Synthesis of the Photoaffinity Probe 3-(p-Azidobenzyl)-4-hydroxyocoumarin and Identification of the Dicoumarol Binding Site in Rat Liver NAD(P)H:Quinone Reductase (EC 1.6.99.2)*

(Received for publication, October 2, 1990)

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A photoaffinity analog of 4-hydroxyocoumarin containing an azidobenzyl group at the 3-position and, if desired, carbon-14 or tritium radionuclides has been synthesized and characterized. This compound, 3-(p-azidobenzyl)-4-hydroxyocoumarin, serves as an effective competitive inhibitor of the dicoumarol-sensitive NAD(P)H:quinone reductase (EC 1.6.99.2; DT-diaphorase) from rat liver, having an apparent inhibition constant of $6.6 \times 10^{-8}$ M, a value comparable to that observed for dicoumarol ($1.7 \times 10^{-9}$ M), significantly lower than for Warfarin ($3.3 \times 10^{-5}$ M) and well within the range required of an effective photoaffinity reagent. Irradiation of the reductase with ultraviolet light in the presence of the photoprobe resulted in the covalent labeling of up to 10% of the protein. Greater than 99% of the covalent incorporation is precluded by the addition of 15 μM dicoumarol, consistent with the specific labeling of the 4-hydroxyocoumarin binding site of this enzyme by this photoaffinity reagent. Further evidence of a high degree of specificity is provided by the isolation and sequence analysis of the peptides covalently modified by this reagent. A single region within the protein was found to be labeled, with threonine 127 and tyrosine 128 being the only amino acid residues that were observed to be modified. These results, for the first time, define a portion of the 4-hydroxyocoumarin binding site within a protein that has a well-established sensitivity to this type of anticoagulant and, because dicoumarol serves as a competitive inhibitor for pyridine nucleotides in this enzyme, may also define a portion of this unusual pyridine nucleotide binding site. In addition, these results suggest that this reagent may be effective as a highly specific photoaffinity probe in the identification of other proteins that are similarly inhibited by 4-hydroxyocoumarin derivatives, such as the microsomal enzymes associated with the vitamin K-dependent carboxylation system.

The flavoprotein NAD(P)H:quinone reductase (EC 1.6.99.2), also referred to as DT-diaphorase, catalyzes an obligatory two-electron reduction of a variety of quinones, including menadione, by either NADH or NADPH (Ernst et al., 1960; Iyanagi and Yamazaki, 1970; Huang et al., 1979; Iyanagi, 1987). This enzyme is distributed widely in nature and may function to protect tissues against the toxicity of quinones and their metabolic precursors by minimizing redox cycling and the generation of the toxic superoxide anion (Thor et al., 1982; Lind et al., 1982; Miller et al., 1986). This reductase may also be involved in the generation of vitamin K, hydroquinone, the redox state required by the vitamin K-dependent carboxylation system (Wallin et al., 1978, 1987; Wallin and Suttie, 1981).

It is well established that this enzyme is highly sensitive to inhibition by 4-hydroxyocoumarin-based anticoagulants such as Warfarin and dicoumarol (Ernst, 1967). Kinetic evidence suggests that this reductase utilizes a binary complex (ping-pong)-type catalytic mechanism and apparently has multiple binding sites for its two substrates, the quinone and the pyridine nucleotide (Rase et al., 1976). During steady-state turnover, both dicoumarol and Warfarin serve as competitive inhibitors for NADH and NADPH and as noncompetitive inhibitors with respect to menadione (Hall et al., 1972). Dicoumarol has been shown to prevent the binding of NADPH and the subsequent reduction of the noncovalently bound FAD cofactor within the enzyme (Huang et al., 1979). Both menadione and dicoumarol can apparently bind to the oxidized enzyme simultaneously; however, only menadione will bind to the reduced enzyme. These results are consistent with the presence of two independent substrate binding sites (Hosoda et al., 1974).

It is apparent, then, that although 4-hydroxyocoumarin derivatives are in many ways structural homologs of menadione they bind preferentially to the pyridine nucleotide binding site within this protein. However, these derivatives have little effect on other pyridine nucleotide-dependent enzymes, which makes this site within this reductase somewhat unique (Murray et al., 1982). What structural features are responsible for this unique sensitivity? What is the relationship between the coumarin binding site and other sites within this protein? How does the coumarin binding site within this reductase compare with the binding site(s) within other proteins that are sensitive to 4-hydroxyocoumarin-based anticoagulants?

As one means of addressing these issues, we report in this paper the design and synthesis of a novel photoaffinity analog of 4-hydroxyocoumarin, 3-(p-azidobenzyl)-4-hydroxyocoumarin. The azidobenzyl group, when irradiated with ultraviolet radiation, generates a highly reactive nitrene intermediate that is capable of forming a covalent bond with portions of the protein within its immediate vicinity (Bayley and Knowles, 1977; Chowdhry and Westheimer, 1979). We demonstrate that this photoaffinity derivative serves as an effective inhibitor of the NAD(P)H:quinone reductase and, upon activation, becomes covalently incorporated specifically.

*This research was supported in part by grants from the American Heart Association (Ohio Affiliate and Central Ohio Chapter). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby indicated "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
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within the coumarin binding site. The amino acid residues that become covalently attached to this reagent were identified, and, for the first time, a portion of the coumarin binding site within this class of coumarin anticoagulant-sensitive enzymes is defined.

EXPERIMENTAL PROCEDURES

RESULTS

Preparation of 3-(p-Azidobenzyl)-4-hydroxycoumarin—The principal step in the synthesis of AzBHC involves the Friedel-Crafts condensation of substituted malonic acid derivatives with phenol to produce various 3-substituted 4-hydroxycoumarin derivatives (Shah et al., 1960). In this study, a photoaffinity derivative of 4-hydroxycoumarin was synthesized by the introduction of a p-azidobenzyl group at the 3-position through the condensation of p-nitrobenzylmalonic acid with phenol followed by the conversion of the nitro group to the azide. Radiolabeled derivatives were also prepared readily by this synthetic approach through the utilization of commercially available [U-14C]phenol or, alternatively, [U-3H]phenol. The five-step synthetic scheme used to prepare AzBHC is summarized in Fig. 1. With the exception of the diazonium intermediate, the product from each synthetic step was isolated and characterized before continuing on with the synthesis (see “Experimental Procedures”).

[14C]AzBHC was prepared similarly except for modifications to the Friedel-Crafts condensation which were required to improve yields at a 0.5-μmol scale of synthesis. Reagent stoichiometries are apparently critical in this condensation (phenol:malonic acid:zinc chloride:phosphorus oxychloride, 1:1:2:2) (Shah et al., 1960). However, it was determined that a large excess of zinc chloride and phosphorus oxychloride could be accommodated, permitting the use of phosphorus oxychloride as a solvent in the transfer and manipulation of the small amounts of radiolabeled phenol with minimal radiochemical dilution by added unlabeled phenol carrier. A final product of high specific activity (121 mCi/mmol) with an overall radiochemical yield of 27% was produced by this approach.

The structure of AzBHC was established by several independent means. Mass spectrometry exhibited a molecular ion with measured mass of 293.0803 (cf. 293.0800 calculated for C16, H11, N3, O3). A large peak at m/e 265 (M–28) was observed which represents the loss of molecular nitrogen, a loss consistent with the presence of an azido group. Other fragment ions present in the mass spectrum further confirmed the proposed structure for AzBHC (Trager et al., 1970; Murray et al., 1982). Infrared spectroscopy revealed the characteristic stretching mode for the azido group at 2,130 cm−1, an absorbance band absent for the 3-(p-aminobenzyl)-4-hydroxycoumarin precursor. The 1H NMR spectrum contains the following resonance peaks (solvent: (CD3)2SO): 3.864 ppm (s, 2H); 7.010 ppm (sextet, 2H); 7.289 ppm (d, 2H, J = 8.4); 7.363 ppm (m, 2H); 7.610 ppm (d, 1H, J = 7.8, Jm = 1.54); and 7.982 ppm (dd, 1H, J = 8.21, Jm = 1.53). These resonance peaks and coupling constants are consistent with the proposed structure (Murray et al., 1982). The ultraviolet absorbance spectrum (220 nm) in acetonitrile is characteristic of 3-substituted 4-hydroxycoumarin derivatives, having an absorbance maximum at 256 nm (εmax = 13,600 M–1 cm–1) with a distinct shoulder centered at 307 nm (ε = 8,500 M–1 cm–1).

The photoactivation of AzBHC by ultraviolet light was confirmed by recording the spectrophotometric changes that occur after regular intervals of irradiation (Fig. 2). The change in absorbance at 254 nm was first order with respect to irradiation time, with a half-time of 14 s under these conditions. Analysis of a 5-min photoactivated mixture by reverse phase HPLC confirmed that AzBHC was completely converted into the azide-activated form.

FIG. 1. Schematic representation of the synthesis of 3-(p-azidobenzyl)-4-hydroxycoumarin from phenol and benzylmalonic acid. BMA, benzylmalonic acid; NBMA, p-nitrobenzylmalonic acid; NBHC, 3-(p-nitrobenzyl)-4-hydroxycoumarin; ABHC, 3-(p-aminobenzyl)-4-hydroxycoumarin.

FIG. 2. Photodecomposition of AzBHC. A 50 μM solution of AzBHC in 50 mM Tris, pH 7.5, contained in a quartz cuvette held at a distance of 2 cm from a short wave Mineralight lamp, was irradiated at 4 °C for 5-s intervals. The ultraviolet absorption spectrum was determined after each interval. A t1/2 of photolysis was obtained by a first-order plot of A254 (inset).
ent retention times.

Inhibition of Purified Rat Liver NAD(P)H:Quinone Reductase—Purified rat liver cytosolic NAD(P)H:quinone reductase was used as a model system in the evaluation of the AzBHC as an effective and specific photoaffinity reagent. This enzyme is well characterized, and its inhibition by 4-hydroxycoumarin-type anticoagulants is well established (Hall et al., 1972; Hosoda et al., 1974). The effectiveness of AzBHC in the reversible inhibition of this reductase was determined in competition assays during the steady-state turnover of the substrate menadione. The results obtained for this compound and other 4-hydroxycoumarin-based inhibitors are shown in Fig. 5. In all cases, inhibition conformed to a single-site binding isotherm. An apparent inhibition constant of $6.6 \times 10^{-5}$ M was determined for AzBHC. This value should be compared with the apparent inhibition constants of $3.5 \times 10^{-5}$ M, $2.7 \times 10^{-5}$ M, and $1.7 \times 10^{-5}$ M for Warfarin, 4-hydroxycoumarin, and dicoumarol, respectively, as determined under identical conditions, values that compare well with previous reports (Hall et al., 1972; Hildebrandt and Sutton, 1982). These results confirm that the addition of the $p$-azidobenzyl substituent at the 3-position of the coumarin did not affect binding significantly.

Covalent Incorporation of AzBHC into Rat Liver NAD(P)H:Quinone Reductase—The extent of the covalent incorporation of the radiolabeled AzBHC into the reductase upon irradiation with ultraviolet light was determined after complete denaturation of the protein and removal of excess reagents by SDS-polyacrylamide gel electrophoresis. Covalent incorporation of [$^{14}$C]AzBHC as a function of irradiation time is shown in Fig. 4A. Maximal incorporation occurred within 2 min of irradiation. Further irradiation led to the photodegradation of the protein, resulting in an apparent decrease in the covalent incorporation of the probe. (Only the full-sized protein band was excised from the gel for the determination of associated radioactivity.) Up to 10% of the protein molecules were covalently labeled under these conditions, levels of incorporation comparable with other aziridinyl-based photoaffinity reagents (Bayley and Knowles, 1977).

Protection from Labeling by Dicoumarol—Dicoumarol is one of the most potent inhibitors of this quinone reductase, displaying an inhibition constant of $1.7 \times 10^{-5}$ M. The specificity of AzBHC for the coumarin binding site in the reductase was demonstrated by the protection from covalent incorporation by dicoumarol (Fig. 4B). A mol ratio of 1:1 (dicoumarol:protein) prevents 90% of the labeling whereas at a ratio of 10:1 greater than 99% of the labeling was precluded.

Characterization of the Site(s) within the Protein Covalently Labeled by AzBHC—The high degree of specificity displayed by AzBHC for the coumarin binding site within the quinone reductase presents the opportunity to identify the specific region within the protein which is covalently modified and, thus, to begin to define a portion of the coumarin binding site within this protein. For these studies, the [$^{14}$C]AzBHC probe was photoincorporated into the reductase as described above but on a larger scale. After exhaustive dialysis to remove unincorporated probe, analysis of a small portion of protein by SDS-polyacrylamide gel electrophoresis indicated that 8.4% of the protein molecules were labeled (Table I), in keeping with earlier results. The remaining sample was digested with TPCK-treated trypsin and the digestion mixture resolved by HPLC under two different chromatographic conditions. Chromatography over a C18 reverse phase medium at neutral pH produced an elution profile that contained seven major radioactive peaks, labeled I-VII, as shown in Fig. 5. Greater than 90% of the radioactivity applied to the column was detected in the effluent, ensuring that the elution profile is representative of the incorporated label. Peaks I-VII accounted for 71% of the total radioactivity applied. The distribution of the modified peptides in each peak as calculated from the radioactivity is summarized in Table I. Because it was unlikely that each of these peaks represented a pure radiolabeled peptide fraction, each was rechromatographed over the same reverse phase medium but at acidic pH. Representative elution profiles from the rechromatography of...
Photoaffinity Labeling of NAD(P)H:Quinone Reductase

FIG. 5. Elution profiles obtained during the first peptide purification step. A tryptic digest of DT-diaphorase modified with AzBHC was fractionated by reverse phase HPLC at neutral pH. The column effluent was monitored at A220 nm (upper tracing). The profile of radioactivity obtained from fractions collected at 1.0-min intervals is shown below. Labels I–VII refer to the major radioactive peaks.

Each radioactive peak, including the absorbance trace (220 nm) and associated radioactivity (shaded peak), are shown in Fig. 6. Under these conditions, the radioactivity within each initial fraction was observed to elute within a single peak having a unique elution time in each case and was generally associated with a discrete and well resolved absorbance peak. The recoveries of each of the seven radioactively labeled peptides purified in this manner are also summarized in Table I.

Each radioactively labeled peptide was subjected to automated peptide sequence analysis using gas-phase Edman degradation chemistry. A single amino acid sequence was observed for each modified peptide fraction except peaks I and II in which case no sequence was detected. The sequence results are summarized in Table II. Comparison of the identified sequence of the peptides covalently modified by AzBHC with the established sequence of NAD(P)H:quinone reductase (Robertson et al., 1986) identifies the peptides unequivocally as belonging to the region-spanning residues 119–134. The extent to which each peptide could be sequenced by the Edman degradation method and the pattern of release of the radioactivity during sequencing (summarized in Table III) both identify the specific amino acids covalently modified in this region and suggest the nature of the covalent reaction itself. These results can be summarized as follows.

Peptides VI and VII represent the entire modified tryptic peptide fragment (residues 119–134). A sequence was identified for the entire length of peptide VI except for cycle 10. A phenylthiohydantoin (PTH)-derivative was not observed at this position, where one should have detected a tyrosine residue (tyrosine 128, based on the published amino acid sequence). Also, a substantial and abrupt decrease in the yield of each PTH-derivative was noted after this cycle. The amino acid sequence of peptide VII terminated after cycle 8, i.e. a PTH-derivative representing threonine 127 was not observed in cycle 9.

Peptides III, IV, and V appear to represent subfragments of this peptide which have been generated by chymotryptic-like activity. Although precautions were taken to minimize such secondary cleavages (e.g. TPCK-treated trypsin was used), lengthy digestion times were required to obtain a limit tryptic digest, which was necessary in order to obtain good recoveries of radioactivity by reverse phase HPLC methods. Peptide IV begins at tyrosine 126 and sequences through to the terminal lysine residue. A significant and abrupt drop in the yield of each PTH-derivative was noted beginning with the second cycle, i.e. at threonine 127. Peptides III and V begin at alanine 125, but, as in peptide VII, the sequence appeared to terminate at threonine 127 in both peptides. Most of the radioactivity associated with peptides III and V was retained on the sequencing filter during the analysis. These results suggest that AzBHC has modified these three peptides at threonine 127 in a way that prevents further Edman degradation.

A detectable sequence was not observed for peaks I and II even though the same amount of radioactivity was loaded.
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For each experiment 30 pmol of peptide was subjected to sequencing based on radioactivity. The amounts of individual amino acids detected in each cycle are shown in pmol in the columns below. The positions within the NAD(P)H:quinone reductase sequence which are modified by AzBHC appear to span the protein region from Val-119 to Asp-134 as shown below.

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* ND, not detected.

onto the sequencing filter as was done for all of the above peptides. Significant amounts of radioactivity were observed in the initial cycles of sequencing for peaks I and II. Thus, these peaks may represent some form of the radioactive probe which either was never covalently associated with a peptide or was released during the preparation of the tryptic digest.

Aromatic nitrenes are extremely reactive electrophiles. Upon its formation, reaction with adjacent polypeptide in the binding site of the photoprobe can occur in a variety of ways (Bayley and Staros, 1984). Interruption of the Edman degradation chemistry during sequence analysis may result from at least two different modes of covalent incorporation of the nitrene within the polypeptide: by modification of an α-carbonyl group, which would interfere with the cleavage step, or by insertion onto the α-carbon atom, which would sterically block cyclization (Rush and Konigsberg, 1990). The inability to sequence through threonine 127 in peptides III, V, and VII suggests that the insertion of the nitrene has occurred at the α-carbon or α-carbonyl of this residue. For peptides IV and VI, some radioactive activity appears to be released with tyrosine 128, and a PTH-derivative of tyrosine 128 is either absent or, like other PTH-derivatives beyond this point, is reduced substantially in yield. These modified peptides suggest that incorporations at tyrosine 128 occur, either within the side chain itself or within the polypeptide backbone at this residue.

Enzyme Inactivation and Inhibition of Photoincorporation of AzBHC by p-Nitrobenzenesulfonyl Fluoride—NAD(P)H:quinone reductase can be inactivated by chemical modification of tyrosine 128 with p-nitrobenzenesulfonyl fluoride (NBSF) (Haniu et al., 1988). The time-dependent inactivation of the reductase by 30 μM NBSF at pH 7.4 is shown in Fig. 7. Enzymatic activity could be recovered by thiolysis of the covalent adduct with β-mercaptoethanol (data not shown). The percent incorporation of photoactivated AzBHC into the reductase that had been preincubated for various times with NBSF closely parallels the percent inhibition of the reductase activity (Fig. 7, dashed line). These results demonstrate conclusively that the prior covalent modification of tyrosine 128 by NBSF precludes the covalent incorporation of AzBHC and confirms that this is the specific site of covalent reaction with activated AzBHC. These results also suggest strongly that AzBHC and NBSF share at least a portion of a common binding site.

Effect of Nicotinamide Nucleotide Coenzymes on Inactivation of NAD(P)H:Quinone Reductase by NBSF—The relationship between the pyridine nucleotide binding site and the site modified by both NBSF and AzBHC was investigated by establishing whether NAD* and NADP* could protect the reductase from covalent inactivation by NBSF. The degree of inactivation by NBSF in 15 min in the presence of various concentrations of each pyridine nucleotide was determined. Because the oxidized coenzymes can inhibit the reductase competitively during steady-state turnover at high concentrations, the NBSF inactivation mixture was diluted 300-fold prior to assay. The percent activity remaining after reaction with NBSF was determined relative to control mixtures containing the oxidized pyridine nucleotide in the absence of NBSF. The results of the protection by NAD* and NADP* are shown in Fig. 8. Both oxidized nucleotides protected the quinone reductase from inactivation by NBSF in a concentration-dependent manner. The level of protection observed for NAD* is less than that observed for an equivalent concentration of NADP*, consistent with a higher binding affinity for NADP* (Hall et al., 1972).

DISCUSSION

A number of mammalian enzymes share a common sensitivity to 4-hydroxycoumarin-type anticoagulants; however, most have yet to be identified, purified, and characterized (Suttie, 1988). It is not known if these proteins share common structural features that are responsible for coumarin binding or even what defines the coumarin binding site itself. Several studies involving the relatively well characterized rat liver NAD(P)H:quinone reductase demonstrate that dicoumarol is a competitive inhibitor with respect to pyridine nucleotides (Hall et al., 1972; Hollander and Ernst, 1975; Rase et al., 1976; and Huang et al., 1979). However, despite the fact that this compound binds with very high affinity to this enzyme, it has little effect on other pyridine nucleotide-dependent enzymes, suggesting that this reductase along with other sensitive enzymes contain special structural features that render them sensitive to this type of anticoagulant. The objective of this study was to develop 4-hydroxycoumarin derivatives to serve as highly specific photoaffinity reagents in the identification of proteins sensitive to this general type of anticoagulant and in the characterization of the structure of the coumarin-binding site within these proteins.

In recent years, numerous successful applications of the photoaffinity labeling technique have been reported (Knowles, 1971; Bayley and Staros, 1984). This approach has been particularly effective in the identification of membrane-associated enzymes and receptors that exhibit well defined ligand binding properties of high affinity (Ruoho et al., 1984; Shalhubhai et al., 1990). The isolation of relevant polypeptide(s) for further characterization is often facilitated by the covalent attachment of a radioactive photoaffinity reagent because the retention of biological activity during the solubilization and purification is no longer necessary.
Despite their many limitations, including substoichiometric incorporation and the tendency of the reactive nitrene to react at multiple sites within the binding pocket, photoaffinity reagents have been used effectively in the determination of polypeptide regions within binding sites or residues within the active site of enzymes in some cases (Evans et al., 1988; Rush and Konigsberg, 1990). Often important amino acid residues within binding sites which are unreactive in ordinary nucleophilic or electrophilic chemical modification reactions can be modified by the reactive nitrene and, thus, identified. In addition, if the protein contains multiple binding sites for various ligands, labeling studies can often provide valuable information as to the relationship between the different binding sites (Potter and Haley, 1983; Dholakia et al., 1989).

There is a precedent for the use of 4-hydroxycoumarin derivatives as photoaffinity reagents. 3-(α-Acetonyl-p-azidobenzyl)-4-hydroxycoumarin (azidowarfarin) was observed to inhibit rat liver NAD(P)H:quinone reductase noncompetitively after photolysis (Almeda et al., 1981). However, definitive evidence for the specific covalent incorporation into the quinone reductase has not been reported. This information is crucial because enzyme inhibition can often result from the reversible binding of photodegradation products of the original photoaffinity reagent, often with high affinity. This type of inhibition was, in fact, reported for the azidowarfarin derivative. This binding limits the use of such reagents under denaturing conditions such as during amino acid sequence analysis or SDS-polyacrylamide gel electrophoresis. Also, the use of this reagent is limited by the difficulty of introducing radioisotopes during synthesis and the relatively poor binding affinity displayed for the quinone reductase.

As an alternative, we report here the design, synthesis, and application of a novel photoreactive derivative of 4-hydroxycoumarin, 3-(p-azidobenzyl)-4-hydroxycoumarin. This derivative offers several significant advantages. First, it is synthesized readily in good yields from commercially available precursors. Second, radioisotopes are introduced easily through the incorporation of commercially available phenol substituted with either tritium or carbon-14. Third, the p-azidobenzyl group incorporated in this derivative exhibits good chemical stability and, upon photoactivation, generates a highly reactive nitrene of short half-life and low rearrangement potential, properties essential for efficient photoincorporation into proteins. Finally, the arylazide is introduced at the 3-position of the 4-hydroxycoumarin molecule. Such substitutions can be accommodated without serious effects on inhibition of the NADP(H) quinone reductase (Johnson et al., 1980).

AzBHC is observed to bind very tightly to this quinone reductase, with an apparent inhibition constant of 6.6 × 10⁻⁸ M, well within the workable range for highly efficient photoaffinity reagents Bayley and Staros, 1984). This value is actually 40-fold lower than that for 4-hydroxycoumarin itself and over 500-fold lower than for Warfarin, despite the fact that AzBHC is a close structural analog of Warfarin. The tight binding of AzBHC to this reductase results in good photoincorporation (10% of the protein molecules are covalently labeled) and excellent specificity for the coumarin binding site within this enzyme (covalent incorporation of AzBHC was completely precluded by low molar excesses of dicoumarol (Fig. 4B1)). This degree of specificity and the dependence of the binding affinity on the substituent at the 3-position suggest that this portion of these coumarin derivatives interacts directly with the coumarin binding site in this protein. Together, these observations significantly strengthen our interpretation of the results of the determination of the site of covalent attachment within the protein and also increase the probability of the specific photoincorporation of this reagent within related reductases.

AzBHC was used effectively in this study to define conclusively a portion of the 4-hydroxycoumarin binding site in the NADP(H) quinone reductase. The results of the sequence analysis of covalently labeled peptides I–VII demonstrate that upon photoactivation AzBHC reacts covalently with a single peptide region within the quinone reductase, residues 119–134. Furthermore, the covalent reaction of the nitrene with the protein appears to be confined to amino acid residues threonine 127 and tyrosine 128 within this region. The pattern of sequencing suggests that the electrophilic nitrene reacts predominantly with the polypeptide backbone atoms of threonine 127 and also reacts to a significant extent with the side chain of tyrosine 128. This type of limited modification might result from the benzylazine portion of the photoprobe interacting with the protein in such a way so as to stack in a coplanar fashion with the tyrosine 128 side chain ultimately positioning the reactive nitrene adjacent to the polypeptide backbone near threonine 127. Some of the time the nitrene may be allowed to react with the tyrosine side chain itself.

NAD(P)H:quinone reductase is proposed to proceed through a binary complex (ping-pong) catalytic mechanism and apparently has multiple binding sites for its two substrates (Rase et al., 1976). An unusual feature of this enzyme is that at high menadione concentrations the two substrates can inhibit the enzyme competitively with respect to one another (Hall et al., 1972). Steady-state kinetic evidence suggests that both dicoumarol and Warfarin are competitive inhibitors for NADH and NADPH and that dicoumarol is noncompetitive with respect to menadione (Hall et al., 1972). More direct evidence suggests that dicoumarol prevents the binding of NADPH and the subsequent reduction of the noncovalently bound FAD cofactor within the enzyme (Huang et al., 1979). Furthermore, oxidized enzyme can apparently bind both menadione and dicoumarol simultaneously whereas the reduced enzyme binds menadione but not dicoumarol, indicating the presence of two independent substrate binding sites (Hosoda et al., 1974). These observations suggest that although 4-hydroxycoumarin derivatives may be structural homologs of menadione, they seem to bind to the pyridine nucleotide binding site within this protein.

NAD(P)H:quinone reductase can be inactivated by chemical modification of tyrosine 128 with the protein-modifying reagent NBSF (Fig. 8 and Haniu et al., 1988). Both modification and inactivation can be prevented by the inclusion of 1-naphthol in the reaction mixture (Haniu et al., 1988). It was demonstrated in this study that the prior modification of tyrosine 128 with NBSF precludes the covalent photoincorporation of AzBHC into the reductase. Thiolysis of the NBSF-tyrosine adduct results in reactivation of the enzyme and resensitization to AzBHC. 1-Naphthol also inhibits the incorporation of AzBHC (data not shown). These observations provide further evidence that the polypeptide region adjacent to tyrosine 128 is the exclusive site of modification by AzBHC and demonstrate that NBSF, 1-naphthol, and AzBHC share a common binding pocket.

Suggestions have been made that the binding site for NBSF is distinct from the pyridine nucleotide site in this protein, based on the observation that NAD⁺ does not prevent modification of the reductase by NBSF (Haniu et al., 1988). These results would appear to be inconsistent with those obtained with AzBHC because its covalent incorporation into the reductase is completely prevented by dicoumarol, a competitive inhibitor for pyridine nucleotides for this enzyme. It is not
possible to evaluate the apparent lack of inhibition of NBSF inactivation by pyridine nucleotides in the original report because the methods and data demonstrating this observation were not presented (Haniu et al., 1988). To resolve this issue, those studies were re-investigated. In contrast, we observed that millimolar concentrations of both NAD+ and NADP+ inhibit the inactivation of the reductase by NBSF (Fig. 8). In fact, the level of protection seen for NADP+ is comparatively higher than that of NAD+ because of the higher binding affinity for NADP+ by the reductase (Hall et al., 1972).

These observations are consistent with the labeling experiments reported here; that is, the covalent modification of the same site by AzBHC and NBSF is inhibited by dicoumarol and pyridine nucleotides, respectively, both of which are mutually competitive ligands for this protein. These results, then, support previous observations that 4-hydroxycoumarin-based anticoagulants such as dicoumarol bind to the pyridine nucleotide site within this reductase and establish definitively a region within the polypeptide sequence which forms a portion of that binding site. It should be noted that AzBHC labels a region within the reductase distinct from that apparently labeled by 5'-p-fluorosulfonfonylbenzoyladenosine, an irreversible inhibitor for NAD(P)H:quinone reductase (Liu et al., 1989). By sequence comparison with other nucleotide binding sites, it was suggested that this reagent may be modifying the pyrophosphate binding portion of the pyridine nucleotide site.

These results, for the first time, identify and define a portion of the co-mamin binding site within NAD(P):quinone reductase, an enzyme with a well established sensitivity to 4-hydroxycoumarin-type anticoagulants. The determination of the region within the protein which is immediately adjacent to a close structural analog of Warfarin will help establish the relationship of this binding site to other sites within this reductase such as the menadione and FAD binding sites, especially when the x-ray crystal structure is eventually established (Ysern and Prochaska, 1989). Photoaffinity reagents of this type (see also Myszka and Swenson, 1990) should be quite useful in the identification of other proteins that are sensitive to 4-hydroxycoumarin-type anticoagulants, such as the dithiol-dependent vitamin K-2,3-epoxide reductase and a dithiol-dependent vitamin K reductase (Whitlon et al., 1978; Fasco and Principe, 1982).

REFERENCES


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Photoaffinity Labeling of NAD(P)H:Quinone Reductase

**Introduction**

The photoaffinity labeling of NAD(P)H:Quinone Reductase (NQO1) with benzylmalonate, 4-hydroxycoumarin, dicumarol, Warfarin, menadione, p-nitrobenzenesulfonyl fluoride, and deuterated solvents was investigated. These reagents were obtained from various suppliers and were used as described in the literature. NADH, NAD+, NADP+, trifluoroacetic acid, and TPCK-treated trypsin were obtained from Sigma Chemical Co. IU-14C Phenol and 15N were purchased from the New England Nuclear. All other solvents were obtained from Aldrich Chemical Co. All solutions were prepared in H2O grade water. All glassware and equipment were treated with concentrated sulfuric acid.

**Materials**

- Sarcosine acid, 3-bromobenzeneacetic acid, difluoroacetic acid, menadione, p-nitrobenzene-4-sulfonyl fluoride, and deuterated solvents were obtained from Aldrich Chemical Co.
- NADH, NAD+, NADP+, trifluoroacetic acid, and TPCK-treated trypsin were obtained from Sigma Chemical Co.
- IU-14C Phenol and 15N were purchased from the New England Nuclear.
- All other solvents were obtained from Aldrich Chemical Co.

**Methods**

1. **Preparation of NAD(P)H:Quinone Reductase**
   - A solution of NQO1 was prepared by the method described in the literature. The enzyme was purified to homogeneity by ammonium sulfate precipitation, affinity chromatography, and gel filtration. The purified enzyme was then used for further studies.

2. **Preparation of Benzylmalonate**
   - Benzylmalonate was prepared by the method described in the literature. The compound was dissolved in D2O and used for the labeling experiments.

3. **Preparation of 4-Hydroxycoumarin**
   - 4-Hydroxycoumarin was prepared by the method described in the literature. The compound was dissolved in D2O and used for the labeling experiments.

4. **Preparation of Dicumarol**
   - Dicumarol was prepared by the method described in the literature. The compound was dissolved in D2O and used for the labeling experiments.

5. **Preparation of Warfarin**
   - Warfarin was prepared by the method described in the literature. The compound was dissolved in D2O and used for the labeling experiments.

6. **Preparation of Menadione**
   - Menadione was prepared by the method described in the literature. The compound was dissolved in D2O and used for the labeling experiments.

7. **Preparation of p-Nitrobenzenesulfonyl Fluoride**
   - p-Nitrobenzenesulfonyl fluoride was prepared by the method described in the literature. The compound was dissolved in D2O and used for the labeling experiments.

8. **Preparation of Deuterated Solvents**
   - Deuterated solvents were prepared by the method described in the literature. The compounds were dissolved in D2O and used for the labeling experiments.

**Results**

- A table was presented showing the results of the labeling experiments. The table included the following columns: Compound, Recovery, and Specific Activity.

**Discussion**

- The results showed that the photoaffinity labeling of NQO1 with benzylmalonate, 4-hydroxycoumarin, dicumarol, Warfarin, menadione, p-nitrobenzenesulfonyl fluoride, and deuterated solvents was successful. The specific activity of the labeled enzyme was determined and compared to the control.

**Conclusion**

- The photoaffinity labeling of NQO1 was successful and provided valuable information about the enzyme's structure and function. Further studies are needed to elucidate the mechanism of action of these agents and their potential therapeutic applications.
Figure 7. Inhibition and Protection of TT-diaphorase with NQD. The right hand panel shows the percent activity of TT-diaphorase (1.0 μM) versus time of exposure to NQD (1 mM). Incubation with NQD was carried out at room temperature in Tris-HCl 50 mM, pH 7.5. The left hand panel shows the percent incorporation of the photolabel with the same conditions of TT-diaphorase, NQD and its time course. Percent incorporation is relative to the amount of labeling achieved in the absence of NQD at time 0 min.

Figure 8. Protection from NQD inactivation of NAD(P)H:quinone reductase by photolabel incorporation. The nucleotides used are NADH (O) and NADP (●). Quinone reductase (1.0 μM) was incubated with increasing concentrations of photolabel for 5 min prior to the addition of NQD (10 μM). Incubation was carried out at 4°C for 15 min at room temperature in Tris-HCl 50 mM, pH 7.5. Each incorporation sample was diluted 500 fold and assayed as outlined in the legend.