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Suggested Mechanism for the Modulation of the Activity of NAD(P)H:Quinone Acceptor Oxidoreductase (DT-Diaphorase) by Menadione: Interpretation of the Effect of Menadione on 5′-[*p*-(Fluorosulfonyl)benzoyl]adenosine Labeling of Rat Liver NAD(P)H:Quinone Acceptor Oxidoreductase

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SUMMARY

5′-[*p*-(Fluorosulfonyl)benzoyl]adenosine (5′FSBA) was previously shown to be an active site-directed affinity label of rat liver NAD(P)H:quinone acceptor oxidoreductase [*Mol. Pharmacol.* 35:818–822 (1989)]. Our recent study revealed that menadione, the substrate of this quinone reductase, had a magnifying effect on inactivation of the enzyme by 5′-FSBA. The dissociation constant for the initial reversible enzyme-inhibitor complex was significantly lower and the rate of inactivation was increased when menadione was present during the incubation. However, [^{14}C]5′FSBA labeling was reduced in the presence of menadione. These results are presented and a possible mechanism for the enzyme is discussed.

NAD(P)H:quinone acceptor oxidoreductase (EC 1.6.99.2; DT-diaphorase) plays an important role in protecting tissues against the mutagenic, carcinogenic, and cytotoxic effects of quinones, which occur widely in nature (1). Protection is accomplished by the unique ability of this quinone reductase to catalyze an obligatory two-electron reduction of several quinones, including vitamin K (e.g., menadione), to hydroquinones, with either NADH or NADPH as electron donor (2, 3). This reaction is thought to bypass the semiquinone free radical state, diminishing the quinone-mediated formation of oxygen free radicals, which are generated by redox cycling of semiquinones in the presence of oxygen.

One feature of the quinone reduction reaction catalyzed by NAD(P)H:quinone acceptor oxidoreductase is that there is an inhibition of the reaction by menadione. The menadione reductase activity is lower when the assay is performed at higher menadione concentrations. Hall *et al.* (4) have demonstrated that NAD(P)H:quinone acceptor oxidoreductase catalyzes a reaction according to a "ping-pong" mechanism, and the electron donor [NAD(P)H] and the electron acceptor (e.g., menadione) inhibit the enzyme in a competitive fashion with respect to one another, suggesting a cooperative relationship between the donor and acceptor binding sites of the enzyme. Furthermore, Hosoda *et al.* (5) have revealed that the quinone reductase can form an enzyme-menadione complex when menadione is present and the FAD prosthetic group is rapidly reduced by NAD(P)H, at a rate almost comparable to that of the enzyme in the absence of menadione. Hosoda *et al.* (5) have also found that the $FADH_2$ formed is oxidized by the bound menadione in a much more sluggish manner, indicating that the reduction of the quinones or the dissociation of the hydroquinones from the enzyme is the rate-limiting step of the reaction. These findings may explain why menadione inhibits this quinone reductase.

We have recently found that menadione has a significant influence on the 5′FSBA labeling of this quinone reductase. The results suggest that menadione may affect the quinone reductase activity in a rather complex fashion. A possible mechanism of this quinone reductase that will explain our results is discussed.

Experimental Procedures

Materials. 5′-FSBA was obtained from Sigma Chemical Co. (St. Louis, MO). 5′-Fluorosulfonylbenzoyl-[*adenine*-8-^{14}C]adenosine (36.2 mCi/mmol) was from New England Nuclear Corp. (Wilmington, DE).

Enzyme preparation. The NAD(P)H:quinone acceptor oxidoreductase was purified from livers of female Wistar rats injected daily for 3 days with 3-methylcholanthrene (4 mg/100 g of body weight), using a procedure described by Haniu *et al.* (6).

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ABBREVIATION: 5′FSBA, 5′-[*p*-(fluorosulfonyl)benzoyl]adenosine.

Enzymatic assays. The NAD(P)H:quinone acceptor oxidoreductase activity was determined spectrophotometrically, by measuring the oxidation of NADH at 340 nm, at 25°, when menadione was used as the substrate (7). The assay mixture (1 ml) contained 50 mM sodium phosphate, pH 7.4, 197 μM NADH, and 160 μM menadione. The reaction was initiated upon addition of the enzyme. The enzymatic assays were always performed in duplicate, and good agreements were always found between the two measurements. The specific activity of this enzyme preparation was 373 μmol of NADH oxidized/min/mg of protein. When potassium ferricyanide was used as the substrate, the activity was determined by measuring the reduction of potassium ferricyanide at 420 nm [$\epsilon_{420} = 1.0 \times 10^3\ M^{-1}\ cm^{-1}$ (8)], at 25°. The assay mixture was identical to that described above, except that 240 μM potassium ferricyanide was used as the substrate instead of menadione.

Results and Discussion

As expected, we found that the NADH-menadione reductase activity of rat liver quinone reductase was reduced upon preincubation of the enzyme with menadione (Fig. 1). However, it was not expected that menadione would enhance 5'FSBA inhibition of the enzyme (Fig. 1). 5'FSBA is an affinity labeling analogue of NAD^+ and has previously been shown to inactivate this quinone reductase by binding to the active site of the enzyme (7). As shown in Fig. 1, a 50% inhibition of the NADH-menadione reductase activity was found after a 15-min incubation in the presence of 1.54 mM 5'FSBA. When menadione was added during the incubation of the enzyme with 5'FSBA, the degree of enzyme inactivation was greatly increased. For example, the enzyme was completely inhibited when 370 μM menadione was present during incubation of the enzyme with 1.54 mM 5'FSBA, whereas 55% of the activity remained for the sample containing menadione alone. The augmentation in activity loss for those samples treated with both 5'FSBA and menadione, compared with the samples treated with only 5'FSBA or menadione, cannot be explained simply by an additive inhibitory effect of menadione and 5'FSBA. If the inhibitions produced by 5'FSBA and menadione were additive, we should have found that the degree of inhibition was unchanged at different menadione concentrations, taking the activities of those samples pretreated with menadione alone as 100%. Instead, we found that the curve was more or less superimposable with the curve plotted by taking the activity of the control (i.e., without treatment with 5'FSBA or menadione) as 100%. These results indicate that menadione has a definite influence on 5'FSBA inactivation of NAD(P)H:quinone acceptor oxidoreductase.

Because the inactivation of the quinone reductase by 5'FSBA involves two processes, firstly, formation of the reversible enzyme-inhibitor complex and, secondly, irreversible incorporation of 5'FSBA into the enzyme (see Ref. 7), the enhancement of 5'FSBA inhibition of the enzyme by menadione could result from facilitation of the formation of the initial reversible enzyme-inhibitor complex or an increase in the rate of inactivation. It was found that, in fact, both the initial binding affinity of 5'FSBA for the quinone reductase and the rate of inactivation were greatly increased when menadione was present. The dissociation constant (K_d) for the initial reversible enzyme-inhibitor complex was estimated to be 0.53 mM, with a k_2 of 0.089 min^{-1} (at pH 7.5 and 25°), when enzyme was preincubated with 0.58 mM menadione, whereas the K_d and k_2 were 2.86 mM and 0.22 min^{-1}, respectively, for enzyme without pretreatment with menadione (Fig. 2). It might also be possible that the enhancement of the 5'FSBA inactivation by menadione resulted in part from the binding of the nicotinamide nucleotide analogue to a negative effector site on the enzyme in the presence of menadione. However, an additional nicotinamide nucleotide binding site, besides the active site of the enzyme, has not been found, and our affinity labeling study using 5'FSBA identified only one binding site (i.e., the active site) per subunit (7). Fig. 3 shows that the influence of menadione on 5'FSBA inhibition of the quinone reductase was pronounced throughout the range of different concentrations of 5'FSBA. In addition, by taking the activities of those samples containing 5'FSBA alone as 100%, we found that the augmentation in activity loss by menadione increased upon increasing of the 5'FSBA concentration.

The findings described above indicate that menadione affects 5'FSBA binding significantly. Because 5'FSBA is a nicotinamide nucleotide analogue and has been shown to bind specifically at the nicotinamide nucleotide binding site, these results suggest that menadione enhances the nicotinamide nucleotide

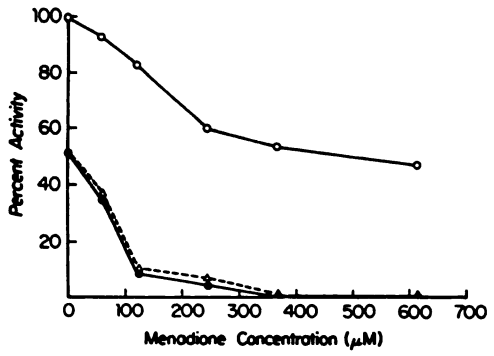

Fig. 1. Enhancement by menadione of 5'FSBA inhibition of NAD(P)H:quinone acceptor oxidoreductase. The quinone reductase (170 μg/ml) was incubated with menadione at the indicated concentrations, in the absence (○) or presence (●) of 5'FSBA (1.54 mM). After a 15-min incubation at 25°, the enzyme activity was determined. The activity of a control without 5'FSBA and menadione treatment was taken as 100%. The percentage activity of samples treated with both 5'FSBA and menadione was also calculated, by taking the activity of the samples containing menadione at same concentrations as 100% (△).

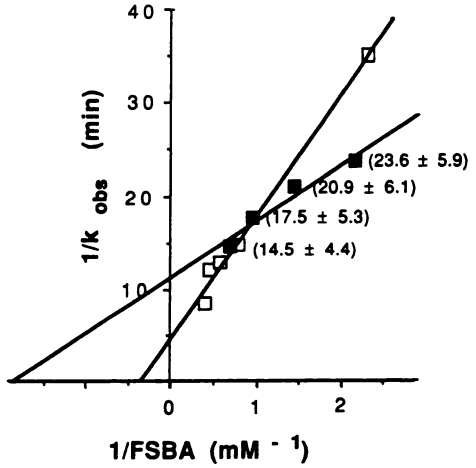

Fig. 2. Dependence of pseudo-first-order rate constants of the inactivation reaction on 5'FSBA concentration. □, Observed first-order constants for the loss of enzyme activity (k_{obs}), calculated from curves as illustrated in Fig. 1 of Ref. 7; ■, k_{obs} determined when 0.58 mM menadione was present during the incubation. The analysis was performed three times. The standard deviation for the data is shown in parentheses.

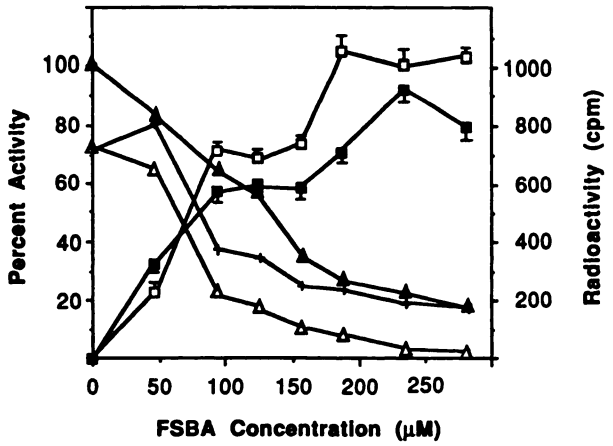

Fig. 3. Effect of menadione on 5'FSBA inhibition and labeling of NAD(P)H:quinone acceptor oxidoreductase. The enzyme (170 μg/ml) was incubated with [^{14}C]5'FSBA (1.33 mCi/mmol) at the indicated concentrations, in the absence (▲) or presence (△) of menadione (0.188 mM). After a 60-min incubation at 25°, the enzyme activity was determined. The activity of a control without 5'FSBA and menadione was taken as 100%. The percentage activity of samples treated with both 5'FSBA and menadione was also calculated by taking the activity of the samples containing 5'FSBA at the same concentrations as 100% (+). The radiolabeled protein was separated from free nucleotide probe by sodium dodecyl sulfate-gel electrophoresis. The amount of radioactivity associated with each sample was determined; □, 5'FSBA labeling in the absence of menadione; ■, 5'FSBA labeling in the presence of menadione. This experiment was performed three times. The standard deviations for the results of enzyme labeling are shown as *error bars*.

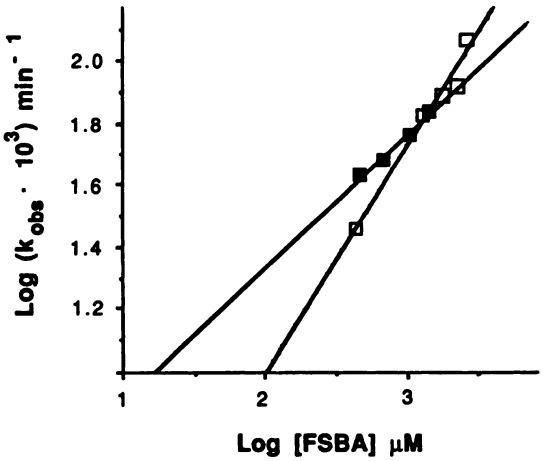

Fig. 4. Kinetic determination of stoichiometry of NAD(P)H:quinone acceptor oxidoreductase inactivation by 5'FSBA in the absence (□) or presence (■) of 0.58 mM menadione.

binding. However, we found that the 5'FSBA incorporation as well was affected by menadione. 5'FSBA labeling decreased upon increasing of the menadione concentration (Fig. 3). Using the data shown in Fig. 2, our inactivation kinetic analysis revealed that NAD(P)H:quinone acceptor oxidoreductase was inactivated by reaction with 0.4 mol of 5'FSBA/mol of active site when 0.58 mM menadione was present during the incubation, whereas 0.8 mol of 5'FSBA/mol of active site was calculated for enzyme without pretreatment with menadione (Fig. 4). These results not only do not agree with the presence of an additional site for nicotinamide nucleotide, besides the active site, but also imply that less than one binding site/subunit is available for nicotinamide nucleotide when menadione is present. These results indicate that menadione modulates the quinone reductase activity in a complex fashion; it enhances nicotinamide nucleotide binding affinity on one hand and decreases nicotinamide nucleotide binding on the other hand. One possible explanation for these results is that there may be a negatively cooperative interaction between the two nicotinamide nucleotide binding sites on two subunits. Our biphasic 5'FSBA binding curve may support such a mechanism (7). With two NAD(P)H binding sites on two subunits, menadione may enhance the nucleotide binding affinity of one binding site but inhibit the binding of the nucleotide to the other site. Hosoda *et al.* (5) measured the menadione binding, obtaining a value of 0.78 mol/mol of enzyme (calculated using a molecular weight of 5.25×10^3). This indicates that there is only one or only one tight menadione binding site/two subunits of the enzyme. With all of the available data, one hypothesis can be put forward, that, during the menadione reduction, the enzyme has only one site/two subunits available for NAD(P)H to bind to, and the binding affinity of NAD(P)H for this site is enhanced when menadione is present. This would be a way for this enzyme to control the efficiency of the electron transfer process, i.e., the menadione molecule receives both electrons from the same NAD(P)H molecule. It was shown by Huang *et al.* (9) that the reduction of 1 mol of quinone to 1 mol of hydroquinone requires only 1 mol of NAD(P)H.

We have performed the analysis using an enzyme assay in the absence of an "activator." We felt that we would deal with a simpler system by leaving out the activator. It has been shown that substances such as Triton X-100, sucrose, and bovine serum albumin abolish the modulation of enzyme kinetics by the relative concentrations of electron donor and acceptor (10, 11). Kinetic analysis in the presence of activators is planned, to determine how these compounds affect the interaction of menadione and 5'FSBA with the enzyme.

Another feature of this quinone reductase is that it is a two-electron transfer quinone reductase. The following mechanism is proposed to explain the nature of the two-electron transfer reaction catalyzed by this enzyme. In general, flavoproteins receive two reducing equivalents from NAD(P)H, through a hydride transfer mechanism, and the reduced flavin donates its reducing equivalents to substrates by two one-electron transfer reactions. As was pointed out previously by Iyanagi (3), the one-electron oxidation-reduction potential is an important factor in the reduction of quinones or other electron acceptors by flavoproteins catalyzing one-electron transfer. During oxidation of enzyme-$FADH_2$, it is thought that the redox potential for enzyme-FAD/enzyme-FADH·for this quinone reductase may be more negative than that for enzyme-FADH·/enzyme-$FADH_2$. Therefore, enzyme-FADH·is very short lived and rapidly converted to enzyme-FAD in the presence of quinone substrates. In addition, quinones, such as menadione, have a high binding affinity for the enzyme, as suggested by Hosoda *et al.* (5). With the efficient electron transfer mechanism of receiving both electrons from the same NAD(P)H molecule (discussed above), the semiquinone may be rapidly converted to hydroquinone upon receiving the second electron, when it is still in the active site of the enzyme. This would explain why no semiquinone is detected during the catalysis and why a one-electron acceptor such as potassium ferricyanide can be used as substrate. Our kinetic analysis revealed that the reduction of potassium ferricyanide catalyzed by this enzyme followed normal Michaelis-Menten kinetics (12). When potassium fer-

ricyanide was used as the substrate, no substrate inhibition was detected, and neither the degree of 5'FSBA inhibition nor the 5'FSBA labeling of the enzyme was affected by preincubation of the enzyme with potassium ferricyanide (data not shown). These results indicate that the mechanism of potassium ferricyanide reduction catalyzed by NAD(P)H:quinone acceptor oxidoreductase is very different from that of quinone reduction.

In conclusion, we suggest that quinones, such as menadione, play an important role in the reaction catalyzed by NAD(P)H:quinone acceptor oxidoreductase. They are substrates of the enzyme and affect the enzyme activity in a complex manner. The proposed mechanism could be physiologically important in controlling the efficiency of the electron transfer process catalyzed by this quinone reductase.

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