0026-895X/89/060825-06$02.00/0

MOLECULAR PHARMACOLOGY, 36:825-830

ACCELERATED COMMUNICATION

Involvement of Sulfhydryl Residues in Aldose Reductase-Inhibitor Interaction

ARUNI BHATNAGAR, SIQI LIU, BALLABH DAS, and SATISH K. SRIVASTAVA

Department of Human Biological Chemistry and Genetics, University of Texas Medical Branch, Galveston, Texas 77550

Received July 3, 1989; Accepted September 18, 1989

SUMMARY

Purification and storage of aldose reductase isolated from human placenta or kidney in β-mercaptoethanol-containing buffers causes a time-dependent decrease in its catalytic activity, as well as in its sensitivity to inhibition by aldose reductase inhibitors such as sorbinil. Dithiothreitol (DTT) slowly regenerated the enzyme activity, as well as reversed the alterations in the sensitivity of the enzyme to sorbinil. In contrast to sorbinil, the inhibition of aldose reductase by tolrestat was less affected by purification and/or storage in β-mercaptoethanol-containing buffers. Kinetic analysis of the rate of increase in sensitivity of the enzyme to sorbinil on incubation with DTT reveals that the reaction follows two kinetically distinct rate constants. Also, sorbinil protected the enzyme from inactivation with sulfhydryl-modifying reagents 5,5′-dithiobis(2-nitrobenzoic acid) and glutathione disulfide. The enzyme stored in β-mercaptoethanol migrates as two distinct bands, one corresponding to molecular weight 36,000 and the other to molecular weight 33,000, on nonreducing sodium dodecyl sulfate-polyacrylamide gel electrophoresis, whereas under reducing conditions the protein migrates as a single discrete band corresponding to molecular weight 36,000. Moreover, the molecular weight 33,000 form of the enzyme could be converted to the molecular weight 36,000 form on reduction with DTT, indicating that the molecular weight 33,000 form of the enzyme is due to intramolecular disulfide bond(s) formed, which presumably cause the protein to assume a more folded conformation and migrate faster through the gel, and not due to proteolysis. These studies indicate that oxidation of sulfhydryl residues, including disulfide bond formation, during purification and storage in β-mercaptoethanol-containing buffers alters the sensitivity of the enzyme to some inhibitors.

Insulin as well as oral hypoglycemic agents have significantly increased the life span of diabetic patients. However, in a majority of patients with diabetes, glucose levels in the insulin-independent tissues fluctuate significantly even with insulin therapy. The periodic hyperglycemia may be the major cause of a number of pathological conditions such as nephropathy, neuropathy, retinopathy, epitheliopathy, and cataractogenesis. Aldose reductase, which reduces aldo-sugars to their corresponding alcohols in the presence of NADPH, has been implicated in the pathogenesis of these diabetic complications (1). A number of synthetic flavonoids and spirohydantoin derivatives, which effectively inhibit aldose reductase *in vitro*, have been shown to reduce or reverse some of the tissue damage under hyperglycemic conditions, providing a strong case for the involvement of this enzyme in the development of diabetic complications (2–4). The obvious therapeutic potential of this class of drugs has stimulated a wide interest in the development of specific aldose reductase inhibitors. A number of compounds that inhibit aldose reductase have been synthesized and a few are in the early stages of clinical trials. However, their therapeutic utility is limited, especially because most of these compounds are nonspecific and also inhibit other enzymes. Therefore, a critical understanding of both the structural requirements of aldose reductase inhibitors and the nature of the enzyme-inhibitor interactions is necessary. Further, because the inhibitors are targeted for eventual human use, it would be desirable to use human aldose reductase in order to eliminate species differences in the inhibition characteristics of the enzyme (3, 5, 6). One of the major problems in the study of enzyme-inhibitor interactions using homogeneous enzyme preparations from human tissues has been the lability of the inhibitor binding site (3, 7). The susceptibility of the enzyme to inhibition has been found to decrease with increased enzyme purification or storage (3). Based on these observations it has been suggested that, for inhibition studies including screening for new drugs, the use of purified enzyme should be avoided (3). The use of the crude enzyme for the study of enzyme-inhibitor interactions could, however, be misleading and liable to lead to erroneous conclusions. In this communication we

This investigation was supported in part by National Institutes of Health Grants EY 01677 and DK 36118.

ABBREVIATIONS: DTT, dithiothreitol; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; NEM, *N*-ethylmaleimide.

present evidence for the involvement of sulfhydryl residues in aldose reductase inhibitor interaction and propose that alterations in the inhibitor binding site of the enzyme on purification and storage are due to the oxidation of the enzyme sulfhydryl residues, including intramolecular disulfide bond formation, and that under fully reducing conditions the inhibitor binding site is stable and unaltered by purification.

Materials and Methods

DTNB, GSSG, DTT, NADPH, and DL-glyceraldehyde were purchased from Sigma Chemical Co. Sorbinil and tolrestat were gifts from Pfizer Chemical Company and Imperial Chemical Industries PLC, respectively. All other chemicals used were of the highest purity available.

Aldose reductase from human placenta or human kidney was purified to homogeneity as described earlier (8). At each step of purification, the enzyme preparation was equilibrated with 10 mM imidazole-HCl buffer containing 5 mM β-mercaptoethanol (Buffer A). Aliquots of the enzyme preparation were removed at every step of purification, i.e., DE-52, chromatofocusing, and affinity chromatography and Sephadex G-100 gel filtration, and were used for K_i determinations in the presence and absence of 5 mM DTT, added to Buffer A. The homogeneity of the enzyme was established by the movement 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 homogeneous enzyme was stored at 4° in 50 mM imidazole buffer (pH 7.0) containing 5 mM β-mercaptoethanol. For sulfhydryl modification studies, the enzyme was dialyzed several times against 100 mM phosphate (pH 7.0) that was saturated with nitrogen in order to remove β-mercaptoethanol. There was no apparent loss of enzyme activity upon dialysis.

The enzyme activity was determined in 50 mM potassium phosphate, pH 6.5, 0.4 M lithium sulfate, 0.1 mM NADPH, 10 mM DL-glyceraldehyde, with an appropriate amount of enzyme, in a total volume of 1 ml at 25°. The reaction was initiated by the addition of enzyme to the reaction mixture and the enzyme activity was determined by monitoring the rate of decrease of NADPH at 340 nm on a Gilford Response spectrophotometer for 4 min. One unit of enzyme catalyzes the oxidation of 1 μmol of NADPH/min at 25°. Thiol compounds such as DTT or β-mercaptoethanol were not included in the assay mixture because they caused rapid reactivation of the inactivated enzyme during the assay period.

In order to determine whether the inhibitors tested react with β-mercaptoethanol or DTT, the absorbance spectrum of 1 mM tolrestat or sorbinil in 0.1 M phosphate (pH 7.0) was studied in the presence and the absence of added thiol compounds, DTT or β-mercaptoethanol, at a concentration of 20 mM. Neither the λ_{max} nor the magnitude of the absorbance peak was affected by the thiol compounds. An absorbance coefficient of 3100 M^{-1} cm^{-1} at 284 nm was used for sorbinil and of 9370 M^{-1} cm^{-1} at 272 nm was used for tolrestat. Equal concentrations of the thiol compounds were added to the reference as well as the sample cuvette to minimize changes in the absorbance due to the thiol compounds themselves.

For inactivation studies, the enzyme was incubated with the indicated concentrations of the modifier in 100 mM phosphate buffer, pH 7.0, at 25°. Aliquots from the incubation mixture were removed at the end of the incubation period and added to cuvettes containing the reaction mixture for enzyme activity determination. In order to study the effect of sorbinil, tolrestat, or NADPH on enzyme modification by sulfhydryl-modifying reagents, aldose reductase was incubated with DTNB or GSSG, in the presence and absence of different ligands, at 25° in phosphate buffer (100 mM, pH 7.0) for up to 30 min. Aliquots were withdrawn at different time intervals for enzyme activity determination. Controls containing no modifying reagents were routinely included and the fractional activity remaining at any given time was calculated relative to the appropriate control. Age of the enzyme is expressed in days, beginning from the day the tissue was homogenized (Day 1). K_i values were calculated using the statistical method of Cleland (9), at saturating concentrations of NADPH (0.1 mM) and DL-glyceraldehyde (10 mM). The curves shown in Fig. 3 were fitted by a nonlinear regression program using Marquardt algorithms.

SDS-PAGE was performed according to the method of Laemmli (10) and the protein concentration was determined by the method of Bradford (11).

Results

Aliquots of the enzyme in Buffer A, removed at each step of purification, showed an increase in the catalytic activity on preincubation with 5 mM DTT (Table 1). When, instead of DTT, additional 5 mM β-mercaptoethanol was added to Buffer A, no increase in the catalytic activity of the enzyme was observed. Storage of the purified enzyme in the presence of 5 mM β-mercaptoethanol-containing Buffer A resulted in a time-dependent loss of catalytic activity of the enzyme. This loss of activity could be reversed by DTT (Fig. 1).The regeneration of the catalytic activity of the enzyme by DTT was slow, and

TABLE 1

Inhibition of human placental aldose reductase at different stages of purification by sorbinil and tolrestat

K_a values were determined without (−DTT) and with 5 mM DTT (+DTT) preincubation in Buffer A at room temperature for 15 min. Figures in parenthesis represent days after homogenization.

Inhibitor	Enzyme activity			
	DE-52 (6)	Chromato-focusing (8)	Affinity (9)	Sephadex (10)
	units/ml			
None				
−DTT	0.027	0.116	0.136	0.043
+DTT	0.036	0.143	0.311	0.100
	K_a			
	DE-52 (6)	Chromato-focusing (8)	Affinity (9)	Sephadex (10)
	μM			
Sorbinil				
−DTT	215 ± 35	251 ± 45	361 ± 51	220 ± 32
+DTT	3.2 ± 0.4	1.7 ± 0.1	0.9 ± 0.1	0.9 ± 0.1
Tolrestat				
−DTT	0.02 ± 0.001	0.022 ± 0.002	0.020 ± 0.003	0.023 ± 0.003
+DTT	0.01 ± 0.001	0.012 ± 0.002	0.007 ± 0.001	0.012 ± 0.001

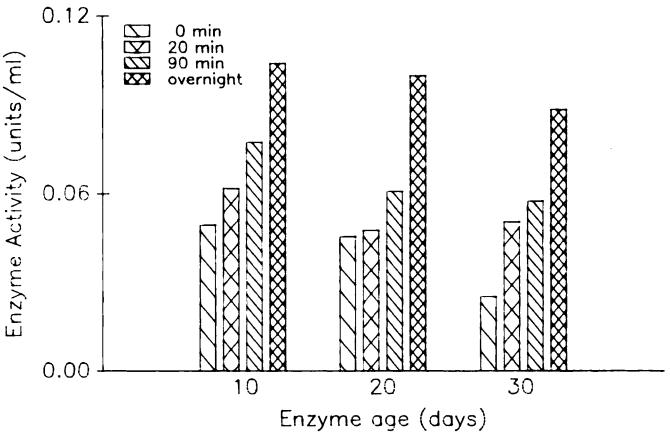

Fig. 1. Regeneration of aldose reductase activity lost during storage by DTT. Homogeneous enzyme obtained 10 days after homogenization was incubated with 5 mM DTT for the indicated time periods.

maximal increase was observed on overnight dialysis with 5 mM DTT. However, the aged enzyme became increasingly resistant to reduction by DTT and less activity could be regenerated after storing the enzyme 30 days, relative to 10 days (Fig. 1). If the enzyme was stored in the absence of β-mercaptoethanol, the catalytic activity of the enzyme was lost rapidly, with a $t_{1/2}$ of 9 min, unless the oxygen dissolved in the storage buffer was completely replaced by nitrogen, in which case it was possible to preserve the enzyme activity for more than 24 hr.

The effect of DTT on the susceptibility of the enzyme, purified and stored in the presence of β-mercaptoethanol, to inhibition was even more striking than that on its catalytic activity. Table 1 shows the K_{ii} values of sorbinil and tolrestat (chemical structures shown in Fig. 2), determined using aldose reductase from different stages of purification, in the absence and the presence of DTT. Little difference in the K_{ii} of sorbinil and tolrestat was observed at different stages of purification. However, when the enzyme aliquots from various stages of purification were treated with DTT, the K_{ii} of sorbinil decreased from approximately 250 μM to 1 to 2 μM, whereas the K_{ii} of tolrestat was reduced by half (Table 1).

Alterations in the inhibition characteristics of aldose reductase on storage with β-mercaptoethanol-containing buffer were not unique to the placental enzyme. Similar changes in the K_{ii} of sorbinil for kidney aldose reductase were observed on storage of the enzyme with 5 mM β-mercaptoethanol. The human kidney enzyme stored for 30 days in Buffer A lost 30% of its catalytic activity. The K_{ii} of sorbinil increased from 2.5 μM on day 10 to 468 μM on day 30 (Table 2). Addition of 5 mM DTT restored the sensitivity of the enzyme to sorbinil. There was no change in the K_{ii} of tolrestat on storage in Buffer A.

To study the kinetics of the effect of DTT on the sensitivity of β-mercaptoethanol to inhibition, 20-day-old aldose reductase

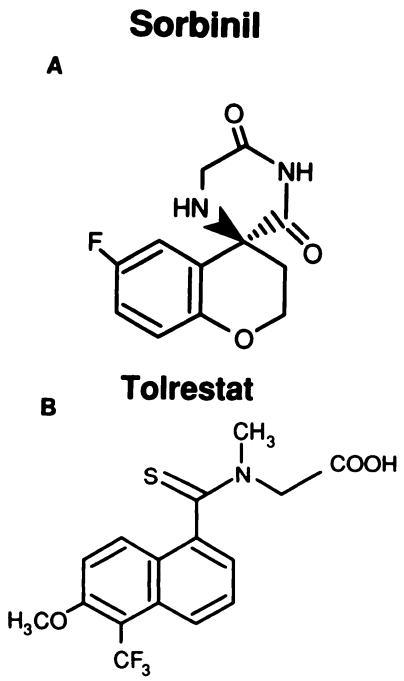

Fig. 2. Chemical structures of aldose reductase inhibitors. A, Sorbinil (CP-45,634, *d*-6-fluro-spiro[chroman-4,4′-imidazolidine]-2′,5′-dione); B, tolrestat (AY27,773, *N*-methyl-*N*-[(5-trifluromethyl-6-methoxyl-naphthalenyl)thioxomethyl]glycine).

TABLE 2

Inhibition of human kidney aldose reductase at different days of storage (at 4–6°) in imidazole-HCl buffer (pH 7.0) with 5 mM β-mercaptoethanol

In the +DTT group, solid DTT was added to the enzyme solution to give a final concentration of 5 mM and the enzyme was incubated with DTT at room temperature for 15 min before the determination of the enzyme activity.

Age of the enzyme[a]	K_{ii}			
	Sorbinil		Tolrestat	
	−DTT	+DTT	−DTT	+DTT
	μM			
10	2.55 ± 0.31	0.89 ± 0.01	0.016 ± 0.002	0.015 ± 0.0001
15	296 ± 16.9	1.23 ± 0.25	0.021 ± 0.004	0.025 ± 0.0030
30	468 ± 90.2	1.10 ± 0.11	0.024 ± 0.004	0.021 ± 0.0020

[a] The total time from homogenization to gel filtration was 10 days.

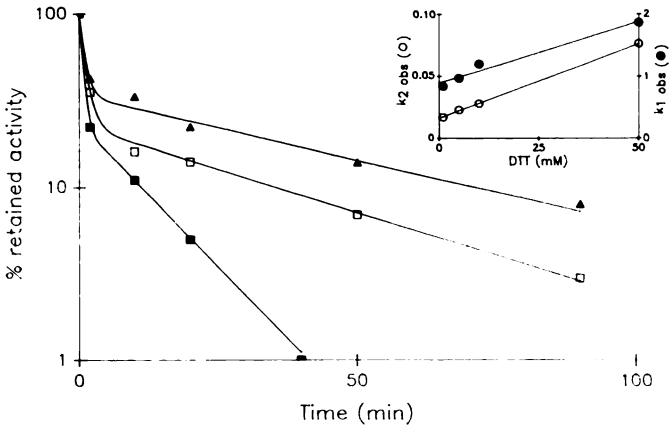

Fig. 3. Effect of sulfhydryl status of aldose reductase on inhibition by sorbinil. The enzyme, exposed to 5 mM β-mercaptoethanol for 20 days was incubated at room temperature with 1 (▲), 5 (□), and 50 (■) mM DTT. Aliquots were withdrawn at different time periods and the enzyme activity was determined with and without sorbinil (1.5 μM). The enzyme activity without sorbinil at each time period was taken as 100%. The curves shown were generated using Eq. 1 and the data are shown as discrete points. *Inset*, the replot of the two k_{obs} calculated from the slopes of the curves shown in the *main panel* except for the curve for 10 mM DTT, which was omitted from the figure for clarity.

was incubated with different concentrations of DTT and inhibition by sorbinil was determined at different incubation times. Fig. 3 shows the inhibition of the enzyme by 1.5 μM sorbinil subsequent to incubation with various concentrations of DTT for up to 90 min. The rate of increase in the sensitivity of the enzyme to inhibition by sorbinil on incubation with DTT was found to be best described by the following equation at all concentrations of DTT tested:

$$y = a_1 e^{-k_{1_{obs}} t} + a_2 e^{-k_{2_{obs}} t} \quad (1)$$

where y is the percentage of retained activity, a_1 and a_2 are the intercept values, t is the time in min, and $k_{1_{obs}}$ and $k_{2_{obs}}$ are the apparent rate constants. At a concentration of 1.5 μM, sorbinil did not inhibit the enzyme stored in β-mercaptoethanol for 20 days. However, more than 50% of the enzyme activity was inhibited by 1.5 μM sorbinil when the enzyme was preincubated with 1 mM DTT for 2 min. On preincubation of the enzyme for 45 min with either 5 or 50 mM DTT, approximately 95% of the enzyme activity was inhibited by 1.5 μM sorbinil (Fig. 3). In order to calculate the actual second-order rate constants of modification of the inhibition characteristics of the enzyme, the apparent rate constants $k_{1_{obs}}$ and $k_{2_{obs}}$ were plotted as a

function of DTT concentration. If each of these two reactions is considered to follow a reversible reaction, then as [DTT] ≫ [E],

$$E \overset{k_1\,[DTT]}{\underset{k_{-1}}{\rightleftharpoons}} E\text{:DTT} \qquad (2)$$

If it is assumed that the concentration of E:DTT at the beginning of the reaction is zero, the rate equation could be integrated and simplified by introducing the equilibrium condition (12) to obtain

$$\ln \{([E] - [E_0])/([E_0]) - [E_e])\} = (k_1\,[\text{DTT}] + k_{-1})t \qquad (3)$$

where $[E_0]$ is the initial concentration of the enzyme, $[E]$ is the concentration at any time t, and $[E_e]$ is the concentration at equilibrium. Because the approach to equilibrium is a first-order rate process, k_{obs}, as obtained above, will be a sum of forward and reverse reaction rate constants and

$$k_{obs} = k_1\,[\text{DTT}] + k_{-1} \qquad (4)$$

Therefore, a plot of k_{obs} as a function of DTT should be linear, with a slope of k_1 and an extrapolated ordinate intercept of k_{-1} (13). The plot of $k_{1_{obs}}$ versus [DTT] is a straight line with a slope (k_1) of $1.9 \pm 0.2 \times 10^{-2}$ mM^{-1} min^{-1}, which intercepts the ordinate at (k_1) 0.96 ± 0.08 mM^{-1} min^{-1}. Similarly, a plot of $k_{2_{obs}}$ versus [DTT] is also linear, with a slope (k_2) of $1.2 \pm 0.013 \times 10^{-3}$ mM^{-1} min^{-1} and an intercept (k_2) of 0.016 ± 0.003 mM^{-1} min^{-1}, indicating that the reduction of enzyme sulfhydryl residues by DTT follows two kinetically distinct rates and that both these reactions proceed with the formation of dissociable E:DTT complexes.

The catalytic activity of aldose reductase was found to be sensitive to sulfhydryl modification. Table 3 shows that incubation of the enzyme with DTNB or GSSG causes a large inhibition of the catalytic activity of the enzyme. Preincubation of the enzyme with 0.1 mM NADPH significantly protected the enzyme from inactivation by GSSG or DTNB. When the enzyme was preincubated with 1 μM sorbinil, the enzyme was also protected from inactivation (Table 3). At low concentrations of GSSG (0.1 mM), sorbinil and NADPH were equally effective in preventing inactivation. However, at 1.0 mM GSSG or at 5 μM DTNB, sorbinil was only half as effective as NADPH in protecting the enzyme against inactivation.

TABLE 3

Protection of the catalytic activity of aldose reductase by sorbinil against inactivation by DTNB and glutathione disulfide

Human placental aldose reductase was completely reduced by incubation with 5 mM DTT for 1 hr at room temperature and was subsequently dialyzed with nitrogen-saturated Buffer A without DTT or β-mercaptoethanol. This enzyme was incubated with the indicated concentrations of the ligands in 10 mM imidazole-HCl buffer, pH 7.0, at room temperature for 45 min. At the end of the incubation period, the enzyme was assayed as described in the text. The control enzyme activity was taken to be 100% and the percentage of retained activity was calculated relative to the control, which consisted of the enzyme incubated under identical conditions but without any additives.

Compound added	Enzyme activity retained
	%
GSSG (0.1 mM)	3.25
GSSG (1 mM)	0.00
GSSG (0.1 mM) + NADPH (1 mM)	99.54
Sorbinil (1 μM)	98.56
Sorbinil (1 μM) + GSSG (0.1 mM)	90.25
Sorbinil (1 μM) + GSSG (1.0 mM)	27.80
DTNB (5 μM)	22.36
DTNB (5 μM) + NADPH (0.1 mM)	82.27
DTNB (5 μM) + sorbinil (1 μM)	47.05

To further study the effects of storage with β-mercaptoethanol on the properties of the enzyme, three aliquots of the placental enzyme sample stored with 5 mM β-mercaptoethanol for 12 days were withdrawn. One of the aliquots was used as such, another was dialyzed overnight without β-mercaptoethanol in Buffer A, and the third was incubated with 5 mM DTT at room temperature for 60 min. Fig. 4 shows the SDS-PAGE of aliquots 1–3 under nonreducing (Fig. 4, *lanes 1–3*) and under reducing (Fig. 4, *lanes 4–6*) conditions. Aliquots 1 and 2 migrate as two distinct bands on the gel, whereas the DTT-treated enzyme aliquot (Fig. 4, *lane 3*) migrates as a single discrete band. All three aliquots migrate as single protein bands when boiled with fresh 5 mM β-mercaptoethanol (Fig. 4, *lanes 4–6*). In Fig. 4, *lanes 1* and *3*, the high molecular weight band corresponds to 36,000, whereas the low molecular weight band to 33,000. We consistently observe two bands on SDS-PAGE of enzyme samples stored with β-mercaptoethanol. Although the fraction of the enzyme present in the high or the low molecular weight form varies with the time of storage of the enzyme with β-mercaptoethanol, the difference between the two molecular weight forms of the enzyme remains unaltered and for 17 such gels a mean difference of molecular weight 3000 ± 40 was observed. No dimer species of the enzyme were observed. It should also be pointed out that the enzyme treated with 5 mM DTT at room temperature migrates at a rate identical to the enzyme boiled with fresh β-mercaptoethanol, indicating that the enzyme treated with 5 mM DTT at room temperature is completely reduced and further denaturation and reduction does not alter the electrophoretic properties of the enzyme. The percentage of inhibition by 1.5 μM sorbinil of the three enzyme aliquots electrophoresed were Fig. 4, *lane 1*, 15%; *lane 2*, 8%; and *lane 3*, 69%. There was no significant difference in the V_{max} of the three enzyme aliquots, nor was the K_m for either NADPH or glyceraldehyde affected (data not

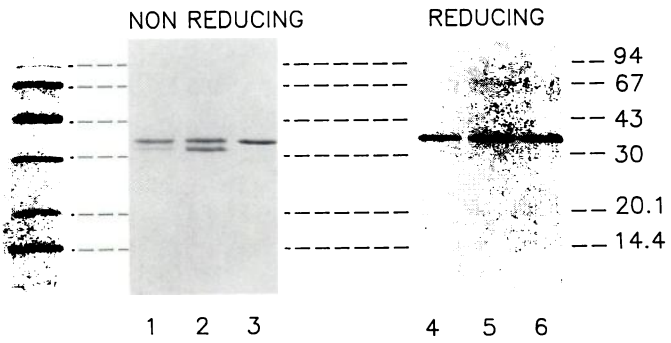

Fig. 4. SDS-PAGE of aldose reductase. *Lane 1*, human placental aldose reductase exposed to 5 mM β-mercaptoethanol for 12 days. *Lane 2*, the same enzyme dialyzed against Buffer A without β-mercaptoethanol. *Lane 3*, the same enzyme sample as in *lane 1*, after incubation with 5 mM DTT for 60 min at room temperature. Samples 1–3 were mixed 1:1 with sample buffer alone (nonreducing), whereas samples 4–6 were mixed with sample buffer plus 5 mM DTT (reducing) and heated to 100°. Samples 4, 5, and 6 are the same as samples 1, 2, and 3, respectively. The protein samples were electrophoresed on 12.5% polyacrylamide gels and stained with Coomassie blue. Molecular weight markers electrophoresed in an adjacent lane (*extreme left*) were: phosphorylase b, 94,000; bovine serum albumin, 67,000; ovalbumin, 43,000; carbonic anhydrase, 30,000; soybean trypsin inhibitor, 20,100; and α-lactalbumin, 14,400. In *lanes 1* and *2*, the high molecular weight band corresponds to molecular weight 36,000 and the apparent low molecular weight band corresponds to molecular weight 33,000.

shown). These results indicate that a decrease in the sensitivity of the enzyme to sorbinil is accompanied by the appearance of an apparent lower molecular weight species, that the two enzyme species could be converted to a single form of molecular weight 36,000 by DTT, and also that the appearance of the low molecular weight form has little effect on the catalytic activity of the enzyme.

Discussion

The results reported here demonstrate the importance of sulfhydryl residues in the inhibition sensitivity of the enzyme and account for the alterations in the susceptibility of the enzyme to inhibition on purification and storage. We report that purification and storage of the enzyme in β-mercaptoethanol-containing buffers alter the catalytic and structural properties of the enzyme as well as its sensitivity to inhibition. It is well known that β-mercaptoethanol oxidizes in solution and forms mixed disulfides with proteins (14, 15) and that these mixed disulfides can further react to form disulfide bridges between conformationally available residues within the same molecule or between two different molecules (16). The observations that the alterations in enzyme properties caused on storage with β-mercaptoethanol could be entirely reversed by DTT strongly suggest that storage of the enzyme with β-mercaptoethanol leads to alkylation and oxidation of the enzyme sulfhydryl residues. These reactions cause a partial loss of the enzyme activity and a sharp decrease in the sensitivity of the enzyme to inhibition, particularly to sorbinil. Reduction of the enzyme with DTT rapidly restores its sensitivity to sorbinil. The K_{ii} values of sorbinil for both the kidney and the placenta enzyme (1 to 2 μM), estimated by us in the presence of DTT, are similar to the reported IC_{50} values for human placenta enzyme purified rapidly in the presence of NADP (7) and for the rat lens enzyme (17), in which the inhibitor binding site is more stable. The high IC_{50} (300–400 μM for human brain) values for sorbinil reported for some human tissues (6) are probably because of the purification and storage in the presence of β-mercaptoethanol. Moreover, although some changes in the K_i of tolrestat were observed on storage of the enzyme with β-mercaptoethanol, the changes were not of the same magnitude as those observed with sorbinil. A straightforward explanation for such a differential sensitivity of these compounds to alterations in sulfhydryl residues could be that these two inhibitors do not bind to the same inhibitor binding site(s) on the enzyme molecule.

In addition to the above-mentioned changes, the structural properties of the enzyme were also altered on storage with β-mercaptoethanol. Our observations that storage with β-mercaptoethanol results in the appearance of an apparent low molecular weight form of the enzyme of 33,000 in addition to the molecular weight 36,000 form, and the appearance of a single protein band of molecular weight 36,000, upon reduction with DTT indicate that under these conditions the apparent decrease in molecular weight is probably due to intramolecular disulfide bond formation. Because the molecular weight 33,000 form could be converted to the molecular weight 36,000 form by DTT, it is unlikely that the altered electrophoretic mobility of the enzyme could be due to proteolysis or hydrolysis of the protein on storage. Therefore, it appears that DTT converts the molecular weight 33,000 form to the molecular weight 36,000 form by reducing the disulfide bond(s) of the protein, which then migrates slower in the gel with an apparent molecular weight of 36,000. Alterations in the electrophoretic mobility of proteins due to disulfide bond formation have been observed for a number of other proteins (16, 18). Besides altering the apparent molecular weight of aldose reductase, reduction by DTT also restores the sensitivity of the enzyme to sorbinil. This would indicate that intramolecular disulfide bond formation may be responsible for the decreased sensitivity of the enzyme to sorbinil.

Aldose reductase contains a total of seven cysteinyl residues (19), two of which are essential for the catalytic activity of the enzyme. Alkylation with DTNB or NEM causes a rapid loss of enzyme activity (20). The loss of the enzyme activity is not a steric effect because the DTNB-inactivated enzyme is not reactivated by CN^- (20). Titration of the fully reduced enzyme with [^{14}C]NEM results in carboxymethylation of all the cysteinyl residues. However, if the enzyme is stored in β-mercaptoethanol only three cysteine residues are alkylated (20). The observation that the number of titratable sulfhydryl residues decrease from seven to three on storage of the enzyme with β-mercaptoethanol is consistent with the oxidation of protein sulfhydryl residues in the presence of this sulfhydryl reagent. The results presented in this communication suggest that a minimum of two functionally distinct sulfhydryl residues are also involved in the enzyme inhibitor interaction, inasmuch as the increase in the sensitivity of the enzyme to sorbinil follows two distinct rate constants. Moreover, both NADPH and sorbinil protect the enzyme against sulfhydryl-oxidizing reagents; therefore, it is quite likely that the same cysteine residues are involved in both *E*:NADPH and *E*:sorbinil interactions, although NADPH and sorbinil do not bind to the same site on the enzyme (3). However, depending on the state of oxidation of these residues, the catalytic activity and the inhibitor sensitivity of the enzyme are affected differently. Thus, storage of the enzyme with β-mercaptoethanol drastically affects the inhibition sensitivity of the enzyme, with little effect on the catalytic activity, and it is possible to generate enzyme forms largely insensitive to sorbinil, without any loss of the catalytic activity. Also, DTNB-alkylated enzyme forms (which are partially active) are as sensitive to sorbinil as the fully reduced form.[1] Although further quantitative experiments will be necessary to estimate the number of disulfide bonds formed, their location(s) within the protein molecule, and the effect of their formation on the properties of the enzyme, it appears that when the cysteinyl residues of the enzyme are in the alkylated state (S-X), as in the presence of DTNB or NEM, the catalytic activity of the enzyme is affected, whereas if these cysteinyl residues are present in the disulfide form (S-S) the sensitivity of the enzyme to inhibition is reduced. Such a hypothesis, although certainly not proven as yet, will be consistent with our results. Nevertheless, our results have important practical applications. The use of supernatants of tissue homogenates has been suggested (3) for aldose reductase-inhibitor interaction studies and such enzyme preparations have been used by a number of investigators (17, 21, 22), because the inhibitor binding site of the enzyme was found to be labile. Because most tissues also contain aldehyde reductase II, which has an overlapping substrate specificity and which is also inhibited by 'aldose reductase inhibitors,' the inhibition studies using tissue

[1] Unpublished observations.

homogenates would invariably lead to inaccurate conclusions. Our studies demonstrate that changes in the K_i of the inhibitors could be eliminated at all stages of purification, including the homogeneous enzyme preparation, by DTT. Therefore, it is recommended that, for inhibitor-aldose reductase interaction studies and screening of aldose reductase inhibitors, the use of β-mercaptoethanol for purification and storage of the enzyme should be avoided.

References

1. Kinoshita, J. H. Aldose reductase in the diabetic eye. XLIII Edward Jackson memorial lecture. *Am. J. Opthamol.* **102**:685–692 (1986).
2. Varma, S. D. Inhibition of aldose reductase by flavonoids: possible attenuation of diabetic complications, in *Plant Flavonoids in Biology and Medicine: Biochemical Pharmacological, and Structure-Activity Relationships*. Cody, V., E. Middleton, Jr., and J. B. Harborne, ed.). Alan R. Liss, New York, 343–358 (1986).
3. Kador, P. F., J. H. Kinoshita, and N. E. Sharpless. Aldose reductase inhibitors: a potential new class of agents for the pharmacological control of certain diabetic complications. *J. Med. Chem.* **28**:841–849 (1985).
4. Dyck, P. J., B. R. Zimmerman, T. H. Vilen, S. R. Minnerath, J. L. Karnes, J. K. Yoa, and J. F. Poduslo. Nerve glucose, fructose, sorbitol, myo-inositol, and fiber degeneration and regeneration in diabetic neuropathy. *N. Engl. J. Med.* **319**:542–548 (1988).
5. Srivastava, S. K., J. M. Petrash, I. J. Sadana, N. H. Ansari, and C. A. Patridge. Susceptibility of aldehyde and aldose reductases of human tissues to aldose reductase inhibitors. *Curr. Eye Res.* **2**:407–410 (1982).
6. O'Brien, M. M., P. J. Schofield, and M. R. Edwards. Inhibition of human brain aldose reductase and hexonate dehydrogenase by alrestatin and sorbinil. *J. Neurochem.* **39**:810–814 (1982).
7. Vander Jagt, D. L., L. A. Stangebye, L. A. Hunsaker, R. P. Eaton, and W. L. Sibbitt. Purification of aldose reductase from human placenta and stabilization of the inhibitor binding site. *Biochem. Pharmacol.* **37**:1051–1056 (1988).
8. Das, B., and S. K. Srivastava. Purification and properties of aldehyde reductases from human placenta. *Biochim. Biophys. Acta* **840**:324–333 (1985).
9. Cleland, W. W. Statistical analysis of enzyme kinetic data. *Methods Enzymol.* **63**:103–138 (1979).
10. Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. *Nature* (*Lond.*) **227**:680–685 (1970).
11. Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**:248–254 (1976).
12. Frost, A. A., and R.G. Pearson. *Kinetics and Mechanisms*, 2nd Ed. John Wiley and Sons, New York, 186 (1961).
13. Cardemil, E. Kinetics of the chemical modification of enzymes. in *Chemical Modifications of Enzymes: Active Site Studies* (J. Eyzaguirre, ed.). John Wiley and Sons, New York, 23–34 (1987).
14. Scopes, R. K. *Protein Purification*. Springer Verlag, New York, 196 (1982).
15. Yu, N.-T., D. C. DeNagel, and C. Slingsby. Raman spectroscopy of calf lens Y-II crystallin: direct evidence for the formation of mixed disulfide bonds with 2-mercaptoethanol and glutathione. *Exp. Eye Res.* **48**:399–410 (1989).
16. Creighton, T. E. Disulfide bond formation in proteins. *Methods Enzymol.* **107**:305–329 (1984).
17. Sarges, R., and M. J. Peterson. Sorbinil: a member of the novel class of spirohydyantoin inhibitors. *Metabolism* **35**:101–104 (1986).
18. Silva, C. M., and J. A. Cidlowski. Direct evidence for intra- and intermolecular disulfide bond formation in human glucocorticoid receptor. *J. Biol. Chem.* **264**:6638–6647 (1989).
19. Kurt, K. M., B. Bullock, B. Wermuth, and K. H. Gabbay. The aldo-keto reductase superfamily. *J. Biol. Chem.* **264**:9547–9551 (1989).
20. Liu, S., A. Bhatnagar, B. Das, and S. K. Srivastava. Functional cysteinyl residues in human placental aldose reductase. *Arch. Biochem. Biophys.*, in press.
21. Inagaki, K., I. Miwa, T. Yashiro, and J. Okuda. Inhibition of aldose reductase from rat and bovine lenses by hydantoin derivatives. *Chem. Pharm. Bull.* **30**:3244–3254 (1982).
22. Poulsom, R. Aldose reductase from human retina and bovine lens: dependence of inhibition upon the choice of enzyme substrate. *Prog. Clin. Biol. Res.* **232**: 341–352 (1987).

Send reprint requests to: Satish K. Srivastava, Department of Human Biological Chemistry and Genetics, University of Texas Medical Branch, Galveston, TX 77550.