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Interaction of Flavones and Their Bromoacetyl Derivatives with NAD(P)H:Quinone Acceptor Oxidoreductase

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SUMMARY

Flavones are a new type of inhibitor of NAD(P)H:quinone acceptor oxidoreductase (DT-diaphorase, EC 1.6.99.2). To further characterize the flavone binding site, three bromoacetyl derivatives of flavones, i.e., 7-bromoacetylflavone, 5-hydroxyl-7-bromoacetylflavone, and 7,8-dibromoacetylflavone, have been synthesized. These compounds have been found to be potent inhibitors that inactivate the rat quinone reductase in a time-dependent manner, suggesting that they can be used as affinity labels for the enzyme. Among the three bromoacetyl derivatives, 7,8-dibromoacetylflavone is the most potent inhibitor; however, its labeling of the quinone reductase is the least stable, so that the enzyme regains activity after a short incubation. In contrast, the inactivation of the quinone reductase by 5-hydroxyl-7-bromoacetylflavone is stable. Accordingly, this flavone derivative is the most suitable compound for labeling the flavone binding site of the enzyme. Electrospray mass spectrometry has been applied to demonstrate that 5-hydroxyl-7-bromoacetylflavone labels this enzyme in a stoichiometric manner.

NAD(P)H:quinone acceptor oxidoreductase (EC 1.6.99.2), also referred to as DT-diaphorase, is a flavoprotein that catalyzes the two-electron reduction of quinones and quinonoid compounds to hydroquinones, using either NADH or NADPH as the electron donor (1, 2). It consists of two identical subunits. Each subunit has a molecular weight of 30,000 and contains one FAD prosthetic group, noncovalently attached to the protein. This quinone reductase reduces vitamin K. It can function physiologically as one of several vitamin K reductases in the vitamin K cycling involved in the hepatic post-translational modification of vitamin K hydroquinone-dependent blood coagulation factors (3). This enzyme is also a target for anticoagulants, such as dicoumarol and warfarin (4, 5).

Four years ago we isolated the glucuronide conjugates of three flavones, i.e., oroxylin A, baicalein, and wogonin, from the Chinese herb *Scutellariae radix* (huang qin) and found oroxylin A and baicalein to be potent inhibitors of NAD(P)H:quinone acceptor oxidoreductase (6). It was suggested that these compounds might be potentially useful anticoagulant drugs. A structure-activity study was recently carried out to determine the important regions of these flavones in inhibiting quinone reductase (7). The 5-hydroxyl, 7-hydroxyl, 8-hydroxyl, and 2-phenyl groups of these compounds were found to be important for their inhibition of the enzyme. The ability to inhibit quinone reductase was significantly decreased for flavones without any of these groups (7).

In addition to the reduction of two-electron acceptors (e.g., menadione), NAD(P)H:quinone acceptor oxidoreductase is unusual in its ability to also reduce one-electron (e.g., potassium ferricyanide) and four-electron (e.g., methyl red) acceptors. The mechanisms of these reduction reactions are being investigated.[1] Because flavones were found to be capable of differentiating among these reactions by inhibiting the NADH-menadione reductase activity, the NADH-potassium ferricyanide reductase activity, and the NADH-methyl red reductase activity of the enzyme to different degrees (7), these flavones were thought to be useful tools for investigating the mechanisms of the three reduction reactions catalyzed by quinone reductase. Moreover, the different effects of these flavones on three types of activities suggest that flavones do not bind to the nicotinamide nucleotide binding site, although they have been shown to be competitive inhibitors with respect to NADH (6). Recent results obtained from this laboratory (7) indicate that these flavones do bind at a site of the enzyme different from that for dicoumarol or phenindone, two known inhibitors of quinone reductase. Thus, the flavones form a new class of inhibitors of NAD(P)H:quinone acceptor oxidoreductase. To further understand the inhibitory mechanism of flavones, we plan to determine the regions of the enzyme that are involved in the binding of flavones. Affinity labeling techniques are a useful approach

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[1] G. Tedeschi, S. Chen, and V. Massey, unpublished observations.

ABBREVIATIONS: MTT, 3-(4,5-dimethylthiazo-2-yl)-2,5-diphenyltetrazolium bromide; HPLC, high performance liquid chromatography.

to determine the locus of interaction of flavones with the enzyme.

In this paper, we report the synthesis of bromoacetyl analogues of three flavones, namely 7-hydroxylflavone, 5,7-dihydroxylflavone, and 7,8-dihydroxylflavone. We have explored their potentials as affinity labels for NAD(P)H:quinone acceptor oxidoreductase.

Experimental Procedures

Materials. 7-Hydroxylflavone, 5,7-dihydroxylflavone (chrysin), and 7,8-dihydroxylflavone were purchased from Aldrich Chemical Co. (Milwaukee, WI). Bromoacetyl bromide was also purchased from Aldrich.

Synthesis and characterization of bromoacetylflavone derivatives. Bromoacetylflavone derivatives were synthesized according to the following procedure. To a stirred solution of flavone (1 mmol) and pyridine (1.5 mmol, 0.12 ml) in methylene chloride (3 ml), cooled to 0°, was added dropwise a solution of bromoacetyl bromide (1.1 mmol, 0.1 ml). After stirring for 30 min, the mixture was poured onto ice and extracted three times with methylene chloride. The organic phase was washed with 20 ml of 5% HCl and 20 ml of water and dried over anhydrous magnesium sulfate. The products were purified by silica gel thin layer chromatography (solvent system of toluene/acetone, 95:5). The structures of these bromoacetylflavone derivatives were determined by mass and NMR spectral analyses. The mass and NMR spectral analyses were performed using a JEOL HX100HF mass spectrometer and a Bruker AM500 spectrometer, respectively.

Enzyme preparation and assay. NAD(P)H:quinone acceptor oxidoreductase was purified from *Escherichia coli* that had been transformed with the quinone reductase expression plasmid pKK DT-2, according to a procedure described by Chen *et al.* (8). The specific activity of this preparation was 7670 μmol of NADH oxidized/min/mg of protein (8). Two quinone reductase mutants, W115F and T148Y, were also used in this study. The preparation and characterization of these mutants will be described in another publication.

The activity of the enzyme was determined spectrophotometrically by measuring the reduction of MTT at 610 nm [$\epsilon(610\ nm) = 11.3 \times 10^3\ M^{-1}\ cm^{-1}$ (9)] at 25°. The assay mixture (1 ml) contained 50 mM potassium phosphate, pH 7.5, 200 μM NADH, 160 μM menadione, and 0.3 mg/ml MTT. In this assay, menadione is the electron acceptor and MTT is included to continuously reoxidize the menadiol formed. All of the assays were initiated with the addition of the enzyme and were performed at least in duplicate.

Inhibition of NAD(P)H:quinone acceptor oxidoreductase by flavones. The effects of flavones and their bromoacetyl derivatives on this quinone reductase were initially determined by carrying out enzyme assays in the presence of various flavones or their bromoacetyl derivatives at different concentrations. Flavones were dissolved in acetonitrile and the maximal volume of acetonitrile was maintained at 10 μl/ml of assay mixture. The activity of the enzyme was not affected by acetonitrile in amounts up to 10 μl/ml. We also performed the enzyme inactivation studies by incubating the enzyme with flavone derivatives first; aliquots of the incubation mixture were then withdrawn after various periods of incubation and assayed for the menadione reductase activity.

Electrospray mass spectrometry was used to confirm the binding of 5-hydroxyl-7-bromoacetylflavone to the quinone reductase upon incubation with the enzyme. The electrospray mass spectral analysis was performed according to the methods described previously by Swiderek *et al.* (10) and Chen *et al.* (8).

Results and Discussion

Synthesis and characterization of bromoacetylflavone derivatives. In our previous study (7), 5,7-dihydroxylflavone (chrysin) and 7,8-dihydroxylflavone were shown to inhibit the quinone reductase with an IC_{50} value of 50–100 nM, a potency similar to that of baicalein (i.e., 5,6,7-trihydroxylflavone, the compound isolated from the Chinese herb *S. radix*). In fact, 7,8-dihydroxylflavone is slightly more potent than baicalein in inhibiting this enzyme (7). Accordingly, affinity labeling analogues of 5,7-dihydroxylflavone and 7,8-dihydroxylflavone should be useful for identifying the flavone binding site in quinone reductase. The bromoacetyl group is a chemically reactive group that can serve as an electrophile toward nucleophilic side chains of amino acids. The aforementioned flavones all contain hydroxyl groups that can be chemically converted to bromoacetyl groups. Bromoacetyl derivatives of 5,7-dihydroxylflavone and 7,8-dihydroxylflavone were synthesized for labeling of the quinone reductase. To evaluate the conditions for the organic synthesis, we initially prepared a bromoacetyl derivative of 7-hydroxylflavone. Because there is only one hydroxyl group in 7-hydroxylflavone, we expected only one product if the synthesis worked as we anticipated.

As expected, only one product was obtained when we used 7-hydroxylflavone as the starting material. The product was confirmed to be 7-bromoacetylflavone by mass spectral and proton NMR analyses. The molecular weight of this product was determined by mass spectral analysis to be 359, agreeing with the calculated value. The proton NMR spectrum indicated the disappearance of the resonance signal from the 7-hydroxyl group and the appearance of a resonance signal that could be assigned to the bromoacetyl methylene group (i.e., 4.1 ppm). The melting point of 7-bromoacetylflavone was determined to be 141–142°. Interestingly, when 5,7-dihydroxylflavone was used as the starting material, again only one product was formed, which was identified to be 5-hydroxyl-7-bromoacetylflavone. The molecular weight of this product was determined by mass spectal analysis to be 375, indicating that only one hydroxyl group was bromoacetylated. As confirmation, only one resonance signal from the bromoacetyl methylene group could be observed at 4.07 ppm in the proton NMR analysis of this product. An extremely low-field resonance signal at 12.8 ppm was assigned to the 5-hydroxyl group. The chemical shift of this signal did not change with concentration, probably because the 5-hydroxyl group can form an intramolecular hydrogen bond with the neighboring 4-keto group. Due to the intramolecular hydrogen bond formed between the 5-hydroxyl group and the 4-keto group and the steric effect of the 4-keto group itself, it is believed that the 5-hydroxyl group is much less reactive or accessible for bromoacetylation. The melting point of 5-hydroxyl-7-bromoacetylflavone was determined to be 181–182°. When 7,8-dihydroxylflavone was used as the starting material, the product was identified as 7,8-dibromoacetylflavone, based on the NMR data. No resonance signal from the free hydroxyl group could be observed and there were two resonance signals from the bromoacetylmethylene groups, at 3.7 and 3.9 ppm, with equal intensity. The melting point of 7,8-dibromoacetylflavone was determined to be 198–202°.

Inhibition of NAD(P)H:quinone acceptor oxidoreductase by 7-bromoacetylflavone. In contrast to the competitive inhibition by 7-hydroxylflavone, the enzyme was inactivated by 7-bromoacetylflavone in a time-dependent manner, as observed during the 1-min assay period (Fig. 1). The initial rates of the reactions catalyzed by the enzyme in the presence of 7-bromoacetylflavone were the same as those for the enzyme treated with 7-hydroxylflavone. However, the time for the inactivation of quinone reductase by 7-bromoacetylflavone was

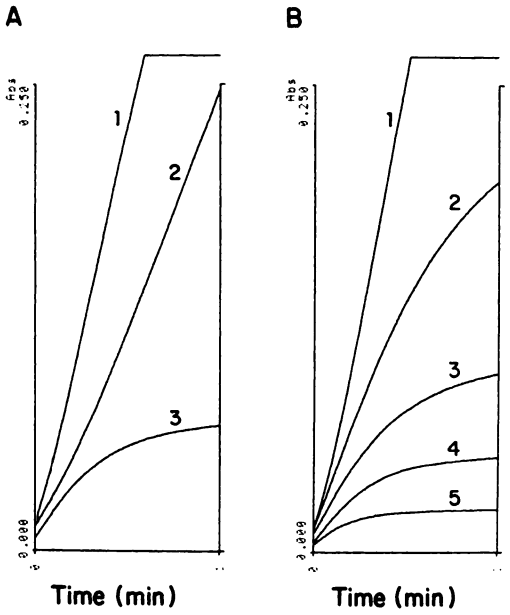

Fig. 1. Inhibition of NAD(P)H:quinone acceptor oxidoreductase by 7-hydroxylflavone or 7-bromoacetylflavone. The 1-min enzyme assay was performed by monitoring the absorption change at 610 nm, as for MTT reduction. A, The assay was performed without inhibitor (*trace 1*), with 1.2 μM 7-hydroxylflavone (*trace 2*), or with 1.2 μM 7-bromoacetylflavone (*trace 3*). B, The assay was performed without inhibitor (*trace 1*) or with 0.4 μM (*trace 2*), 0.8 μM (*trace 3*), 1.2 μM (*trace 4*), or 2 μM 7-bromoacetylflavone (*trace 5*).

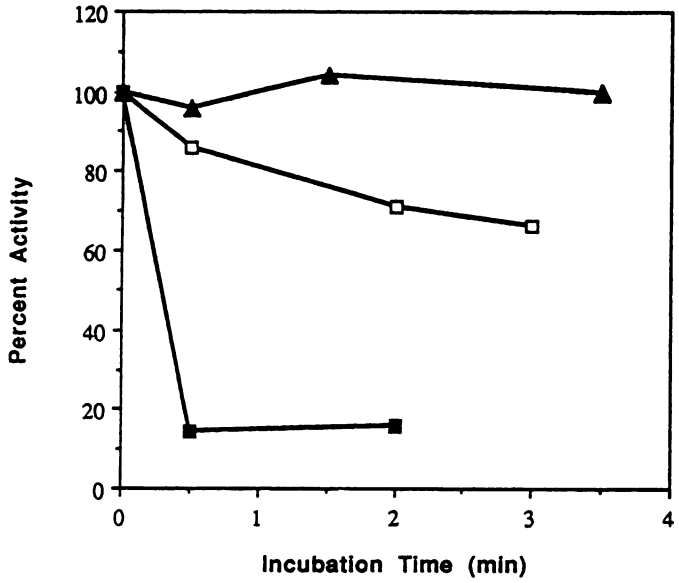

Fig. 2. Time-dependent inhibition of NAD(P)H:quinone acceptor oxidoreductase by 7-bromoacetylflavone. The enzyme (59 μg/ml) was incubated at room temperature in the presence of 7-bromoacetylflavone (2 μM) (■), 7-hydroxylflavone (2 μM) (▲), or 7-bromoacetylflavone (2 μM) plus NADH (50 μM) (□). At the indicated times, 2.5-μl aliquots were withdrawn and assayed for quinone reductase activity.

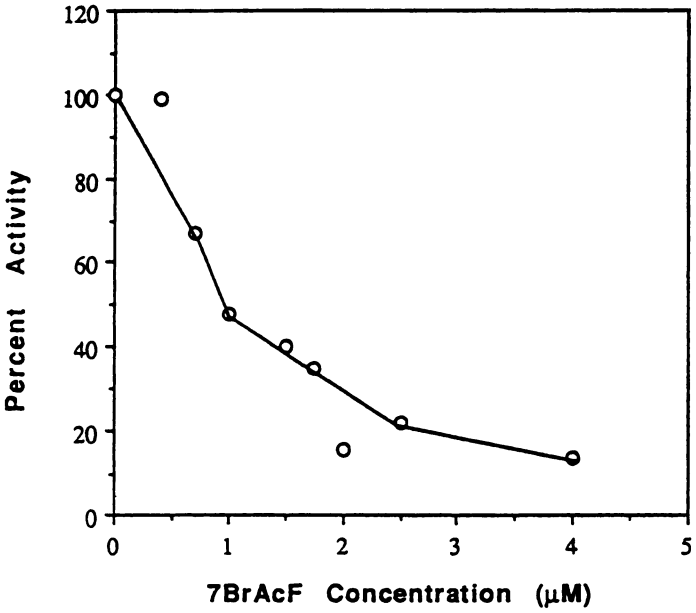

Fig. 3. Concentration dependence of 7-bromoacetylflavone (*7BrAcF*) inhibition of NAD(P)H:quinone acceptor oxidoreductase. The indicated concentrations were those during the incubation. After a 30-sec incubation, 2.5-μl aliquots were withdrawn and assayed for quinone reductase activity.

directly related to the concentration of the bromoacetyl derivative. We found that the enzyme was inactivated more rapidly by the 7-bromoacetylflavone at a higher concentration (Fig. 1B).

To show that the inhibition did not develop during enzyme turnover, i.e., mechanism-based inhibition, the following experiment was performed. The enzyme was incubated with 7-bromoacetylflavone (2 μM) initially, and aliquots of 2 μl of the mixture were withdrawn for enzyme assay after different periods of incubation. The inactivation process was very rapid, so that maximal inhibition was reached at the first time point (30 sec after addition of the inhibitor) (Fig. 2). In this experiment, the actual concentration of 7-bromoacetylflavone during the assay was 5 nM, and no inhibition of quinone reductase was detected when the assay was performed in the presence of the inhibitor at this concentration. Flavones are competitive inhibitors of the quinone reductase with respect to NADH (6). NADH protected the enzyme from inactivation by 7-bromoacetylflavone when it was present during the incubation (Fig. 2). As expected, no time-dependent inhibition was observed when the enzyme was incubated with 7-hydroxylflavone at the same concentration.

Fig. 3 shows that the inactivation of quinone reductase by 7-bromoacetylflavone was concentration dependent. The actual concentration during the assay was 1/400 of the "incubation" concentrations.

Concentration dependence of the inhibition of NAD(P)H:quinone reductase by bromoacetylflavone derivatives. Fig. 4 shows the inhibition profiles of 7-hydroxylflavone, 7-bromacetylflavone, 5,7-dihydroxylflavone, 5-hydroxyl-7-bromoacetylflavone, 7,8-dihydroxylflavone, and 7,8-dibromoacetylflavone with the quinone reductase. The measurements were performed with the addition of the various inhibitors during the enzyme assay. Therefore, the indicated concentrations of inhibitors were those during the enzyme assay. The activity calculation was based on the initial rate when the assay was performed in the presence of the inhibitors. As indicated above (see Fig. 1), there was no difference in the initial reaction rate when the enzyme was assayed in the presence of 7-hydroxylflavone or 7-bromoacetylflavone, at the same concentration. The IC_{50} value for both 7-hydroxylflavone and 7-bromoacetylflavone was estimated to be 0.74 μM (Fig. 4). The initial reaction rates were also identical when the enzyme

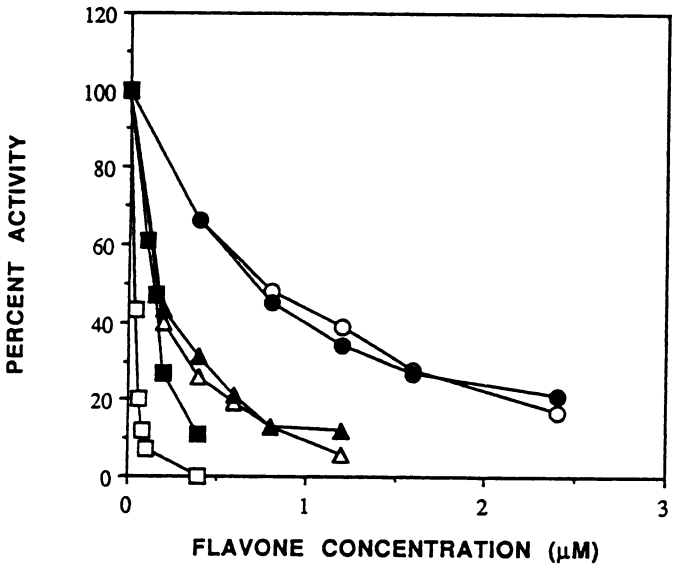

Fig. 4. Inhibition of NAD(P)H:quinone acceptor oxidoreductase by 7-hydroxylflavone (●), 7-bromoacetylflavone (○), 5,7-dihydroxylflavone (▲), 5-hydroxyl-7-bromoacetylflavone (△), 7,8-dihydroxylflavone (■), or 7,8-dibromoacetylflavone (□). The indicated concentrations of flavones are the assay concentrations. The activity calculation was based on the initial rate when the assay was performed in the presence of the inhibitors, taking that of the control as 100%.

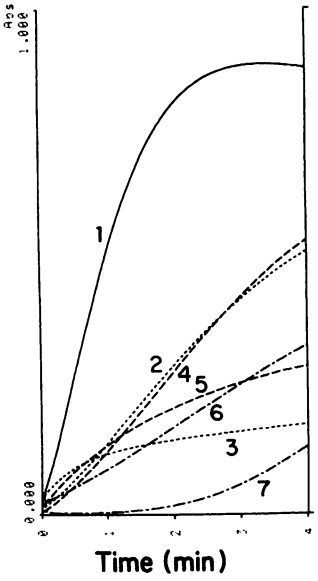

Fig. 5. Quinone reductase assay in the presence of different inhibitors. The assay was initiated with the addition of 2 μl of enzyme (59 μg/ml). *Trace 1*, no inhibitor; *trace 2*, 1.6 μM 7-hydroxylflavone; *trace 3*, 1.6 μM 7-bromoacetylflavone; *trace 4*, 0.4 μM 5,7-dihydroxylflavone; *trace 5*, 0.4 μM 5-hydroxyl-7-bromoacetylflavone; *trace 6*, 0.4 μM 7,8-dihydroxylflavone; *trace 7*, 0.4 μM 7,8-dibromoacetylflavone.

was assayed in the presence of 5,7-dihydroxylflavone or 5-hydroxyl-7-bromoacetylflavone, and both of these compounds were more potent inhibitors (with a IC_{50} value of 0.18 μM) than 7-hydroxylflavone and 7-bromoacetylflavone (Fig. 4). The results indicate that the binding affinities of the flavones are probably not changed upon conversion of the 7-hydroxyl group to the 7-bromoacetyl group. On the other hand, 7,8-dibromoacetylflavone was found to be a more potent inhibitor (IC_{50} = 0.03 μM) than 7,8-dihydroxylflavone (IC_{50} = 0.15 μM), probably resulting from very rapid inactivation of the enzyme by 7,8-dibromoacetylflavone. The time-dependent inhibition of the quinone reductase was achieved between the time of addition of the inhibitor and the time of initiation of recording of the reaction. Therefore, conversion of the 8-hydroxyl group of flavones to the 8-bromoacetyl group probably has a significant effect on the inhibition of quinone reductase.

Stability of the binding of three bromoacetylflavones to NAD(P)H:quinone acceptor oxidoreductase. Quinone reductase was rapidly inactivated by 7,8-dibromoacetylflavone, but within a short time the activity recovered to the level achieved with 7,8-dihydroxylflavone (Fig. 5). These results indicate that 7,8-dibromoacetylflavone labels the enzyme in a rather unstable manner. To further evaluate the stability of the labeling, quinone reductase was incubated with 7-bromoacetylflavone (4 μM), 5-hydroxyl-7-bromoacetylflavone (5 μM), or 7,8-dibromoacetylflavone (5 μM). At different times, aliquots of the mixture were withdrawn and assayed for activity. As expected, the labeling of 7,8-dibromoacetylflavone was rather unstable, and the enzyme activity rapidly recovered after an incubation of 1.5 min (Fig. 6). The enzyme activity also recooered significantly after 30 min of incubation of the enzyme with 7-bromoacetylflavone. Among the three compounds, the labeling of 5-hydroxyl-7-bromoacetylflavone appeared the most irreversible (Fig. 6). These results led us to conclude that, among the three bromoacetylflavone derivatives, 5-hydroxyl-7-

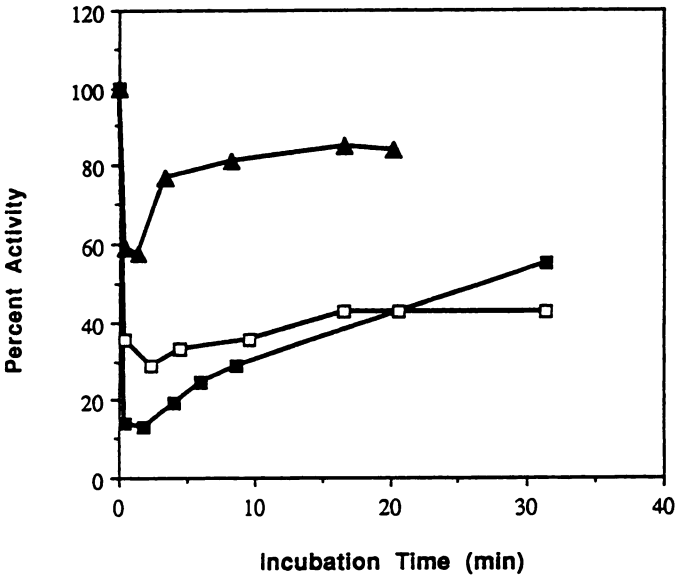

Fig. 6. Stability of the inactivation of NAD(P)H:quinone acceptor oxidoreductase by bromoacetylflavones. The enzyme was incubated with 4 μM 7-bromoacetylflavone (■), 5 μM 5-hydroxyl-7-bromoacetylflavone (□), or 5 μM 7,8-dibromoacetylflavone (▲). At the indicated times, 2.5-μl aliquots were withdrawn and assayed for quinone reductase activity.

bromoacetylflavone is the best compound for the labeling study of quinone reductase.

The bromoacetyl group forms stable derivatives with the side chains of the amino acids cysteine and histidine and forms less stable derivatives with those of hydroxy-amino acids and acidic amino acids. The difference in the stability of the binding of the three bromoacetylflavones to quinone reductase is probably a result of interaction with different amino acids. Because the rat quinone reductase contains only one cysteine residue per subunit and this residue has been found not to be important

for the catalytic function of the enzyme,[2] 5-hydroxyl-7-bromoacetylflavone may react with a histidine residue, forming a stable derivative. However, the nature of the modified amino acid residue remains to be identified experimentally.

Interaction of flavones with quinone reductase mutants W115F and T148Y. Trp-115 and Thr-148 of this quinone reductase have been suggested to be present at the flavin and nicotinamide nucleotide binding sites, respectively (11).[3] The binding of the FAD prosthetic group and NAD(P)H are significantly decreased for the quinone reductase mutants W115F and T148Y.[4] We found that the inhibition profiles for 7-hydroxylflavone with the wild-type enzyme, W115F, and T148Y were very similar, indicating that these mutations do not affect the binding of this flavone (data not shown). The results again indicate that flavones probably do not bind to the NAD(P)H or FAD binding site.

Confirmation by electrospray mass spectrometry of the incorporation of 5-hydroxyl-7-bromoacetylflavone into NAD(P)H:quinone acceptor oxidoreductase. The UV/visible spectrum of reverse phase HPLC-purified, 5-hydroxyl-7-bromoacetylflavone-modified, quinone reductase has an absorbance shoulder, with a maximum at 320 nm, that is not seen in the spectrum of the unmodified enzyme, suggesting the binding of the flavone derivative to the enzyme (Fig. 7). The spectrum of 5-hydroxyl-7-bromoacetylflavone is also shown in Fig. 7 for comparison.

The final confirmation of the labeling of the quinone reductase by 5-hydroxyl-7-bromoacetylflavone was provided by electrospray mass spectrometry. The molecular weight of the quinone reductase is 30,846. We expected a molecular weight

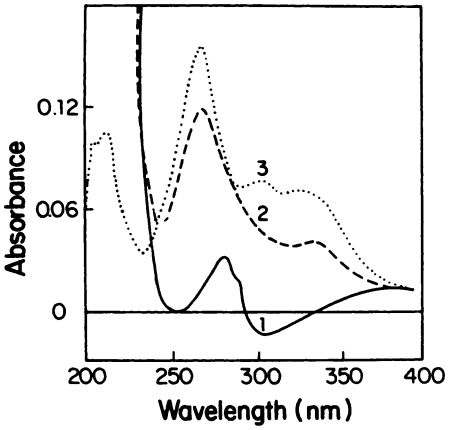

Fig. 7. UV/visible spectral analysis of 5-hydroxyl-7-bromoacetylflavone-modified NAD(P)H:quinone acceptor oxidoreductase. The enzyme samples were initially subjected to reverse phase HPLC to remove unreactive flavone. A Pierce Aquapore octyl RP300 column (3 cm × 2.1 mm) was used. A 30-min gradient program was run from 100% solvent A (0.1% trifluoroacetic acid) to 100% solvent B (trifluoroacetic acid/water/acetonitrile, 0.1:9.9:90, v/v/v), at a flow rate of 0.8 ml/min. The spectra of the HPLC fractions containing the enzyme were recorded using an Hitachi U-3110 spectrophotometer. *Trace 1*, spectrum of the fraction obtained from HPLC separation of 400 μl of unmodified quinone reductase (0.59 mg/ml); *trace 2*, spectrum of the fraction obtained from HPLC separation of 400 μl of quinone reductase treated with 0.14 mM 5-hydroxyl-7-bromoacetylflavone; *trace 3*, spectrum of 6.9 μM 5-hydroxyl-7-bromoacetylflavone in 100% HPLC solvent B.

[2] H. Yuan and M. Haniu, unpublished observations.
[3] H. Chen, G. Forrest, and S. Chen, unpublished observations from site-directed mutagenesis studies.
[4] S. Chen and P. Cheng, unpublished observations.

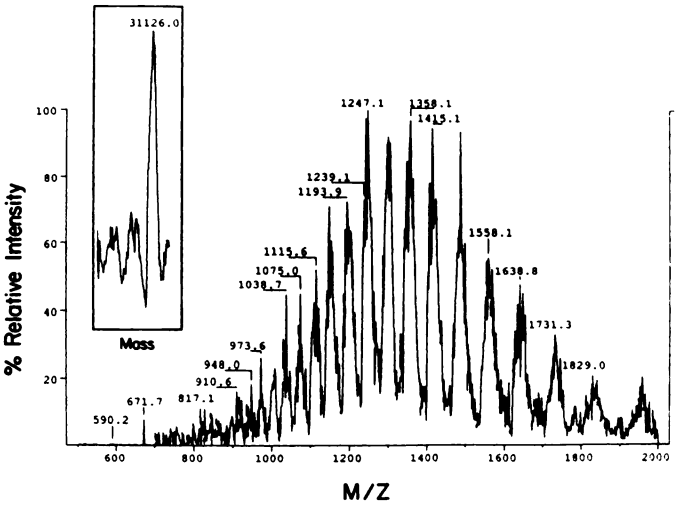

Fig. 8. HPLC-linked electrospray mass spectral analysis of 5-hydroxyl-7-bromoacetylflavone-modified NAD(P)H:quinone acceptor oxidoreductase. Mass spectra were recorded in the positive ion mode using a TSQ-700 triple-quadrupole instrument (Finnigan-MAT, San Jose, CA) with an electrospray ion source operating at atmospheric pressure. The electrospray needle was operated at a voltage differential of 3–4 kV, and a sheath flow of 2 μl/min methoxyethanol was used. Scans were continually acquired every 3 sec. The chromatography was performed with a micro-capillary HPLC system built at the City of Hope. Fused silica columns with an inner diameter of 250 μm were packed with Vydac 3-μm C_{18} reverse phase support. Twenty four picomoles of modified quinone reductase (8 pmol/μl) were injected and eluted with a gradient from 22% solvent A (0.1% trifluoroacetic acid) to 82% solvent B (90% acetonitrile/0.07% trifluoroacetic acid/9.93% water) in 30 min, with a flow rate of 2 μl/min. An electrospray spectrum of the modified enzyme is shown; *inset*, deconvolution of this spectrum.

increase of 294 for the enzyme molecule with one molecule of flavone associated, yielding a molecular weight of 31,140. The 5-hydroxyl-7-bromoacetylflavone-treated enzyme had a molecular weight of 31,126, as determined by electrospray mass spectral analysis (Fig. 8). The error of this measurement is 0.04%. The treated enzyme had only minimal activity. These results indicate that 5-hydroxyl-7-bromoacetylflavone labels the enzyme at its binding site in a stoichiometric manner. Because the electrospray mass spectrum of the labeled preparation revealed microheterogeneity, it is thought that the affinity probe probably reacts with more than one amino acid residue and that intramolecular rearrangements take place within the flavone binding site. One point that is worthy of mention is that this mass spectral analysis of enzyme labeling was carried out without the need for a radioactive derivative.

In conclusion, evidence has been presented that 5-hydroxyl-7-bromoacetylflavone is a potentially useful affinity label that labels NAD(P)H:quinone acceptor oxidoreductase in a stable fashion. Experiments are being carried out to identify the modified amino acid(s). The protein structural information will be very important for a further understanding of the inhibitory mechanism of flavone with this quinone reductase.

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