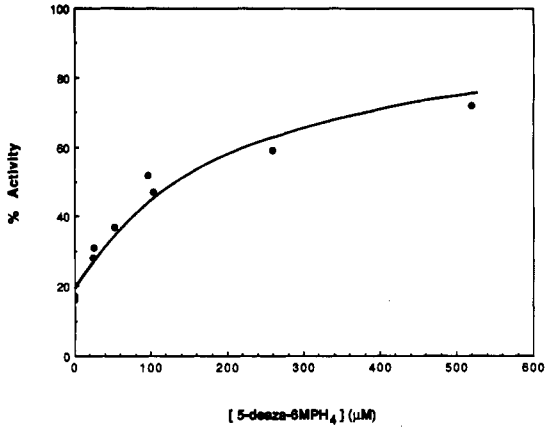


FIGURE 2: Protection of PAH against ANBADP photolabeling by 5-deaza-$6MPH_4$. The reaction mixtures contained 4 μM PAH (active-site concentration) and 40 μM ANBADP in 0.1 M potassium phosphate buffer, pH 6.8. Photolysis was for 1 min at 302 nm as described under Experimental Procedures. The solid line is the expected value for activity under the conditions of the experiment. The amount of 5-deaza-$6MPH_4$ bound compared to ANBADP was calculated as described under Experimental Procedures.

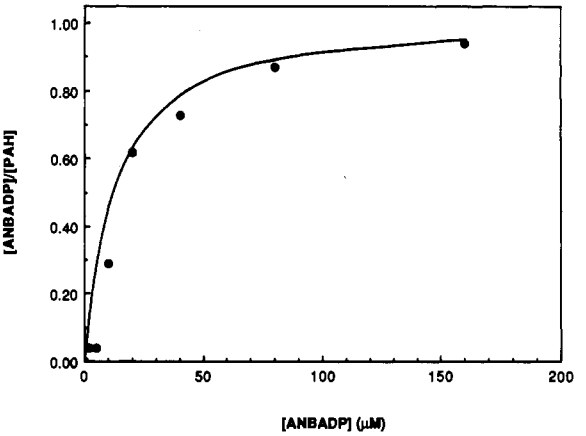


FIGURE 3: Concentration-dependent photolabeling of PAH by ANBADP. The reaction mixture contained 4.4 μM PAH (active-site concentration), 30% DMF, and 0.1 M potassium phosphate buffer, pH 6.8. Photolysis was at 302 nm for 2 min as described under Experimental Procedures. The solid line is the expected value for incorporation of the label (see Experimental Procedures).

pterins are known to be good photosensitizers (Chahidi et al., 1981), and it would follow that an oxidation product of $6MPH_4$ would also be a good photosensitizer. 5-Deaza-$6MPH_4$, however, is inert toward molecular oxygen and is a good competitive inhibitor of PAH (Moad et al., 1979). Increasing concentrations of 5-deaza-$6MPH_4$ protected PAH from photoinactivation by ANBADP (Figure 2). The results in Figure 2 show that the actual protection by 5-deaza-$6MPH_4$ matched the predicted values (solid line).

Photolabeling of PAH with [^3H]ANBDAP. The optimal conditions for photolabeling of PAH were determined with respect to time of photolysis and ANBADP concentration. The extent of labeling was dependent on the time of photolysis with maximum labeling obtained after 2 min. Further irradiation did not increase the amount of label bound. These results correlate with observed spectral changes for ANBADP photolyzed in solution (data not shown). At photolysis times greater than 2 min, no further changes were observed in the spectrum. The amount of label bound to PAH was dependent on ANBADP concentration (Figure 3). The data in Figure 3 show that ANBADP modifies a saturable site with the stoichiometry of label incorporated closely following the

Table I: Nature of Attachment of ANBADP to PAH

	[ANBADP]/[PAH][a]
rat liver PAH	
$+h\nu$[b]	0.92
$-h\nu$	0.08
−urea[c]	0.93
+urea[c]	0.89
C. violaceum PAH	
−urea[c]	0.38
+urea[c]	0.07

[a] Calculated as described under Experimental Procedures. Blanks and standards for the protein assays contained urea to correct for urea in the sample. [b] Enzyme plus label irradiated at 302 nm for 2 min. [c] Enzyme denatured by incubating in 8 M urea at 37 °C for 1 h after enzyme was irradiated at 302 nm for 2 min in the presence of label. The control (−urea) was also irradiated in the same manner.

predicted values. The amount of label incorporated is independent of whether or not PAH is preactivated with phenylalanine (data not shown). This suggests that phenylalanine does not affect the binding of ANBADP to PAH.

The specificity of ANBADP modification was further examined by using $6MPH_4$ and 5-deaza-$6MPH_4$ as competing ligands against the photoaffinity label. Increasing concentrations of $6MPH_4$ reduced the amount of label incorporated from 0.9 mol/mol of subunit to 0.1 mol/mol of subunit at a final concentration of 3.4 mM $6MPH_4$. The presence of 5-deaza-$6MPH_4$ also reduced the amount of label incorporated. The amount of label incorporated was reduced from 0.9 to 0.35 mol/mol of subunit in the presence of 750 μM 5-deaza-$6MPH_4$. The experimental values agreed with the predicted values for both ligands. This is further evidence that ANBADP is uniquely modifying the pterin binding site.

The modification of rat liver PAH was determined to be covalent by two criteria: the amount of label bound in the absence of photolysis and the amount bound after denaturing of the protein (Table I). In the absence of light, less than 10% of the predicted amount of label remains associated with the enzyme. This suggests that label associated with PAH after photolysis is covalently bound. To discount the possibility that the photoproducts of ANBADP are tightly but noncovalently bound, photolabeled PAH was incubated in 8 M urea at 37 °C for at least 1 h. Any nonbound species after that time were removed from the protein by gel filtration. Treatment with urea did not significantly alter the amount of label bound indicating that labeling is covalent. PAH from *Chromobacterium violaceum* was also photolabeled with ANBADP. The extent of photolabeling was significantly less than with the rat liver enzyme. After incubation with urea only a small amount of label remained bound, suggesting that labeling is not covalent (Table I).

Isolation of [^{3}H]ANBADP-Labeled Tryptic Peptides and Identification of the Labeled Amino Acid Residue. Tryptic digestion of [^{3}H]ANBADP-labeled PAH followed by fractionation of the resulting peptides by HPLC yields the chromatograph in Figure 4. The radioactivity was monitored by collecting 1-mL fractions and counting a small aliquot. Five preparative injections were made and peaks 1 and 2 (Figure 4) consistently appeared in all five runs. Incubation of label alone under the conditions used for proteolysis resulted in 50% of the radioactivity applied to the column eluting in the void volume. This material had no defined spectrum and was assumed to be tritiated water. The tryptic digest of labeled PAH is carried out under basic conditions where abstraction of tritium by a hydroxide ion would be probable. No unbound ANBADP or tritiated hydrolysis product, 3-azido-6-nitrobenzaldehyde, which elute at 39 and 40 min into the gradient, respectively (data not shown), was observed in the HPLC isolation of the labeled PAH tryptic digest. Further purification of peaks 1 and 2 did not yield any unbound radioactivity in the void, suggesting that degradation occurs during proteolysis. Peak 1 accounted for 10% and peak 2 for 45% of the radioactivity put on the HPLC column. The void peak contained ~40% of the counts.

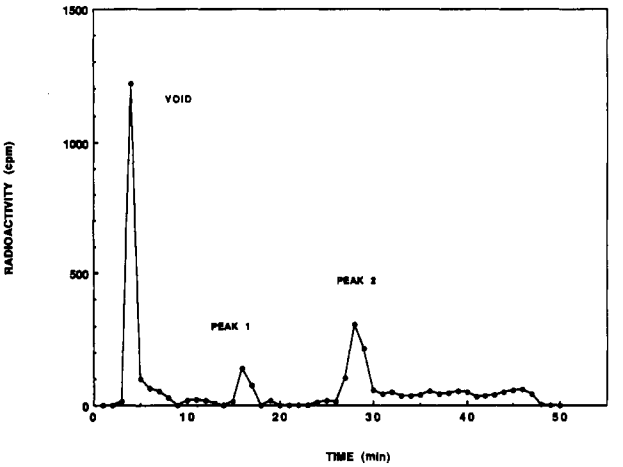


FIGURE 4: HPLC fractionation of peptides derived from trypsin digestion of [^{3}H]ANBADP-photolabeled PAH. Photolabeled PAH was digested with trypsin and fractionated as described under Experimental Procedures. The gradient used was 0% B for 5 min and then 1% B/min over the next 100 min. Fractions were collected every minute and had a volume of 1 mL. This plot is the sum of four different injections. Values for cpm were corrected for background.

Table II: Amino Acid Sequence Analysis of Peptides 1, 2a, and 2b

cycle	amino acid	peptide 1 (pmol)	peptide 2a (pmol)	peptide 2b (pmol)
1	Thr	130.9	309.1	98.2
2	Leu	126.1	352.6	109.4
3	Lys	27.9	281.8	63.1
4	Ala	8.5	318.6	169.8
5	Leu	7.6	322.8	129.1
6	Tyr		332.7	147.8
7	Lys		55.3	4.0
8				
9				
10	end of sequence			

Further purification of peak 1 by HPLC yielded one radioactive peptide, while peak 2 yielded two radioactive peptides that could be separated by HPLC. Approximately 670 pmol (total) of labeled peptide was recovered. All three peaks were submitted for N-terminal amino acid sequence analysis and the results are shown in Table II. The sequence identifies the tryptic peptide as containing amino acid residues Thr192–Lys198. In cycle 7 for peptides 2a and 2b, the amount of Lys198 drops off to background levels. There is also a decrease in the amount of Lys194 recovered but not as dramatic a change as with Lys198. No further residues are observed after Lys198. One reason may be that trypsin still recognizes the residue and cleaves there. More likely, however, is that because of insertion of the label no further degradation can occur. For peptide 1, the first three amino acids are clearly identified. After that, however, the level of amino acids recovered drops off to background. There is a sharp drop in the amount of Lys194 recovered. The sequencing data for peptide 1 suggest that labeling occurs at Lys194.

The majority of the radioactivity was isolated in peaks 2a and 2b. Peak 2a contained 0.093 μCi of tritium and peak 2b contained 0.044 μCi. These correspond with 311 and 147 pmol

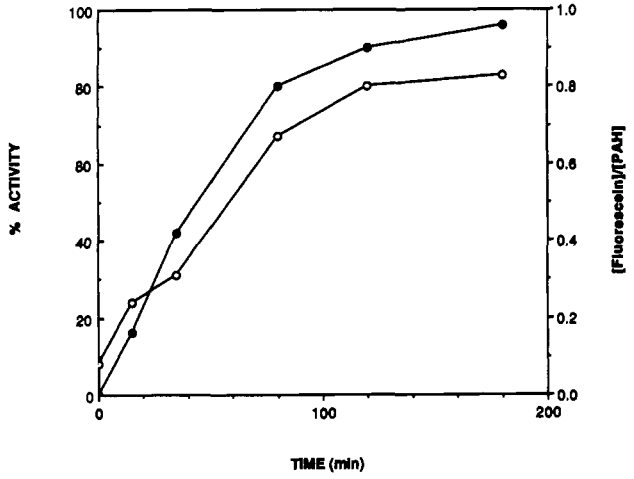


FIGURE 5: Time course of fluorescein incorporation and stimulation of PAH activity. The reaction mixture contained 20 μM PAH and 40 μM 5-IAF in 0.1 M potassium phosphate buffer, pH 6.8. The activity (O) and extent of fluorescein incorporation (●) were determined as described under Experimental Procedures.

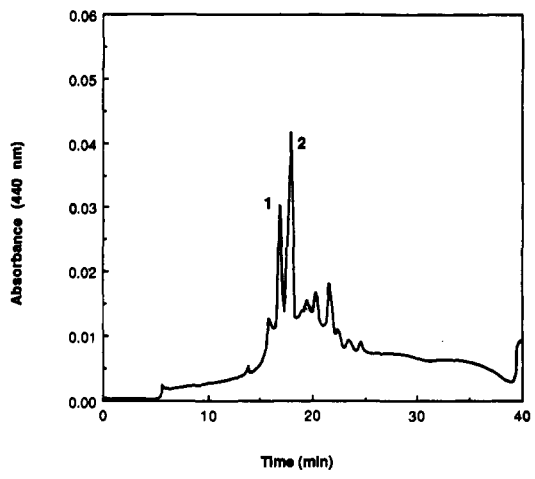


FIGURE 6: HPLC fractionation of peptides derived from chymotrypsin digestion of fluorescein-labeled PAH. Photolabeled PAH was digested with chymotrypsin and fractionated as described under Experimental Procedures. Fluorescein-containing peptides were monitored at 440 nm.

of label recovered in peaks 2a and 2b, respectively. The ratio of label to peptide was 0.89 for peak 2a and 0.87 for peak 2b. Peak 1, however, contained only 0.016 μCi of tritium or 53 pmol of label. The ratio of label to peptide was 0.41, significantly lower than that for peaks 2a and 2b. The data suggest that the majority of labeling occurs at Lys198, while there is a smaller but significant amount of labeling at Lys194.

Stoichiometry of the Reaction of 5-IAF with Phenylalanine Hydroxylase. When native rat liver PAH was incubated with a hydrophobic sulfhydryl-modifying agent such as 5-IAF, one sulfhydryl per subunit was modified. The amount of label incorporated was determined spectrophotometrically as a function of time (Figure 5). As the sulfhydryl was modified there was a concomitant increase in the activity of the labeled PAH in the absence of activation by phenylalanine (Figure 5). The activity increased from 8% with no activation to a maximum of 82% upon labeling with 5-IAF compared to 100% activation for phenylalanine-activated PAH. The data in Figure 5 show that the increase in enzyme activity parallels the extent of modification of the reactive sulfhydryl by 5-IAF. Similar results have been reported with *N*-ethylmaleimide as the sulfhydryl reagent (Parniak & Kaufman, 1981).

The fluorescein-labeled PAH was subjected to limited proteolysis by α-chymotrypsin to produce an active 35 000-dalton fragment. After isolation of this species, the amount of fluorescein bound was found to be 0.97 fluorescein/35 000-dalton fragment. However, unlike unlabeled PAH, where the activity increases after limited proteolysis (Abita et al., 1984), no further increase in activity was observed with modified PAH.

Identification of the Modified Cysteine. After either tryptic or chymotryptic digestion of fluorescein-labeled PAH, the resulting peptides were fractionated by reverse-phase HPLC. The resulting chromatograph from the chymotryptic digestion is shown in Figure 6. The fluorescein-labeled peptides can be readily identified by their absorbance at 400 nm and characteristic spectra. The maximum wavelength of fluorescein is pH dependent, and under the conditions used for separation (pH 3–5), the wavelength maximum shifts from 495 to 440 nm. As shown in Figure 6, there are two major peaks that fit this criterion. Peak 2 has a retention time (17 min) and electronic spectrum identical with those of 5-IAF incubated with chymotrypsin in the absence of PAH. This was confirmed when after subsequent purification by HPLC

Table III: Amino Acid Sequence Analysis of Fluorescein-Labeled Peptides

	chymotryptic peptide		tryptic peptide	
cycle	amino acid	pmol	amino acid	pmol
1			Glu	13.0
2			Asp	8.8
3			Asn	8.9
4			Ile	15.4
5			Pro	15.8
6			Gln	15.0
7			Leu	11.4
8			Glu	14.8
9			Asp	14.6
10			Val	7.9
11			Ser	6.8
12			Gln	10.2
13			Phe	10.4
14	Leu	83.8	Leu	11.2
15	Gln	110.7	Gln	9.3
16	Thr	116.6	Thr	9.2
17				
18	Thr	55.6	Thr	4.6
19	Gly	73.6	Gly	5.2
20	Phe	59.9	Phe	4.3
21			Arg	3.8

no amino acids were observed with N-terminal amino acid sequence analysis. Peak 2 contained 60% of the fluorescein label while peak 1 accounted for 31%. The remaining 9% of label could have been lost in peak isolation or incomplete digestion of the native PAH. Similar results were obtained with the tryptic digestion (data not shown). Peak 1 from the chymotryptic digestion and the peak from the tryptic digestion were further purified by HPLC. In each case a single fluorescein-containing species was isolated. Both the chymotryptic and tryptic peaks were submitted for N-terminal amino acid sequence analysis and the results are shown in Table III. The sequence identifies the tryptic fragment as spanning amino acid residues Glu220–Arg240. The chymotryptic peptide sequence, Leu233–Phe239, overlaps with the tryptic sequence. The absence of any PTH-amino acids eluting in either cycle 4 of the chymotryptic peptide or cycle 17 of the tryptic peptide identifies the modified cysteine as Cys236.

Discussion

Phenylalanine hydroxylase is covalently modified at the pterin binding site by the pterin analogue ANBADP. The

following criteria have been used to show that labeling is specific for this site. First, the photolabel is a competitive inhibitor of the enzyme with respect to 6,7-DMPH$_4$. The K_i of ANBADP, 8.8 μM, is only 2-fold higher than the K_m of 3 μM for 6-phenyltetrahydropterin (Bailey & Ayling, 1978, 1980). Molecular modeling of ANBADP shows that it is a planar molecule identical in overall conformation with 6-phenyl-7,8-dihydropterin and 6-phenyl-PH$_4$. Energy minimization studies indicate that there is very little rotation of the phenyl ring of ANBADP, suggesting that it should bind to the pterin binding site in the same manner as the pterin substrates. In addition, the bulky nitro and azido substituents did not significantly affect the binding to PAH. Secondly, phenylalanine hydroxylase is protected against photolabeling with ANBADP by 5-deaza-6-MPH$_4$ and 6-MPH$_4$. Finally, the presence of phenylalanine does not affect the stoichiometry of labeling. It is possible that the phenyl substituent of ANBADP would bind at either the allosteric activation site or the phenylalanine binding site.

A complication in the interpretation of the photolabeling experiments is the concomitant photoinactivation of PAH. Phenylalanine hydroxylase is significantly photoinactivated during photolysis in the absence of ANBADP at 302 nm. The presence of three tryptophans and 22 tyrosines as well as the iron probably plays a role in the inactivation. Removal of all oxygen by alternately evacuating the sample and flushing with argon did not reduce the photoinactivation of PAH. However, the presence of the photoaffinity label protects the enzyme against this photoinactivation, as observed with lower concentrations of the label (Figure 1). This was confirmed with the fluoro analogue FNBADP as well as 5-deaza-6MPH$_4$. Protection against photoinactivation by a substrate ligand has been observed for thymidine kinase (Cysyk & Prusoff, 1972). The presence of thymidine decreased the amount of photoinactivation by almost 30% in thymidine kinase. Although the exact mechanism of protection by ANBADP is not known, there are several possibilities. Bound ANBADP may act as an "inner filter", protecting amino acids at the binding site that are necessary for the attachment of the substrate or play a role in catalysis. The label has an absorbance maximum at 283 nm, close to the absorbance maxima of both tryptophan and tyrosine. Alternatively, protection may result from a substrate-induced conformational change in the protein structure that alters the UV sensitivity of a residue that is critical for maintaining activity. There is evidence that a conformational change does occur in PAH upon binding of tetrahydrobiopterin and 6-MPH$_4$ (Koizumi et al., 1988).

The amount of protection afforded by both ANBADP and FNBADP against photoinactivation of PAH is much greater than predicted on the basis of the K_i values and the concentrations of label and enzyme. At an ANBADP concentration of 1 μM, approximately 10% of the label will be bound to the enzyme, and yet there is a 36% increase in the amount of activity recovered. This increased protection may be the result of a conformational change that affects not only the subunit that has label bound. PAH is a tetrameric enzyme and the binding of phenylalanine is positively cooperative (Shiman et al., 1990). There is evidence for a conformational change upon binding of pterin to PAH (Koizumi et al., 1988). A cooperative conformational change upon binding may explain the increased protection against photoinactivation.

The peptide isolated from tryptic digestion of the ANBADP-labeled PAH had the sequence Thr$_{192}$-Leu-Lys-Ala-Leu-Tyr-Lys$_{198}$. The labeled residues were identified as Lys198 and Lys194, with a majority of the label being associated with Lys198. These results are consistent with the observation that nitrenes preferentially insert into the more nucleophilic O–H and N–H bonds (Bayley & Knowles, 1977; Fleet et al., 1972). Three labeled peptides were isolated, accounting for 52% of the total radioactivity injected onto the column. The remainder of the radioactivity was found in the void and was presumed to be the result of degradation of the label during proteolysis. There was no radioactivity associated with any other peptide sequence.

There are several features of this peptide that are of interest. The first is the overall hydrophobicity of the peptide. Four of the seven amino acids are nonpolar residues. Bailey et al. (1989) propose a pterin cofactor side-chain binding domain that includes a limited region of nonpolar amino acid residues for tyrosine hydroxylase. Their results are based on kinetic studies with a series of tetrahydropterin derivatives substituted in the 6-position. Studies were also carried out with PAH and similar results obtained (Bailey et al., 1989). A hydrophobic binding domain for the cofactor side chain is consistent with the K_m for 6-phenyltetrahydropterin being 10-fold lower than that for 6-MPH$_4$ (3 μM and 35 μM, respectively) (Bailey & Ayling, 1978, 1980).

The second feature of interest is the location of the peptide in relation to the total sequence. It is found toward the N-terminus of the PAH sequence and only 40 residues in from the site of proteolytic cleavage that forms the active 35-kDa species (Abate et al., 1988). This is consistent with the model proposed by Bailey et al. (1989) for tyrosine hydroxylase and PAH, where upon activation there is a large conformational change in the pterin binding site.

The sequence identified for the pterin binding site shows a high degree of homology with sequences from both human and mouse PAH (Ledley et al., 1990), rat tyrosine hydroxylase (TYH), and, to a lesser extent, rabbit tryptophan hydroxylase (TRH) (Grenett et al., 1987). In all the enzymes Leu193, Leu196, and Tyr197 are conserved. This striking homology between the various pterin-requiring hydroxylases further supports this sequence as being part of the pterin binding site. One other similarity in the sequences also was striking. Immediately following this sequence is a sequence containing His-X-X-X-Glu-X-X-His which is also highly conserved in the various hydroxylases. This corresponds with the results of Cox et al. (1988) suggesting that the non-heme iron is bound by a triad consisting of two imidazoles and one carboxylate.

rat PAH	Thr-Leu-Lys-Ala-Leu-Tyr-Lys
human PAH	Thr-Leu-Lys-Ser-Leu-Tyr-Lys
mouse PAH	Thr-Leu-Lys-Ala-Leu-Tyr-Lys
rat TYH	Thr-Leu-Lys-Gly-Leu-Tyr-Ala
rabbit TRH	Glu-Leu-Asn-Lys-Leu-Tyr-Pro

One surprising result was that PAH from *C. violaceum* was not covalently modified by ANBADP. Comparison of the sequences, however, shows no homology in the region of interest (Onishi and Benkovic, personal communication):

rat liver	Thr-Leu-Lys-Ala-Leu-Tyr-Lys
C. violaceum	Arg-Gln-Cys-Lys-Leu-Leu-Pro

The corresponding sequence for the bacterial PAH shows less nonpolar residues and the absence of the tyrosine. The Tyr197 may play a role in the tight binding of ANBADP through interaction with the phenyl ring. This would also account for the extensive modification of Lys198. It should be noted that the natural pterin cofactor for *C. violaceum* PAH is unknown. The binding domain for the 6-position side chain may be very different. In various species of nonmethanogenic archaebacteria, tetrahydropterins were identified that contained bulky, negatively charged side chains in the 6-position (Lin

& White, 1988). Jennings and Cotton (1990) have shown structural similarities among pterin binding sites of various enzymes, using monoclonal antibodies produced to a pterin binding site. Their work, however, was limited to mammalian PAH and tyrosine hydroxylase.

The free sulfhydryl associated with activation was labeled with the chromophore fluorescein and the labeled residue identified as Cys236. A concomitant increase in PAH activity with fluorescein bound was similar to that observed for modification by *N*-ethylmaleimide (Parniak & Kaufman, 1981). Cys236 is located in a part of the sequence that contains a significant number of nonpolar residues. Activation of PAH results in full exposure of the sulfhydryl (Fisher & Kaufman, 1973) as well as an enhanced ability to bind to hydrophobic surfaces (Shiman & Gray, 1980). Cys236 is also conserved in both human and mouse PAHs (Ledley et al., 1990). In rat tyrosine hydroxylase and rabbit tryptophan hydroxylase, Cys236 is replaced by an arginine. There is a high degree of homology between all the hydroxylases in the region of Cys236. As there is no evidence that Cys236 is directly involved in activation of the enzyme, it is not surprising that it is not conserved.

Affinity labeling has allowed us to begin to probe the structure of PAH. Further studies are necessary, however, to determine the relationship between the metal binding site, the catalytic phenylalanine binding site, and the pterin binding site.

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