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Photoaffinity Inhibition of Rat Liver NAD(P)H Dehydrogenase by 3-(α-Acetonyl-*p*-azidobenzyl)-4-hydroxycoumarin†

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ABSTRACT: NAD(P)H dehydrogenase was purified in four steps from a homogenate of rat liver. The final step was affinity chromatography on Sepharose coupled to 3,3′-(*m*-hydroxybenzylidene)bis(4-hydroxycoumarin). The purified enzyme was inhibited competitively with respect to NADH by 3-(α-acetonyl-*p*-nitrobenzyl)-4-hydroxycoumarin (acenocoumarin) (K_i = 1.7 μM). The acenocoumarin was converted into an azide which was used to photoaffinity inhibit the enzyme. Following photolysis in the presence of the azide, the enzyme was inactivated in proportion to the concentration of azide present during irradiation. A maximum of 35–40% inhibition could be achieved by a single irradiation at 254 nm for 1.5 min. This inhibition was noncompetitive with respect to NADH. The inactivation was shown to be specific as acenocoumarin afforded complete protection against inactivation, irradiation was required to achieve inactivation, and the enzyme was unaffected by irradiation alone.

Affinity labeling is a technique which has the potential to identify and distinguish specific binding or catalytic sites on proteins (Singer, 1967). The photoaffinity labeling reagents represent a unique class of affinity labeling reagents (Knowles, 1972; Creed, 1974) since the reactive group is created in situ after the protein has been reacted with a ligand. Thus, in partially characterized multienzyme systems such as that involved in the vitamin K dependent γ-glutamyl carboxylation of the coagulation zymogens (Suttie, 1978), photoaffinity labeling reagents potentially could be used to identify and dissociate enzyme activities associated with the various steps in the process. There is mounting evidence that the epoxide reductase is the enzyme in this multienzyme carboxylation

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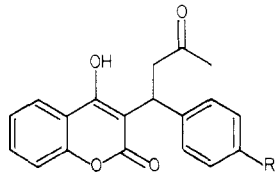
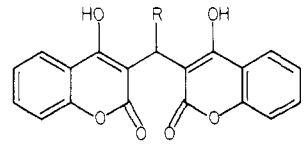
system which is the target acted upon by the widely used indandione and coumarin anticoagulants. We have developed from acenocoumarin[1] (**1**, R = NO_2) a photoaffinity labeling



1, R = H (warfarin)
R = NO_2 (acenocoumarin)
R = N_3 (azidowarfarin)



2, R = H (dicoumarol)
R = 3-OH-C_6H_4
[3,3′-(*m*-hydroxybenzylidene)-bis(4-hydroxycoumarin)]

reagent, 3-(α-acetonyl-*p*-azidobenzyl)-4-hydroxycoumarin (**1**, R = N_3, "azidowarfarin"), which structurally resembles warfarin (**1**, R = H). This reagent could potentially be used to study the active site(s) and mechanism of the epoxide reductase as well as any other enzyme inhibited by **1** (R = H).

Since the mechanism of inhibition of such enzymes by warfarin is unknown and the epoxide reductase has only been partially purified, we have chosen to utilize the liver cytosol enzyme NAD(P)H dehydrogenase (EC 1.6.99.2, also called DT-diaphorase) in the characterization of this photoaffinity labeling reagent. This enzyme was described first by Ernster and co-workers (Ernster & Navazio, 1958; Ernster et al., 1962; Ernster, 1967; Hall et al., 1972), and its isolation in purified form has been reported by several investigators (Ernster et al., 1960, 1962; Hosoda et al., 1974; Rase et al., 1976; Wallin et al., 1978; Wallin, 1979a,b). The enzyme is inhibited by the indandione and coumarin anticoagulants, and it has been suggested that NAD(P)H dehydrogenase or an enzyme very much like it catalyzes the conversion of vitamin K naphthoquinone to vitamin K hydroquinone, a reaction which must occur prior to vitamin K dependent carboxylation of the precursor of prothrombin (Lind & Ernster, 1974; Friedman & Shia, 1976; Sadowski et al., 1976; Girardot et al., 1976; Wallin, 1979a,b; Suttie, 1980). In this report, we describe the development of a novel affinity matrix based on dicoumarol (**2**, R = H), utilizing 3,3′-(*m*-hydroxybenzylidene)bis(4-hydroxycoumarin) (**2**, R = 3-OH-C_6H_4) as a specific ligand for NAD(P)H dehydrogenase as well as the synthesis of azidowarfarin which has been used to photoaffinity inhibit the enzyme.

Materials and Methods

Chemicals. Acenocoumarin, 3-(α-acetonyl-*p*-nitrobenzyl)-4-hydroxycoumarin, was a gift of Ciba-Geigy. Tween 20, BSA, warfarin, NADH, cytochrome *c*, DCIP, and menadione were obtained from Sigma Chemical Co. (St. Louis, MO). All other organic chemicals were obtained from Aldrich Chemical Co. (Metuchen, NJ). Solvents and buffer salts were of ACS reagent grade.

Organic Syntheses. Melting points were determined on a Thomas-Hoover capillary melting point apparatus and are uncorrected. Infrared spectra were obtained on a Perkin-Elmer 137B infracord spectrometer, and proton magnetic resonance spectra were obtained on a Varian T-60 instrument. High-pressure liquid chromatography (HPLC) utilized a Waters Associates instrument equipped with a μBondapak C18 column and a Gilson HM holochrome ultraviolet monitor. Products were detected at 308 nm. Elemental analysis was performed by Spang Microanalytical Laboratory, Eagle Harbor, MI. Mass spectra were obtained through Dr. Catherine Costello at the Massachusetts Institute of Technology, Cambridge, MA.

Synthesis of 3,3′-(m-Hydroxybenzylidene)bis(4-hydroxycoumarin) (HBBHC). A mixture of 3-hydroxybenzaldehyde (1.22 g; 10 mmol) and 4-hydroxycoumarin (3.20 g; 20 mmol) in ethanol (50 mL) was heated to reflux, giving a yellowish solution. After 2 h at reflux, the solution was filtered by gravity while hot to remove a small amount of insoluble material. The filtrate was concentrated under reduced pressure on a rotary evaporator to a syrup, which was dissolved in acetic acid. When this solution was chilled, the product crystallized and was collected by suction filtration and dried in vacuo over KOH. The yield of crude 3,3′-(*m*-hydroxybenzylidene)bis-(4-hydroxycoumarin) (mp 241–243 °C) was 1.9 g (45%). An analytical sample was prepared by evaporative crystallization from acetic acid at ambient temperature and was dried in vacuo at 100 °C for 24 h prior to analysis: mp 242–243 °C. Anal. Calcd for $C_{25}H_{16}O_7$: C, 70.08; H, 3.77. Found: C, 70.12; H, 3.76.

In repeating this procedure on a larger scale, it was found advisable to recrystallize the 3-hydroxybenzaldehyde from ethanol prior to use (mp 100–102 °C). In this case, refluxing 25 mmol of aldehyde with 50 mmol of 4-hydroxycoumarin in the same volume (50 mL) of ethanol for 2 h resulted in crystallization of the crude product (mp 240–242 °C) in 68% yield directly from the chilled ethanolic reaction solution.

Synthesis of 3-(α-Acetonyl-p-azidobenzyl)-4-hydroxycoumarin ("Azidowarfarin"). Acenocoumarin (1.4 g; 4 mmol) was dissolved by warming in about 250 mL of ethanol and shaken for 1 h under an atmosphere of hydrogen (3-atm pressure) in the presence of 0.1 g of 10% palladium on charcoal catalyst. The catalyst was removed by gravity filtration, and the filtrate was concentrated to dryness under reduced pressure on a rotary evaporator, leaving a golden-brown solid which was used without further purification.

The crude amine (1.3 g; 4 mmol) was dissolved by warming in 140 mL of 10% HCl, and the solution was filtered by gravity and the filtrate chilled in an ice bath. To this stirred, cold solution was added dropwise an ice-cold solution of $NaNO_2$ (0.4 g; 5.8 mmol). Stirring was continued for 10 min at 0 °C following addition of the nitrite. Excess nitrous acid was destroyed by addition of 1 mL of ice-cold 8 M urea, and then an ice-cold solution of NaN_3 (0.26 g; 4 mmol) in 2 mL of H_2O was added. The reaction mixture was stirred in dim light for 1 h on ice and for 90 min at room temperature. The product separated as a light yellow floating precipitate and was protected from light as much as possible during subsequent manipulations.

The crude azide was collected by suction filtration, washed thoroughly with water, and dried in vacuo over $CaSO_4$. The azidowarfarin (yield 0.7 g; 50%) melted at 80–95 °C and appeared homogeneous by TLC (silica gel, benzene/ethyl acetate 4:1; R_f 0.53). The infrared spectrum (chloroform) exhibited a strong azide absorbance at 2120 cm^{-1}, and other infrared and proton magnetic resonance spectral features were consistent with the assigned structure. Attempts to purify the crude azide by recrystallization or silica gel chromatography were unsuccessful, presumably due to decomposition of the compound. The structure was confirmed by high resolution mass spectrometry: M^+ *m/e* 349.10744 (calcd 349.10625).

[1] Abbreviations used: warfarin, 3-(α-acetonylbenzyl)-4-hydroxycoumarin; acenocoumarin, 3-(α-acetonyl-*p*-nitrobenzyl)-4-hydroxycoumarin; azidowarfarin, 3-(α-acetonyl-*p*-azidobenzyl)-4-hydroxycoumarin; dicoumarol, 3,3′-methylenebis(4-hydroxycoumarin); BSA, bovine serum albumin; DCIP, 2,6-dichloroindophenol; mS, millisiemens; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; DMF, dimethylformamide.

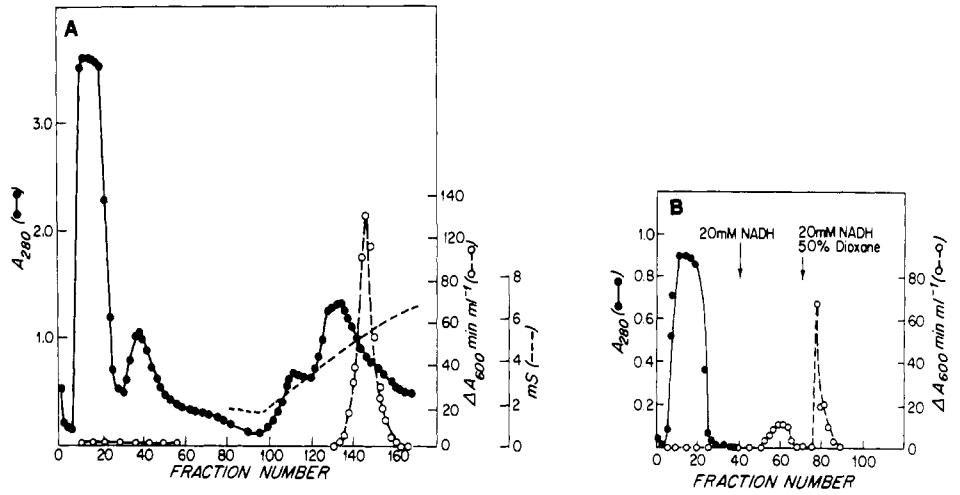


FIGURE 1: (A) Chromatography of NAD(P)H dehydrogenase on hydroxylapatite. Chromatography was performed as described under Materials and Methods. Enzyme activity was detected by assay with DCIP and NADH. (B) Chromatography of NAD(P)H dehydrogenase on HBBHC-Sepharose.

Preparation of Sepharose-3,3'-[m-[(ethylsulfonyl)ethoxy]benzylidene]bis(4-hydroxycoumarin) (HBBHC-Sepharose). About 50 mL of Sepharose CL-4B (Pharmacia Fine Chemicals, Piscataway, NJ) was washed on a Büchner funnel with about 500 mL of water. The filtered resin (50 g) is suspended in 50 mL of 1 M Na_2CO_3, pH 11, and mixed with 5 mL of divinyl sulfone on a magnetic stirrer in a fume hood for 60 min. The resin was then filtered by vacuum filtration on a Büchner funnel (S&S filter paper 273) and was successively washed with 500 mL of 1 M Na_2CO_3, pH 11, H_2O, and 1 M Na_2CO_3, pH 11. To the resin was added 250 mg of 3,3'-(*m*-hydroxybenzylidene)bis(4-hydroxycoumarin) dissolved in 5 mL of DMF and 45 mL of 1 M Na_2CO_3, pH 11. The resin was stirred 20 h at room temperature and washed successively with approximately 500 mL of 1 M Na_2CO_3, pH 11, H_2O, methanol, and H_2O. It was equilibrated with 20 mM potassium phosphate buffer, pH 7.0, prior to use. A given lot of resin was used only once for purification of NAD(P)H dehydrogenase from the hydroxylapatite eluant.

Assays. NAD(P)H dehydrogenase was assayed in two ways. After a purification step, the enzyme was incubated in 50 mM Tris-HCl buffer, pH 7.5, with 0.1 mM NADH and 40 μM 2,6-dichloroindophenol (DCIP); the activity was assayed spectrophotometrically at room temperature by measuring the rate of decrease in absorbancy at 600 nm (Dallner, 1963). For kinetic experiments, DCIP was replaced by menadione (2-methyl-1,4-naphthoquinone) plus cytochrome *c* as the terminal electron acceptor. The incubation mixture contained 75 μM cytochrome *c* in 50 mM potassium phosphate buffer, pH 7.5, while the menadione and NADH concentrations were varied from 1 to 5 μM and 25 to 400 μM, respectively; the rate of increase in absorbancy at 550 nm was assayed at room temperature (Hollander et al., 1975). A Gilford 240 spectrophotometer was used for all enzymatic assays. The concentrations of DCIP and of cytochrome *c* were determined by using molar extinction coefficients of 21 000 and 18 500, respectively. Enzyme activity was expressed in IU mL^{-1} min^{-1} (1 μmol of product mL^{-1} min^{-1}) where NADH was 0.1 mM and DCIP was 40 μM (Ernster, 1967).

Protein was measured by the method of Lowry et al. (1951) with BSA as a standard. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was performed on 10% gels according to the method of Weber & Osborn (1972). Samples in 0.1% sodium dodecyl sulfate were reduced with 20 mM dithiothreitol for 60 min at 37 °C, alkylated with 40 mM iodoacetamide for 20 min at 37 °C, and heated at 100 °C for 60 s prior to electrophoresis. Protein bands were located by staining with 0.125% Coomassie brilliant blue (R250) in acetic acid/50% methanol (1:9 v/v) following fixation in 20% sulfosalicylic acid. Molecular weight markers were obtained from Pharmacia Fine Chemicals.

Gel filtration chromatography on Sephadex G-25 (medium) was used to remove excess reagents in the inhibitor experiments. A 2.5 × 20 cm calibrated column was used and a flow rate of 1 mL/min maintained.

Purification of NAD(P)H Dehydrogenase. A modification of the method of Rase et al. (1976) was used. Two male CD rats (250 g) were starved overnight and then decapitated. All subsequent procedures were done at 0–4 °C unless indicated otherwise. The livers (17.6 g wet weight) were removed and homogenized in 0.25 M sucrose (30% w/v) with several passes of a Potter-Elvehjem motor-driven homogenizer rotating at 2000 rpm. This homogenate was centrifuged at 10000*g* for 15 min to remove insoluble material, and the resulting supernatant then was centrifuged at 100000*g* for 60 min. The supernatant (28 mL) from this centrifugation was adjusted to pH 5.9 with 0.1 N HCl and adsorbed with 10 g of CM-cellulose (Whatman CM-32) which had been equilibrated with 10 mM potassium phosphate buffer, pH 5.9 The resin was removed by centrifugation at 2000*g*, washed once with 28 mL of the same buffer, and recentrifuged. The light yellow supernatants were combined and adjusted to pH 7.0 with 1 M NaOH. This solution was applied to a 4 × 3 cm hydroxylapatite column equilibrated with 20 mM potassium phosphate buffer, pH 7.0. Nonadsorbed protein was washed from the column with the same buffer, and the enzyme was eluted at a flow rate of 15 mL/h, utilizing a linear gradient of 20–150 mM potassium phosphate buffer, pH 7.0 (total volume 400 mL; Figure 1A). Fractions of 5 mL were collected, and those with enzyme activity, which eluted between 4 and 6.5 mS (see Figure 1), were pooled and applied to a 29-mL column of the affinity resin equilibrated with 20 mM potassium phosphate, pH 7.0. Unbound protein was removed by washing with the same buffer until A_{280nm} was less than 0.02 and the enzyme eluted in 5-mL fractions with 50 mL of 20 mM NADH in 20 mM potassium phosphate, pH 7.0, followed by 20 mM NADH in 10 mM potassium phosphate, pH 7.0, and 50% dioxane (scintillation grade) at a flow rate of 1 mL/min (see Figure 1B). All fractions containing activity were pooled and concentrated in an Amicon 50-mL cell on a PM-10 membrane to 5 mL. Dioxane and NADH were removed by passage over Sephadex G-25 (Pharmacia PD-10 column) equilibrated in

Table I: Purification of NAD(P)H Dehydrogenase from Rat Liver

purification step	vol (mL)	protein mg/mL	protein total	activity[a] IU[b]/mL	activity[a] total	sp act. (IU/mg)	purification (fold)	recovery (%)
100000*g* supernatant	28	45.8	1282	123.8	3466	2.7	1	100
CM-cellulose supernatant	46	16.3	750	66.7	3068	4.1	1.5	88.5
hydroxylapatite eluant	58	0.73	42.3	37.1	2152	50.9	18.8	62.1
HBBHC–Sepharose	7	0.24	1.7	185.7	1300	765	283	37.5

[a] NAD(P)H dehydrogenase was measured with DCIP and NADH as described under Materials and Methods. [b] IU is international units; see Materials and Methods.

10 mM potassium phosphate, pH 7.0. The protein was pooled and stored frozen at −80 °C until used. The entire procedure was performed within 48 h to minimize loss of enzymatic activity. Because the enzyme contains FAD, it was shielded from light throughout the procedure.

Irradiation of Enzyme. Samples were placed in a 1- or 3-mL quartz cuvette located 1 cm from the source in the dark, and photolysis was accomplished by irradiating for 1.5 min with a 254-nm Mineralight. Control experiments in which the enzyme was irradiated alone showed no loss of enzyme activity during this time, although prolonged irradiation (5 min) resulted in about 25% loss of activity. A preliminary experiment with 1 mM azidowarfarin in methanol and analysis of the reaction mixture by HPLC revealed 90% disappearance by 1 min and complete disappearance of the azide by 2 min. While these conditions were different than that for photolysis in the presence of enzymes, this experiment did provide useful information regarding the stability and lifetime of the nitrene. No Tween 20 or BSA was present during photolysis, as these were found to interfere with the labeling due to the UV-absorbing properties of both materials at 254 nm. All photolysis was done in the presence of Tris buffer which has been suggested as a scavenger for photoaffinity labeling reagents (Baley & Knowles, 1977).

Results

Purification of NAD(P)H Dehydrogenase. The results of the purification of NAD(P)H dehydrogenase are presented in Table I. Maximum recovery and yields were obtained by sequential elution with 20 mM NADH followed by 20 mM NADH–50% dioxane. Elution with only NADH did not give a maximal yield. Elution with the NADH–dioxane mixture without the first elution with NADH alone resulted in lower specific activity. On the basis of activity, the enzyme was purified 283-fold with an overall recovery of 37.5%. The fold purification is about half that reported by others, but the initial activity of the enzyme used in the present studies was almost 4 times greater than that of similar preparations in other reports (Rase et al., 1976; Hosoda et al., 1974; Wallin, 1979a). This may be why the specific activity of our purified NAD(P)H dehydrogenase is 2–10 times higher (765 IU or 1.28×10^4 nkat/mg) than that of any other previous preparation. The high degree of purity of the enzyme was confirmed by analysis of reduced and alkylated purified protein on 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Figure 2). There is a major staining band at an M_r of 33 000. This is a somewhat higher molecular weight for NAD(P)H dehydrogenase than reported by other workers (Rase et al., 1976; Wallin, 1979a; Hosoda et al., 1974) and may be due to differences in the method used for sodium dodecyl sulfate–polyacrylamide gel electrophoresis analysis, the method used for purification, as well as strain differences in the rats used.

Attempts to use the resin to purify the enzyme from the CM-cellulose or 100000*g* supernatant fraction were not successful, presumably due to nonspecific binding of other proteins

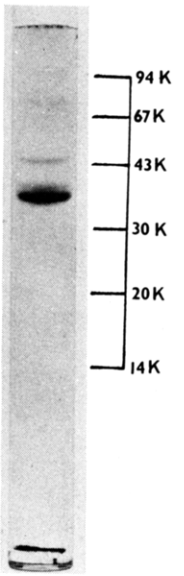


FIGURE 2: Electrophoresis of purified NAD(P)H dehydrogenase. The gel was 10% polyacrylamide and contained 40 μg of reduced and alkylated protein.

in those fractions to the HBBHC–Sepharose. On the other hand, the specificity of the HBBHC–Sepharose was demonstrated because divinyl sulfone treated Sepharose CL-4B failed to bind any NAD(P)H dehydrogenase from the hydroxylapatite eluant (data not shown). These results indicate that the NAD(P)H dehydrogenase obtained was suitable for use in affinity-labeling studies with azidowarfarin.

Inhibition of NAD(P)H Dehydrogenase. We first confirmed that the enzyme exhibited ping-pong kinetics toward NADH and menadione when cytochrome *c* was used as the terminal electron acceptor (Hosoda et al., 1974; Hollander et al., 1975; Hollander & Ernster, 1975). It has been shown previously that warfarin inhibits the NAD(P)H dehydrogenase competitively with respect to reduced pyridine nucleotide (Hosoda et al., 1974). We established that acenocoumarin and azidowarfarin as well as warfarin were competitive inhibitors of NAD(P)H dehydrogenase with respect to NADH. For these experiments, the enzyme eluted from the hydroxylapatite column was used and the type of inhibition evaluated by the graphical method described by Dixon & Webb (1979). Inhibition was measured with NADH as the substrate and DCIP as the terminal electron acceptor. The $K_{m(app)}$ for NADH was determined to be 88 μM (Figure 3), and the K_i's for acenocoumarin (Figure 3A) and azidowarfarin (Figure 3B) were 6 and 1.7 μM, respectively. This compares favorably with warfarin (K_i = 17 μM, Figure 3C). The best competitive inhibitor was the photolysis product(s) of azidowarfarin (K_i = 100 nM, Figure 3D), which was prepared by irradiating a 1 mM solution of azidowarfarin in 50 mM Tris-HCl buffer, pH 7.5, at 254 nm for 1.5 min. As seen in Figure 3D, this solution contained an effective competitive inhibitor. No attempt was made to isolate the photolysis product(s). Col-

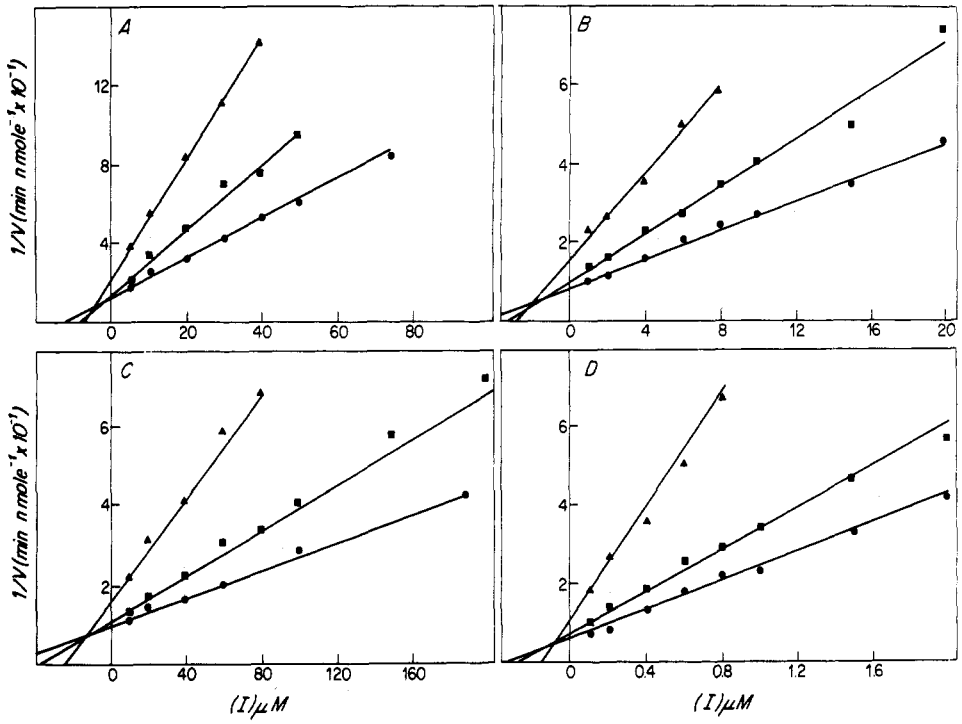


FIGURE 3: Determination of K_i of acenocoumarin (A), azidowarfarin (B), warfarin (C), and the photolysis product of azidowarfarin (D). For the experiments, 10 units of enzyme was used, and the DCIP was 40 μM. The experiments were done at 23 °C. The points are experimental, and the lines were fitted by unweighted linear regression analysis; the correlation coefficient for each line was 0.99 or greater. NADH: 50 μM (▲); 100 μM (■); 150 μM (●). V_{max} was determined as 1.88 mmol min^{-1} mL^{-1}.

lectively, these results demonstrated that all of the intermediates, including the parent acenocoumarin and final product(s) produced by photolysis of azidowarfarin, were potent competitive inhibitors of NAD(P)H dehydrogenase.

In contrast, the photolysis for 1.5 min of 300 μM azidowarfarin at 0 °C 1 cm from a 254-nm Mineralight in the presence of 0.17 IU/mL of enzyme (hydroxylapatite eluant) resulted in noncompetitive inhibition of the enzyme (Figure 4). For this experiment, the photolysis reaction products were removed by passage of the photolyzed mixture over Sephadex G-25, and residual enzyme activity eluting in the void volume was assayed with menadione and cytochrome *c* as the terminal electron acceptor. On the basis of the change in V_{max}, 40% inhibition of the enzyme was achieved. These data show that the enzyme is inhibited noncompetitively, as predicted for an affinity reagent, and that both nonreacted and inhibited enzyme exhibit ping-pong kinetics with respect to NADH.

Azidowarfarin was established as a specific affinity inhibitor reagent for NAD(P)H dehydrogenase by the following experiment. Affinity-purified enzyme (62 μg/mL) was diluted 10-fold in 50 mM Tris-HCl buffer, pH 7.5, and treated as follows: one aliquot was kept as 0 °C; a second was irradiated as described under Materials and Methods; a third and fourth were irradiated identically with the second but in the presence of 1 and 2 mM azidowarfarin. A fifth aliquot was pretreated at 0 °C for 15 min with 2 mM acenocoumarin, and azidowarfarin was added to 600 μM and irradiated identically with the second aliquot. All samples were made 0.01% in BSA and 1% in Tween 20 and chromatographed on G-25 to remove noncovalently bound reaction products. The enzyme was assayed with NADH and menadione with cytochrome *c* as the terminal acceptor. These results are summarized in Figure 5. Whereas 1 and 2 mM azidowarfarin caused noncompetitive inhibition, neither irradiation alone nor irradiation in the presence of acenocoumarin caused any inhibition (<2%).

The degree of inhibition exhibited a concentration dependence with maximal inhibition (~35%) occurring at 1 mM azidowarfarin (Figure 6). Percent inhibition was determined in terms of the change in V_{max}. These experiments were performed identically with those described in Figure 5 with affinity-purified enzyme (62 μg/mL). In other experiments, it was noted that up to 40% inhibition could be obtained (see Figure 4).

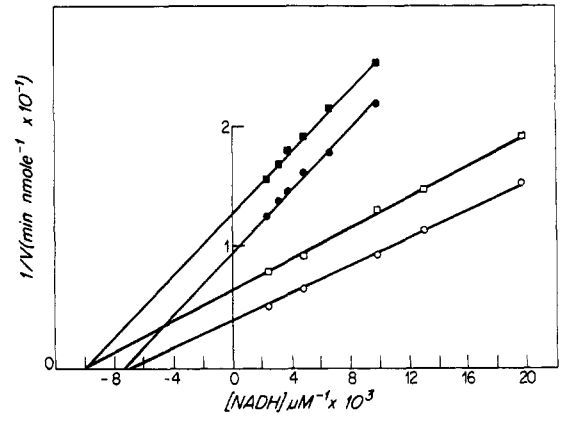


FIGURE 4: Noncompetitive inhibition of NAD(P)H dehydrogenase by azidowarfarin. The enzyme was reacted with azidowarfarin and photolyzed as described under Results. Unbound reaction products were removed by gel filtration over a Sephadex G-25 and assayed at 5 (○, ●) and 2 μM (□, ■) menadione with NADH varied from 25 to 400 μM and cytochrome *c* as the terminal electron receptor. Closed symbols are the azidowarfarin-treated enzyme, and open symbols are enzyme treated identically in the absence of azidowarfarin. The points are experimental and the lines fitted by unweighted linear regression. The correlation coefficient was 0.97 or greater for each line.

Discussion

This study was undertaken to determine the feasibility of developing photoaffinity labeling reagents for the warfarin-inhibitable enzyme involved in the vitamin K dependent carboxylation of proteins. NAD(P)H dehydrogenase was chosen for this study as it is an enzyme which can be extracted

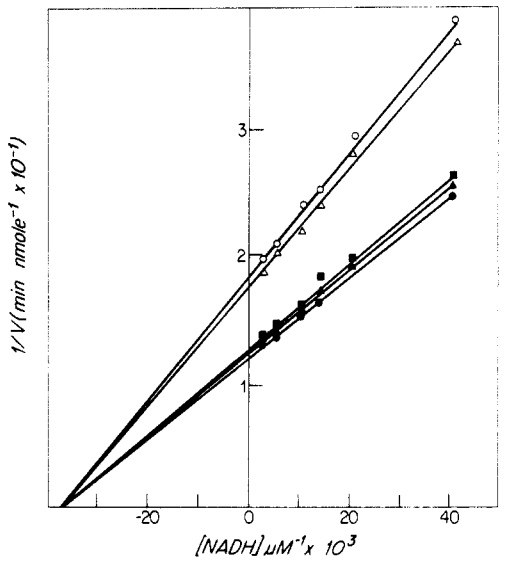


FIGURE 5: Inhibition of NAD(P)H dehydrogenase by azidowarfarin. Irradiation and separation of the enzyme was performed as described under Results and Discussion: 2 mM (○) and 1 mM (△) azidowarfarin, V_{max} = 0.537 and 0.574 IU mL^{-1} min^{-1}, respectively; nonirradiated enzyme (●), V_{max} = 0.807 IU mL^{-1} min^{-1}; irradiated enzyme (▲), V_{max} = 0.807 IU mL^{-1} min^{-1}; acenocoumarin-treated enzyme followed by irradiation in the presence of azidowarfarin (■), V_{max} = 0.794 IU mL^{-1} min^{-1}. The points are experimental and lines fitted by unweighted linear regression analysis. The correlation coefficient of all lines was 0.99.

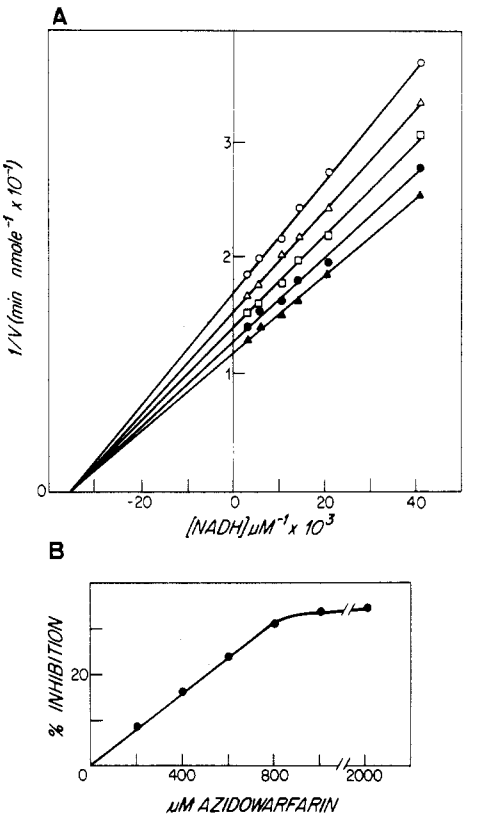


FIGURE 6: Inhibition of NAD(P)H dehydrogenase by increasing concentrations of azidowarfarin. (A) Assay of azidowarfarin reacted enzyme with menadione (2 μM) and NADH from 25 to 400 μM with cytochrome *c* as the terminal acceptor. Azidowarfarin was 800 (○), 600 (△), 400 (□), 200 (●), and 0 μM (▲). The points are experimental and the lines fitted by unweighted linear regression. The correlation coefficients were 0.993 or greater. (B) Percent inhibition of NAD(P)H dehydrogenase based on a decrease in V_{max}. The last two points at 1 and 2 mM are taken from Figure 5.

and purified from liver cytosol and because of its possible involvement in the reduction of vitamin K naphthoquinone, a reaction which must occur before vitamin K dependent carboxylation. Several authors have reported the purification of NAD(P)H dehydrogenase in a series of steps (Hosoda et al., 1974; Rase et al., 1976; Wallin et al., 1978). We have used principally the method of Rase et al. (1976) but have included as the last step an affinity chromatography step which uses a novel resin, HBBHC–Sepharose. The ligand 3,3′-(*m*-hydroxybenzylidene)bis(4-hydroxycoumarin) was chosen to couple to the resin because of the reported high anticoagulant activity of related compounds (Dezelic & Trkovnik, 1964; Arora et al., 1967). The apparent stability of such affinity resins prepared with divinyl sulfone is due to the chemical inertness of the ether bonds which covalently bind the coumarin derivative; the excellent flow properties of the resin result from extensive cross-linking of the Sepharose by the divinyl sulfone (Porath & Sundberg, 1972). The fact that a single protein elutes in two different fractions (Figure 1B) probably represents heterogeneity in the resin due to the impurities of the Sepharose or local high and low concentrations of the ligand within the matrix.

It was demonstrated that acenocoumarin and derivatives of it were effective competitive inhibitors of NAD(P)H dehydrogenase with respect to NADH, with affinities about 3–10 times greater than that of warfarin (K_i = 1.7–4.0 vs. 17 μM). The most effective inhibitor was the product of azidowarfarin following photolysis (K_i = 100 nM). No attempt was made to isolate and characterize this (these) compound(s). As reviewed by Baley & Knowles (1977), arylnitrenes can undergo a variety of intra- and intermolecular rearrangements as well as insertion into O–H or N–H bonds and hydrogen abstraction reactions. It is unknown, however, to what extent such side reactions occur when the nitrene is present in the enzyme receptor site, and it also has been reported that as opposed to alkyl azides, aryl azides exhibit low susceptibility to intramolecular rearrangements following photolysis (Nielsen et al., 1975). The formation of this (these) photolysis product(s) in the presence of enzyme generates an effective competitive inhibitor in situ, and this could account for the apparent saturation with regard to affinity inhibition (i.e., noncompetitive inhibition) occurring at 35–40% inhibition with the purified enzyme (Figures 4 and 6B). It was clearly established, however, that photolysis of azidowarfarin in the presence of enzyme converted it to a noncompetitive inhibitor (Figure 4). This inhibition was irreversible as passage of the enzyme over G-25 did not result in restoration of enzyme activity, whereas if the azidowarfarin was not irradiated, gel filtration resulted in full recovery of NAD(P)H dehydrogenase activity (Figure 5).

That azidowarfarin fulfills all other criteria for a specific photoaffinity inhibitor for NAD(P)H dehydrogenase can be summarized as follows: First, photolysis of the enzyme has no effect on the enzyme (Figure 5). Second, prephotolysis of the reagent leads to a competitive inhibitor (Figure 4) which can be removed from the enzyme. Third, the enzyme does not irreversibly react with the reagent unless photolysis occurs (Figure 6). Fourth, the labeling site shows saturation kinetics for photochemical labeling (Figure 6). Because of the small amounts of enzyme used, no attempt was made to increase the degree of inhibition by isolating unlabeled enzyme and rephotolyzing in the presence of azidowarfarin. The observed 40% maximum (Figure 4) inhibition is comparable to other similar systems in which only a single photolysis reaction is used to inactivate the enzyme (Campbell & Gioannini, 1979). Fifth, the parent ligand, acenocoumarin, afforded full protection against labeling by the photoaffinity inhibitor. Based

on these observations, there is no evidence for nonspecific inhibition, although certainly nonspecific covalent labeling of proteins is possible with such reagents. We have thus fulfilled all criteria for an affinity labeling process (Wofsy et al., 1962). Photoaffinity labeling represents a special case of affinity labeling reagents because they can potentially act by generating in the active site free radicals which can inactivate the enzyme and result in noncompetitive inhibition. It thus still remains necessary to demonstrate incorporation by use of a radiolabeled ligand or chemical identifiction of the enzyme–ligand covalent complex. We feel, however, that collectively these results show that the azidowarfarin can be used to investigate the mechanism and role of NAD(P)H dehydrogenase and other similar enzymes which are involved in vitamin K dependent carboxylation reactions.

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