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Interaction of a photolabile NADH analog with rat liver dihydropteridine reductase*

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Dihydropteridine reductase (DHPR,† EC 1.6.99.7), an essential participant in the metabolic hydroxylation reactions of phenylalanine, tyrosine and tryptophan [1–3], catalyzes the NADH-promoted conversion of quinonoid dihydrobiopterin to its 5,6,7,8-tetrahydro derivative. Previous communications from this laboratory have described the isolation of the homogeneous enzyme from rat liver [4], its interaction with NADH [5], its crystallization and preliminary X-ray analysis [6], and recently its cloning and total sequence [7]. To assist in structurally defining the nucleotide binding site, the photolabile NADH analog, A3′-*O*-(3-[*N*-(4-azido-2-nitrophenyl)amino]propionyl)-NADH [8], has been reacted with DHPR, and the resultant derivatized enzyme has been analyzed by proteolytic degradation and peptide purification. This report presents a description of our preliminary observations.

Materials and methods

Chemicals and other agents were obtained from the following sources: rat livers from Pel-Freeze Biologicals, Rogers, AR; NAD^+, NADH, 1,1′-carbonyldiimidazole, and yeast alcohol dehydrogenase from the Sigma Chemical Co., St Louis, MO; β-alanine and 4-fluoro-3-nitroaniline from the Aldrich Chemical Co., Milwaukee, WI; 2-amino-4-hydroxy-6,7-dimethyl-5,6,7,8-tetrahydropteridine hydrochloride from Calbiochem, San Diego, CA; Sephadex G-50 from Pharmacia Inc., Piscataway, NJ; and [3-^{3}H]β-alanine (sp. radioact. 40–85 Ci/mmol) from New England Nuclear, Boston, MA. Arylazido-β-alanyl NAD^+ was synthesized as described by Chen and Guillory [8]; however, the procedures outlined by these workers for the preparation of the tritiated derivative gave very low yields and a scaled-down version of the non-radioactive preparative procedure was employed, e.g. [^{3}H]β-alanine (1.0 mCi) supplied in 0.01 N HCl (1 ml) was treated with 1 M NaOH (0.01 ml), and β-alanine (3.63 mg, 40.7 μmol) plus Na_2CO_3 (10.7 mg, 101 μmol) were added. Ethanol (0.5 ml) and 4-fluoro-3-nitrophenyl azide (9.90 mg, 54.4 μmol) were introduced to the resulting solution, and the mixture was stirred in the dark for 18 hr at 65°. The red product was concentrated and then taken up in H_2O (10 ml). The aqueous phase was extracted two times with ether (5 ml), acidified to pH 2 with 2.5 N HCl, and then extracted three times with ether (5 ml). The ether extract was washed three times with saturated NaCl (5 ml), dried with anhydrous $MgSO_4$, and the ether evaporated to afford *N*-4-azido-2-nitrophenyl-[3-^{3}H]β-alanine (2.62 mg, 10.4 μmol, 25.5%, sp. radioact. 36 Ci/mol). This compound was taken up in dry dimethylformamide (0.1 ml), carbonyldiimidazole (17.1 mg, 106 μmol) was added, and the mixture was stirred in the dark for 15 min at room temperature. NAD^+ (3.71 mg, 5.4 μmol) in H_2O (0.2 ml) was added, and the mixture was stirred overnight. The solvent was evaporated, and the residue was washed repeatedly with acetone and then dried under argon. The solid, readily soluble in H_2O (0.2 ml) was subjected to descending chromatography on Whatman 3MM paper, elution being performed with *n*-butanol/H_2O/acetic acid, 5/3/2. Aqueous elution of the colored band, $R_f = 0.35$, afforded homogeneous arylazido-β-alanyl NAD^+ (0.69 mg, 0.76 μmol, 14.1% relative to NAD^+, sp. radioact. 24 Ci/mol).

Arylazido-β-alanyl NADH. An aqueous solution (0.4 ml) of arylazido-β-alanyl NAD^+ (0.22 mg/ml) was diluted with an equivalent volume of 0.2 M Tris–chloride, pH 7.6, followed by the addition of ethanol (0.2 ml) and yeast alcohol dehydrogenase (~1 mg). The reaction was carried out in the dark at room temperature and monitored by increasing absorbance at 340 nm, which showed quantitative conversion after 3 hr. The mixture was then centrifuged and the clear supernatant applied at 4° to a column of Sephadex G-50 (0.7 × 30 cm) pre-equilibrated with deaerated 0.01 M Tris–chloride, pH 7.6. The NADH analog was eluted as the principal component with further quantities of the same buffer solution. Average yields of ~60–70% were recovered assuming $\varepsilon_{475\,(pH7)} = 4700$ as has been determined for the NAD^+ analog [8]. The product could now be used directly or lyophilized and stored. When stored in solution at −5°, the oxidation rate was ~16% per week when estimated by the loss of absorbance at 340 nm, assuming $\varepsilon_{340} = 6.56 \times 10^3$ in accordance with that reported for NADH [9]. The radioactively labeled NADH analog was prepared by the same procedure, to afford material of sp. radioact. 24 Ci/mol.

DHPR. The enzyme was isolated from rat livers by the procedure of Shahbaz *et al.* [7] and was assayed spectrophotometrically [10] by observing the absorbance change at 340 nm which accompanied the NADH-dependent reduction of the quinonoid dihydropteridine substrate. Preparations were homogeneous by SDS polyacrylamide electrophoresis with specific activity >300 units/min/mg. The substrate was generated in the assay mixture by the oxidation of 2-amino-4-hydroxy-6,7-dimethyl-5,6,7,8-tetrahydropteridine with an excess of potassium ferricyanide. Inhibition constants were obtained from plots of $1/v$ against I at various substrate concentrations, where v is the initial rate of reaction and I is the inhibitor concentration. Regression analyses of the experimental results were carried out with an IBM PC XT computer employing the program of Cleland [11].

Photolysis. Solutions (~0.5 ml) were irradiated in flat-bottomed quartz tubes (0.6 × 9.5 cm) suspended in a refrigerated 5% copper sulfate bath such that the sample was 10 cm from a 660 W, 120 V Acme Lite with approximately 2 cm of the copper sulfate solution in the light path. Irradiation times were for 8 min, unless indicated otherwise, during which time the bath temperature was maintained at 5–10°. Both the external bath and the samples were stirred throughout the irradiations. With these conditions, enzyme degradation could be minimized during irradiation and, therefore, any enzyme activity losses could be directly attributed to photo labeling. If, however, irradiation were carried out for a longer period, significant non-reproducible loss of enzymatic activity occurred leading to variable and inaccurate estimates of ligand insertion.

In experiments where enzyme samples were irradiated in the presence of tritiated NADH analogs, the enzyme-bound radioactivity was determined after exhaustive

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† Abbreviations: DHPR, dihydropteridine reductase; arylazido-β-alanyl NAD^+, A3′-*O*-(3-[*N*-(4-azido-2-nitrophenyl)amino]propionyl)NAD^+; TPCK, L-1-tosylamido-2-phenylethyl chloromethyl ketone; and SDS, sodium dodecyl sulfate.

dialysis against 0.5 M Tris–Cl^-, pH 7.5. The protein concentrations of the dialysates were determined by the method of Lowry *et al.* [12] and the radioactivity by counting in 10 ml Budget-Solve (R.P.I. Corp., Mount Prospect, IL) using a Beckman LS-233 scintillation counter. Absorbance spectra were recorded with a Cary model 219 spectrophotometer. HPLC separations were carried out with a Beckman model 332 liquid chromatograph equipped with a Hitachi model 100-40 variable wavelength detector. The effluent was monitored at 210 nm. Samples (20 μl) were applied to a C_{18} μ-Bondapak (Waters) column (0.39 × 30 cm), and elution was carried out with a linear gradient of 0–70% acetonitrile in 0.1% phosphoric acid applied over 90 min at a flow rate of 1 ml/min. To locate radioactively labeled effluent, 1-ml samples were collected automatically with a FRAC-100 programmable collector (Pharmacia) and counted.

Protein digestion. Samples (0.5 to 1.0 ml) from the irradiation experiments containing approximately 2–5 μM reductase were exhaustively dialyzed against 0.2 M NH_4HCO_3, pH 8.1, and then reacted for 30 hr in a wrist shaker at ambient temperature with TPCK-treated trypsin (Worthington Biochemicals, Freehold, NJ) dissolved in 0.001 N HCl. The molar ratio of reductase to trypsin was 20:1. After lyophilization, the digested products were dissolved in 50 μl of 0.1% phosphoric acid containing 2 M urea, subjected to centrifugal microfiltration, and then analyzed by HPLC.

Results and disscussion

Arylazido-β-alanyl NADH was a good inhibitor of DHPR (Fig. 1) with inhibition being expressed relative to both the nucleotide cofactor ($K_i = 0.14\ \mu$M) and dihydropteridine substrate ($K_i = 0.35\ \mu$M). Kinetic analysis showed the inhibitor to be competitive with the former and mixed or non-competitive with the latter. Optimal conditions for interaction between the photolabile derivative and DHPR required an 8-min irradiation of a solution

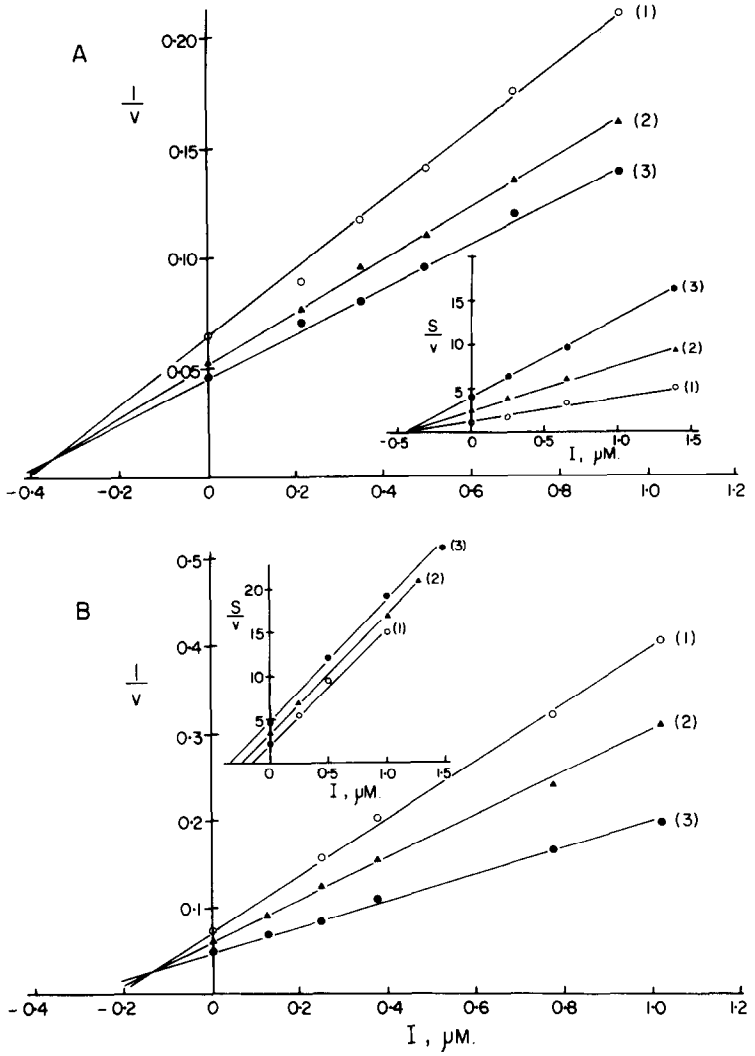

Fig. 1. (A) Inhibition of dihydropteridine reductase by arylazido-β-alanyl NADH measured in the presence of: (1) 18.4; (2) 49.0, and (3) 79.6 μM 2-amino-4-hydroxy-6,7-dimethyl-5,6,7,8-tetrahydropteridine. Enzymatic assays were performed as described in Materials and Methods with an NADH concentration of 87.5 μM. (B) Inhibition measured in the presence of: (1) 33.7, (2) 59.0, and (3) 84.3 μM NADH with a pteridine concentration of 54.3 μM. Results are presented as both the inverse of the initial velocity ($1/v$) and (inset) the ratio of the variable substrate concentration and initial velocity (S/v) against the inhibitor concentration (I).

of 0.1 M Tris–Cl^-, pH 7.0, containing 1 μM enzyme, a 20- to 30-fold molar excess of arylazido-β-alanyl NADH, and 0.2 mM β-mercaptoethanol. With these conditions a loss of 25% enzymatic activity was observed repeatedly. When the photolabile derivative alone was irradiated under these conditions, it exhibited a spectral half-life of 1.4 min. β-Mercapthethanol was present to help stabilize the enzyme; however, at concentrations greater than 1 mM the photolytic interaction of NADH analog and enzyme was eliminated, presumably via the known scavenging action of thiols for nitrenes [13]. When the irradiation experiment was carried out in the presence of NADH at a 25-fold elevated concentration over analog, only a 3–4% loss of enzymatic activity was observed, suggesting that the natural substrate protects the nucleotide binding site.

An experiment was carried out with radioactively labeled arylazido NADH to determine whether the uptake of nucleotide analog corresponded to loss of enzymatic activity. Using a 30-fold excess of the affinity label, a 26.5% loss of enzymatic activity was observed after the usual 8-min irradiation. The irradiated sample was dialyzed exhaustively against 0.1 M Tris–Cl^-, pH 7.5, containing 1 mM β-mercaptoethanol until no radioactivity was observed in the dialysate. Aliquots of the labeled enzyme were then removed for both protein estimation and measurement of radioactivity. It was calculated that approximately 25% labeling of the enzyme had occurred, a figure which correlates well with the observed enzymatic activity loss. Without irradiation, no uptake of radioactivity by the enzyme could be detected.

With higher concentrations of affinity label greater covalent substitution of the enzyme was not observed, probably because of greater absorption of incident irradiation by arylazido NADH molecules not at the active site. This was suggested by the observation that elevation of the apparent $T_{1/2}$ occurred when increased concentrations of the photolabile compound were irradiated alone. Disparity between the observed inhibition in kinetic experiments and the level of labeling achieved is a common feature in this type of experiment [14–16] and probably reflects a distinction between binding at the enzyme active site of molecules of inhibitor which possess the active nitrene and those which interact at the site but are not activated by the irradiation. Both classes of molecule will inhibit the enzyme but labeling will occur with the former only.

To examine the pattern of radioactive labeling in the protein by HPLC, two 600-μl samples, each containing 300 μl reductase (0.0014 μmol) and 300 μl arylazido-β-alanyl NADH (0.065 μmol, sp. radioact. 64.5 Ci/mol), were irradiated in the usual way and then dialyzed exhaustively (four times) against 0.2 M NH_4HCO_3, pH 8.1. The protein concentration and radioactive uptake were measured to confirm the expected 25% enzyme labeling. The sample was then subjected to trypsin digestion and lyophilized, and the residue was taken up in 50 μl of 0.1% phosphoric acid containing 2 M urea. The retention of the major part of the bound radioactivity through these manipulative steps was confirmatory evidence that most of the label was covalently bound. After centrifugal microfiltration, 20-μl aliquots containing approximately 50 μg protein were subjected to HPLC. Application of a linear gradient of 0–70% acetonitrile in 0.1% phosphoric acid at an elution rate of 1 ml/min over a period of 90 min afforded the profile illustrated in Fig. 2. The distribution of the radiolabel was obtained by collecting 1-ml fractions of the effluent and measuring the tritium content. Analysis of the radioactive and absorbance profiles indicated that, in addition to a run-through peak coincident with the major urea absorbance, three peptides could be clearly associated with elevated radioactivity.

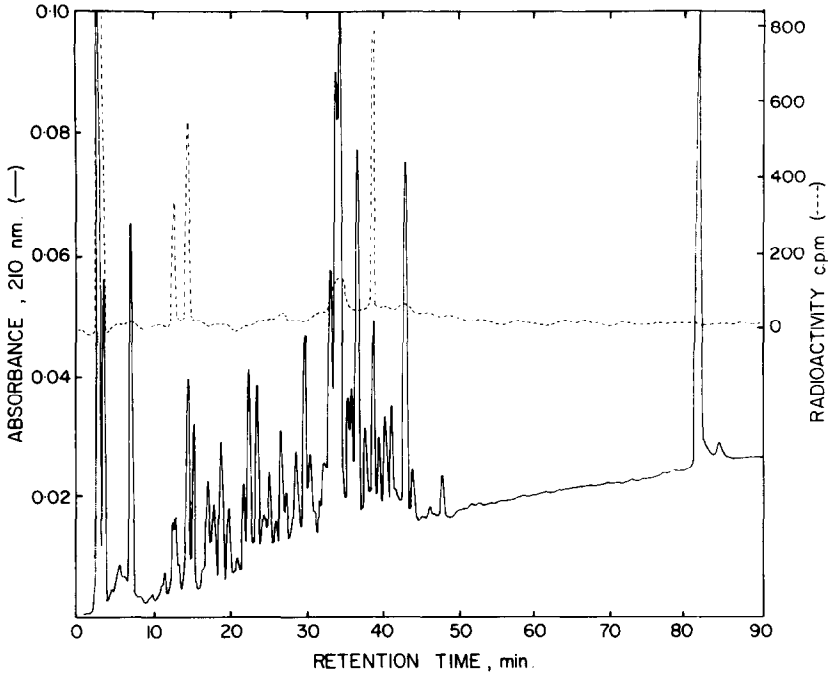

Fig. 2. HPLC and bound radioactivity traces of the tryptic digest of dihydropteridine reductase after photoaffinity labeling with [^{3}H]arylazido-β-alanyl NADH. Peaks attributable to trypsin recorded in a blank run have been excluded from the diagram. The 20-μl samples were prepared as described in Materials and Methods and then applied to a Waters C_{18} μ-Bondapak column which was eluted with a linear gradient of 0–70% acetonitrile in 0.1% phosphoric acid at a flow rate of 1 ml/min. The peaks at 3 and 82 min correspond to the urea employed in the redissolution of the lyophilized trypsin-treated enzyme.

The presence of more than one radioactive peptide was expected since the continuous mobility of the enzyme molecule, the flexibility of the arylazido-β-alanyl substituent on the nucleotide, and the finite lifetime of the nitrene could each contribute to the probability of numerous interactions. The possibility that incomplete tryptic digestion had generated several labeled peptides was discounted because of the lengthy digestion period employed, and an alternate explanation involving the presence of more than one specific nucleotide binding site in an enzyme of subunit size ~25,000 was considered unlikely. The additional possibility of bacterial contamination occurring during the 30-hr digestion was negated by the consistency observed in the HPLC profiles of both labeled and unlabeled enzyme samples digested for times varying from 8 to 36 hr. The specificity exhibited by the photoaffinity NADH analog suggests that any of the three labeled peptides may reside in the vicinity of the active site and, therefore, contribute towards the binding affinity of enzyme and nucleotide. Analysis of the composition and sequence of these peptides will allow selection of the most probable contributors to the nucleotide envelope and prediction of possible configurations for its structure.

In summary, therefore, arylazido NADH was examined as a photoaffinity probe for the nucleotide binding site of rat liver dihydropteridine reductase. When a 30-fold excess of the nucleotide analog was irradiated with the reductase, a 26.5% loss of enzymatic activity was observed and 25% uptake of radioactivity occurred if the tritiated derivative was employed. Irradiation in the presence of NADH led to minimal enzymatic activity loss. Inhibition constants of 0.14 and 0.35 μM were determined for the arylazido-β-alanyl NADH derivative relative to NADH and 2-amino-4-hydroxy-6,7-dimethyl-5,6,7,8-tetrahydropterine respectively. The results suggested specific interaction of the NADH analog at the reductase nucleotide binding site, and the radioactive arylazido-β-alanyl NADH-labeled enzyme was digested and subjected to HPLC analysis to afford radiolabeled peptides as a prelude to active-site structural characterization.

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Effects of drugs on the activity of histamine-*N*-methyltransferase from guinea pig skin

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The principal metabolic pathway of histamine in cutaneous tissue is mediated by histamine-*N*-methyltransferase (HMT) [1–3]. We have succeeded recently in purifying the enzyme from guinea pig skin and have demonstrated that biogenic amines which contain a CH_2—CH_2—NH_2 moiety next to a hydrophobic group (serotonin, tryptamine, dopamine, tyramine,) have intense inhibitory effects on the enzyme activity [4]. Although numerous kinds of drugs containing such a structure are now widely used clinically, and inhibitory effects of antihistaminics, tranquilizers, local anesthetics and antimalarials on HMT activity have been demonstrated [5–7], the molecular mechanism has not been elucidated. We decided, therefore, to examine the structure–inhibition relationship as a possible explanation for the inhibitory effects of these drugs, and to ascertain whether other drugs containing such a structure, whose effects on the enzyme activity have not yet been reported, also inhibited the activity of HMT purified from guinea pig skin.

Materials and methods

Materials. The following chemicals were used: histamine dihydrochloride (Wako, Tokyo, Japan), *S*-adenosyl-L-methionine (SAM; Boehringer, Mannheim West Germany), and *S*-[*methyl*-^{3}H]adenosyl-L-methionine (15 Ci/mmol, Radiochemical Centre, Amersham, Bucks, UK). DEAE-cellulose was from Whatman, Clifton, NJ, U.S.A. Diphenhydramine, diphenylpyraline, promethazine, pyrilamine, tripelennamine, cimetidine, chloropromazine, haloperidol, imipramine, trifluoperazine, dibucaine, lidocaine, procaine, chloroquine and quinacrine were from Sigma, St. Louis, MO, U.S.A. The generous