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Mechanism of Inhibition of Aldose Reductase by Menadione (Vitamin K3)

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SUMMARY

Incubation of human placental aldose reductase (EC 1.1.1.21) with menadione (0.5–3.0 mM) resulted in time-dependent loss of the catalytic activity of the enzyme. Kinetic analysis of the data suggests that the inactivation process follows a single apparent rate constant that displays hyperbolic dependence on menadione concentration, indicating that menadione forms a kinetically significant, dissociable complex with the enzyme before the formation of an inactive enzyme-menadione complex. The inactivation of the enzyme with menadione was reversed upon dialysis of the inactivated enzyme against buffer containing 10 mM dithiothreitol suggesting that menadione reacts with enzyme sulfhydryl residue(s). Inactivation of the enzyme was significantly prevented by dithiothreitol (5 mM), NADPH (0.1 mM), and DL-glyceraldehyde (10 mM). Correlation of the fractional remaining activity with the extent of modification indicates that loss of catalytic activity corresponds to the modification of a single amino acid residue of the enzyme protein. Recombinant human aldose reductase, obtained by overexpression in *Escherichia coli*, and aldose reductase in which Cys-80 or Cys-303 was replaced by serine were also inactivated by menadione. However, enzyme in which Cys-298 was replaced by serine was insensitive to menadione. On the basis of these observations, it is suggested that menadione forms a thiodione-like adduct with Cys-298, leading to inactivation of the enzyme.

Aldose reductase catalyzes the NADPH-dependent reduction of aldo-sugars to their corresponding alcohols, which is the first and the rate-limiting step of the polyol pathway (1, 2). The enzyme has been found to be present in most tissues examined and is a member of an oxido-reductase superfamily (3). The observation that exposure of tissues that do not require insulin for glucose uptake to high glucose concentrations results in the activation of the polyol pathway has led to the suggestion that activation of aldose reductase may be responsible for the manifestation of hyperglycemic cell injury associated with secondary complications of diabetes mellitus (1–5). This hypothesis is supported by the observations that inhibition of aldose reductase by a variety of structurally nonrelated compounds leads to attenuation, prevention, and in some instances reversal of diabetic tissue pathology (3–5). The ability of aldose reductase inhibitors to prevent diabetic complications has instigated an extensive search for specific and selective aldose reductase inhibitors that could be used for the therapeutic management of diabetic complications. Nevertheless, most of these compounds inhibit other enzymes also (6, 7) and the therapeutic efficacy of these compounds remains uncertain (8, 9).

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In spite of the proposed involvement of aldose reductase in the etiology of diabetic complications, little is known about the active site of the enzyme, and its substrate and inhibitor binding sites have not been identified. Most aldose reductase inhibitors show non- or uncompetitive inhibition patterns with the varied substrates in the direction of aldehyde reduction (7, 10) and have been proposed to bind to a unique inhibitor binding site on the enzyme, distinct from the substrate binding sites (10). The only competitive inhibitor versus the aldehyde substrate is menadione (11, 12). It has also been suggested (11) that the observed increase in sorbitol levels in muscle of patients with Duchenne muscular dystrophy (13) may be a result of a decrease in the concentration of a natural inhibitor such as vitamin K. However, neither the mechanism of inhibition of aldose reductase by K group vitamins nor the binding site of these compounds has been identified. In this communication we provide evidence that menadione inhibits the enzyme by forming covalent adducts with its sulfhydryl residues. Our studies using site-directed mutagenesis further suggest that inhibition by menadione results from thiodione-like formation with Cys-298.

Materials and Methods

Menadione bisulfide, NADPH, and DL-glyceraldehyde were purchased from Sigma Chemical Co. All other chemicals were of the highest purity available.

ABBREVIATIONS: SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; DTT, dithiothreitol.

Expression, isolation, and mutagenesis of human aldose reductase. Expression of human aldose reductase in shaker flask cultures of *Escherichia coli* will be described elsewhere.[1] The reductase was extracted from host cells by an osmotic shock treatment and was purified to homogeneity. Briefly, the purification scheme included ammonium sulfate fractionation (50–80%), chromatofocusing on polybuffer exchanger 74 (Pharmacia), and hydroxylapatite (Bio-Rad) chromatography. Purity of the final enzyme fraction was assessed by SDS-PAGE, which showed a single polypeptide band (35 kDa) on Coomassie staining.

Oligodeoxyribonucleotide-directed mutagenesis (14) was used to prepare aldose reductase cDNA sequences encoding serine substitutions at positions Cys-80 (ARC80S), Cys-298 (ARC298S), and Cys-303 (ARC303S). Briefly, wild-type aldose reductase sequences carried in a pBluescript vector (Stratagene) were treated with individual mutagenic oligonucleotide primers. Wild-type aldose reductase sequences in the expression plasmid pMON5997[1] were then exchanged with the mutant sequences. Before expression in *E. coli*, complete structures of the mutant coding sequences were verified by nucleotide sequence analysis. Mutant reductases were purified to homogeneity using conditions identical to those used for wild-type aldose reductase. Mutant and wild-type reductases co-migrated on SDS-PAGE and cross-reacted with antibodies directed against bovine lens aldose reductase (15).

Aldose reductase was purified from human placenta by the method of Das and Srivastava (16), except that ammonium sulfate was used as the first step in the purification protocol. The homogeneity of the enzyme was established by the presence of a single protein band on reducing SDS-PAGE at pH 8.6 and by the appearance of a single protein peak coincident with the enzyme activity peak on Sephadex G-100 gel filtration. The enzyme was stored at a concentration of 300 μg/ml at 4° in 50 mM imidazole-HCl, pH 7.0, containing 5 mM β-mercaptoethanol.

Enzyme assays and inactivation studies. The enzyme activity was determined at 25° in 50 mM phosphate buffer containing 0.4 M lithium sulfate, pH 6.0, 0.1 mM NADPH, and 10 mM DL-glyceraldehyde, with an appropriate amount of the enzyme, by monitoring the rate of disappearance of NADPH by using a Gilford Response II spectrophotometer. One unit of the enzyme is defined as the amount of enzyme required to oxidize 1 μmol of NADPH/ml/min.

Because purification and storage of aldose reductase, even in thiol-containing buffers, leads to the formation of mixed and intramolecular disulfides (17), in order to obtain a single species the enzyme was incubated with 0.1 M DTT for 1 hr at 37°. The reduced enzyme was filtered through a Sephadex G-10 column to remove excess DTT and was then incubated with the indicated concentrations of menadione in 0.1 M Tris·HCL, pH 8.0. Aliquots were withdrawn from the incubation mixture at different times for determination of the enzyme activity.

In order to quantitate the number of sulfhydryl residues modified with menadione, the reduced enzyme was concentrated by ultrafiltration through a Centricon microconcentrator with a 10-kDa cut-off filter. The concentrated enzyme was incubated with menadione bisulfide, and the thiodione formation was quantitated at 412 nm using a molar coefficient of 1000 M^{-1} cm^{-1}, after correction for unreacted menadione (molar coefficient at 412 nm, 60 M^{-1} cm^{-1}). The molar absorbance coefficient of thiodione was determined by stoichiometric titration of menadione bisulfide and disodium glutathione in 0.1 M Tris·HCl, pH 8.0.

Protein was determined by the method of Bradford (18) and SDS-PAGE was performed in accordance with the procedure of Laemmli (19).

Data analysis. Inactivation curves were fitted to either single- or double-exponential equations, with or without a constant intercept term, using NFIT (Island Products, Galveston, TX). The best fits to the data were chosen on the basis of the lowest χ^2 and highest r^2 values.

Results

Inactivation kinetics of the native enzyme. Menadione (0.5–3.0 mM) caused a time-dependent loss of catalytic activity of aldose reductase purified from human placenta (Fig. 1). At each menadione concentration a fraction of the enzyme, which does not change upon prolonged incubation, remains uninhibited. The time course of this type of inactivation is best described by the following equation:

$$y = a + b \cdot \exp^{-kt} \quad (1)$$

where y is the fractional remaining activity, a is the uninhibited fraction of the catalytic activity, and k is the apparent first-order rate constant for inactivation. All inactivation curves were well fitted to a single-exponential equation (r^2 varied from 0.996 to 0.999). There was no further increase in the goodness of fit upon addition of another exponential term to the equation. When the concentration of menadione was increased, the inactivation rate increased proportionally (Table 1). The initial rates of inactivation (k) showed a hyperbolic dependence on menadione concentration (Fig. 1, *inset*), suggesting that a significant amount of dissociable complex is formed between the enzyme and menadione before the formation of the inactivated enzyme-menadione complex. In such a reaction scheme the observed rate constant (20) is described as:

$$k = (k_1 \cdot k_2[I])/(k_1[I] + k_{-1}) \quad (2)$$

where k_1 and k_{-1} are the rate constants for the formation and dissociation of the dissociable complex, respectively, and k_2 is the rate constant for the formation of the inactivated complex. A fit of the data to eq. 2 is shown in Fig. 1, *inset*.

In contrast to the rate constant k, the intercept factors did not show large changes on increasing of the menadione concentration, and the reactive fraction of the enzyme activity was 0.83 ± 0.03. The enzyme inactivated by menadione could be

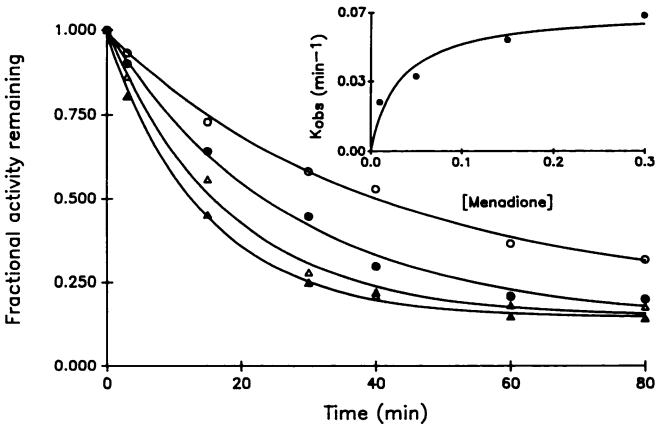

Fig. 1. Inactivation of human placental aldose reductase by menadione. Reduced human placental aldose reductase (200 μg/ml) was incubated with 0.05 (○), 0.1 (●), 0.15 (△), or 0.3 (▲) mM menadione bisulfide in 0.1 M Tris·HCl, pH 8.0. At the indicated times, aliquots of the enzyme were withdrawn and the catalytic activity of the enzyme was determined as described in Materials and Methods. Data are shown as discrete *points*, and the *curves* are best fits of eq. 1 to the data. *Inset*, concentration dependence of k. Data are shown as discrete *points*, and the *curve* is the best fit of the data to eq. 2, in which $1/k_2 = 17.1$ min and $k_{-1}/(k_1 \cdot k_2) = 0.24$ mM·min^{-1}.

[1] J. M. Petrash, T. M. Harter, C. Devine, P. Olins, A. Bhatangar, S.-Q. Liu, and S. K. Srivastava. Involvement of cysteine in catalysis and inhibition of human aldose reductase: site-directed mutagenesis of cysteine residues 80, 298, and 303. *J. Biol. Chem.* In press.

TABLE 1

Kinetic parameters for the rate of inactivation of human placental aldose reductase by menadione

Enzymes were reduced upon incubation with 0.1 mM DTT. Excess DTT was removed by filtration on Sephadex G-25. The enzymes were incubated with the indicated concentration of the additives in 0.1 M Tris·HCl, pH 8.0. Appropriately diluted enzyme incubated under identical conditions was used as the control.

Additives	Time constant (1/k)	a	b
	min		
Menadione			
0.01 mM	40.7 ± 5.7	0.20 ± 0.05	0.79 ± 0.04
0.05 mM	27.0 ± 3.1	0.13 ± 0.03	0.86 ± 0.04
0.15 mM	17.9 ± 2.0	0.15 ± 0.03	0.85 ± 0.03
0.30 mM	14.6 ± 1.0	0.14 ± 0.11	0.84 ± 0.02
Menadione, 0.05 mM			
+100 μM NADPH	18.2 ± 3.0	0.78 ± 0.02	0.23 ± 0.03
+10 mM DL-Glyceraldehyde	35.1 ± 4.0	0.52 ± 0.07	0.46 ± 0.06

reactivated (>90% recovery) by overnight dialysis against 10 mM DTT, suggesting that menadione was reacting primarily with the sulfhydryl residues of the enzyme.

Pre-equilibration of the enzyme with DTT (10 mM) or with saturating concentrations of NADPH (0.1 mM) or DL-glyceraldehyde (10 mM) afforded significant protection against inactivation of the enzyme by menadione (Fig. 2). No significant loss of activity was observed in the presence of DTT. With NADPH, the unreactive fraction of the enzyme activity increased from 0.20 to 0.80 (Table 1). However, in the presence of NADPH the reactive fraction reacted with menadione at a faster rate than it does with the unliganded enzyme, possibly because the reduction of the number of reactive sites in the presence of NADPH increases the effective molar excess of menadione over the reactive sites on the enzyme. DL-Glyceraldehyde also protected the enzyme against inactivation. The unreactive fraction in the presence of DL-glyceraldehyde rose from 0.2 to 0.5, suggesting that DL-glyceraldehyde was less effective in occluding the menadione-reactive sites than was NADPH.

Stoichiometry of modification. In order to quantitate the number of sulfhydryl residues modified by menadione, 550 μg of the native enzyme protein, purified from human placenta, were incubated with 0.15 mM menadione and the modification reaction was monitored as an increase in absorbance of the mixture at 412 nm, as described in Materials and Methods. After various time intervals aliquots of the enzyme were withdrawn, to assay the enzyme activity. Fig. 3 shows the correlation between the fractional remaining activity and the number of cysteine residues modified. The relationship appears linear (r^2 = 0.995), suggesting that incubation of the enzyme with menadione results in the modification of a single cysteine residue on the enzyme protein.

Identification of the modified cysteine. Wild-type aldose reductase and aldose reductase in which Cys-80, Cys-298, or Cys-303 was replaced with serine were used to identify the cysteine residue modified by menadione. Complete sequence analysis of the mutant cDNA revealed that the only changes to the wild-type coding sequences had occurred at the intended mutation site. Complete kinetic characterization of these mutants has been presented elsewhere.[1] Incubation of the wild-type enzyme, AR-C80S, or AR-C303S with 0.15 mM menadione led to time-dependent decreases in the catalytic activity of these enzymes, but AR-C298S was not inactivated by menadione (Fig. 4). The rates of inactivation of the recombinant enzymes were similar to the rate of inactivation of the native enzyme purified from human placenta, except that a larger fraction of the enzyme activity remained unreactive with menadione (Table 2). These results suggest that Cys-298 is the major site of menadione binding and that substitution of this group with serine renders the enzyme insensitive to menadione.

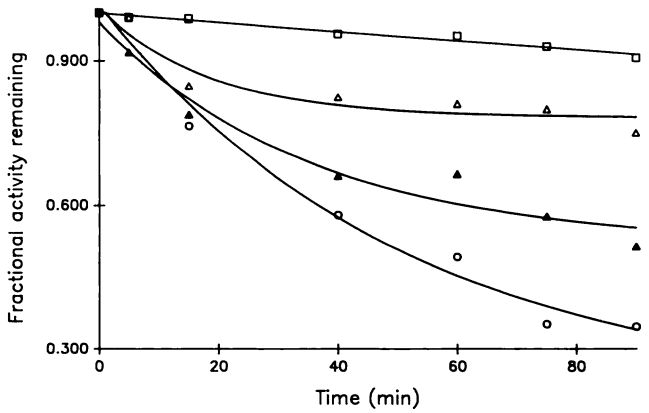

Fig. 2. Protection of aldose reductase from inactivation by menadione. Reduced aldose reductase was incubated with 0.15 mM menadione bisulfide (○) or 0.15 mM menadione bisulfide containing 0.1 mM NADPH (△), 5 mM DTT (□), or 10 mM DL-glyceraldehyde (▲). Aliquots were withdrawn at the indicated times and the enzyme activity was determined. Data are shown as discrete *points*, and the *curves* are best fits of eq. 1 to the data.

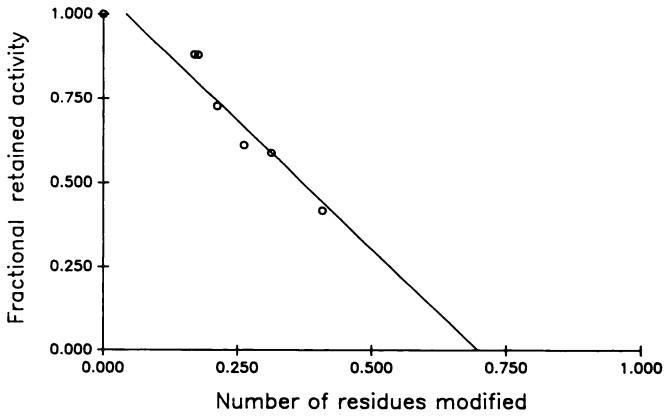

Fig. 3. Correlation between the loss of catalytic activity and the extent of modification of aldose reductase by menadione. Reduced (native) aldose reductase (580 μg/ml) was incubated with 0.15 mM menadione in 0.1 M Tris·HCl, pH 8.0. The extent of modification was determined by following the absorbance of the reaction mixture at 412 nm. The *line* shown is the best fit of the data to a linear function ($y = ax + b$), where a = 1.53 ± 0.2 and b = 1.06 ± 0.05 (r^2 = 0.995).

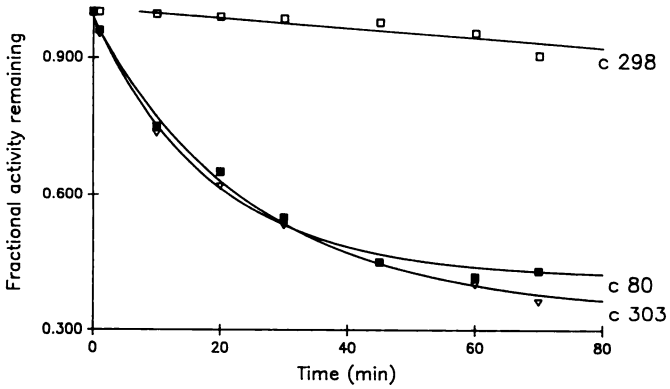

Fig. 4. Inactivation of recombinant human placental aldose reductase and aldose reductase mutants in which Cys-80 (■), Cys-298 (□), or Cys-303 (▽) has been substituted with serine. The enzymes were reduced and incubated with 0.15 mM menadione bisulfite, and aliquots were withdrawn at the indicated times and assayed for enzyme activity. The inactivation curve for the wild-type enzyme has been omitted for clarity. Data are shown as discrete *points*, and the *curves* are best fits of eq. 1 to the data.

TABLE 2

Kinetic parameters for the rate of inactivation of human recombinant aldose reductase (wild-type) and aldose reductase mutants in which Cys-80 (C-80), Cys-298 (C-298), or Cys-303 (C-303) has been replaced by serine

The enzymes were incubated with 0.15 mM menadione under experimental conditions identical to those used to obtain the data shown in Table 1. See Materials and Methods for details.

Enzyme	Time constant (1/*k*)	*a*	*b*
	min		
Wild-type	9.31 ± 3.2	0.42 ± 0.03	0.52 ± 0.04
C80S	18.8 ± 4.9	0.41 ± 0.04	0.57 ± 0.05
C298S	>80	ND[a]	ND
C303S	25.1 ± 4.5	0.34 ± 0.04	0.64 ± 0.04

[a] ND, not determined.

Discussion

Menadione is one of the few inhibitors that inhibit aldose reductase competitively with respect to the aldehyde substrate (11, 12). Being a quinone, this compound is expected to act as a substrate analog and, therefore, studies on the mechanism of inhibition of the enzyme by menadione and related analogs could yield potentially useful information about the structure and the environment of the active site of the enzyme. However, little is known about the mechanism of inhibition of the enzyme by menadione or other vitamin K analogs, and the binding site for these compounds, which presumably is the substrate binding site of the enzyme, has not been identified.

As shown in Fig. 1, simple exponential inactivation of the enzyme indicates a unique set of reactive sites on the enzyme with identical reactivity towards menadione. Statistical correlation between the fractional activity lost and the fraction of residues modified, in accordance with the method of Tsou (21), shows a linear relationship, suggesting that loss of activity of the enzyme corresponds to modification of a single amino acid residue. The likelihood that this residue is a sulfhydryl is suggested by the reversibility of inactivation by DTT.

Location of the site that binds menadione is indicated by the protection experiments. The ability of both NADPH and DL-glyceraldehyde to prevent inactivation of the enzyme by menadione suggests that the menadione-binding residue may be present at the active site of the enzyme. Binding of NADPH causes a distinct conformational change in the enzyme and, therefore, it could be argued that NADPH protection of the enzyme from inactivation may be due to a conformational shift in the position of the reactive cysteine. Alternatively, NADPH binding may result in steric masking of the reactive cysteine. Because DL-glyceraldehyde also protected the enzyme from inactivation by menadione, albeit to a much lesser extent, it is possible that the substrate binding site is in close proximity to the reactive cysteine. Human placental aldose reductase displays random addition of substrates (12) and, therefore, DL-glyceraldehyde could bind to the free enzyme, although the affinity of the enzyme for DL-glyceraldehyde is expected to be much lower than that for NADPH. Simultaneous protection of the enzyme by both the substrates indicates that menadione binds to a single sulfhydryl residue located near the active site of the enzyme. However, because AR-C298S is catalytically active, it is likely that Cys-298 does not directly participate in catalysis.

Of the total of seven cysteine residues present in aldose reductase, only three are accessible to 5,5′-dithiobis(2-nitrobenzoic acid) (22). A structural model of porcine aldose reductase complexed to 2′-monophosphoadenosine 5′-diphosphoribose has revealed that Cys-80, Cys-298, and Cys-303 are located near the cofactor binding site (23). In order to identify which cysteine residue of the enzyme binds menadione, we generated three different mutants of the enzyme, AR-C80S, AR-C298S, and AR-C303S, in which Cys-80, -298, and -303, respectively, were substituted with serine. The susceptibility of each of these mutants to inactivation by menadione was determined, and of these only AR-C298S was found to be insensitive to inhibition by menadione; the other mutants displayed reactivity to menadione similar to that of the wild-type or the native enzyme. Moreover, the modified cysteine residues, 298 and 303, are expected to be close together in the enzyme sequence. The ability of menadione to inhibit AR-C303S, but not AR-C298S, suggests that binding of menadione to Cys-298 represents a specific interaction.

The reactivity of quinones with sulfhydryl compounds is well known (24, 25). Position 2-substituted 1,4-napthoquinones undergo nucleophilic substitution reactions with glutathione and cysteine (25) and with sulfhydryl residues of proteins (26). Reaction of cysteine with menadione forms 5-methyl-6-hydroxy-3*H*-naphtho[2,2-*b*][1,4]thiazine-2-carboxylic acid (27), whereas reaction of glutathione with menadione forms a 3-glutathionyl derivative of menadione termed thiodione (25). Thus, for the formation of carboxylic acid (the former reaction) cysteine must be at the amino terminus, and for the latter reaction cysteine should not be at the amino terminus of a peptide. Because menadione reacts with Cys-298 (which does not have a free amino group), it is likely that menadione forms a thiodione-like adduct with Cys-298 of aldose reductase. It is, therefore, suggested that Cys-298 is a part of the active site of aldose reductase.

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