Modification of Aldose Reductase by *S*-Nitrosoglutathione†

Animesh Chandra,‡ Sanjay Srivastava,‡ J. Mark Petrash,§ Aruni Bhatnagar,|| and Satish K. Srivastava*,‡

Departments of Human Biological Chemistry & Genetics and Physiology & Biophysics, University of Texas Medical Branch, Galveston, Texas 77555-0647, and Department of Ophthalmology and Visual Sciences, Washington University, St. Louis, Missouri 63110

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ABSTRACT: Kinetic and structural changes in recombinant human aldose reductase (AR) due to modification by *S*-nitrosoglutathione (GSNO) were investigated. Incubation of the enzyme with 10−50 μM GSNO led to a time- and concentration-dependent inactivation of the enzyme, with a second-order rate constant of 0.087 ± 0.009 M^{-1} min^{-1}. However, upon exhaustive modification, 30−40% of the enzyme activity was retained. The non-inactivated enzyme displayed a 2−3-fold change in K_m for NADPH and K_m for DL-glyceraldehyde, whereas the K_m for the lipid peroxidation product, 4-hydroxy-2-*trans* nonenal (HNE), was comparable to that of the untreated enzyme. The residual activity of the enzyme after GSNO treatment was less sensitive to inhibition by the active site inhibitor sorbinil or to activation by sulfate. Significantly higher catalytic activity was retained when the enzyme was modified in the presence of NADPH, suggesting relatively low reactivity of the E−NADPH complex with GSNO. The modification site was identified using site-directed mutants in which each of the solvent-exposed cysteines of the enzyme was replaced individually by serine. The mutant C298S was insensitive to GSNO, whereas the sensitivity of the mutants C303S and C80S was comparable to that of the wild-type enzyme. Electrospray ionization mass spectroscopy of the GSNO-modified enzyme revealed a major modified species (70% of the protein) with a molecular mass that was 306 Da higher than that of the untreated enzyme, which is consistent with the addition of a single glutathione molecule to the enzyme. The remaining 30% of the protein displayed a molecular mass that was not significantly different from that of the native enzyme. No nitrosated forms of the enzyme were observed. These results suggest that inactivation of AR by GSNO is due to the selective formation of a single mixed disulfide between glutathione and Cys-298 located at the NADP-(H)-binding site of the enzyme.

Aldose reductase (AR) is a monomeric, NADPH-dependent enzyme that is a member of the aldo−keto reductase superfamily. The enzyme catalyzes the reduction of aldo sugars and other saturated and unsaturated aldehydes (*1*−*3*). This enzyme constitutes the first and the rate-limiting step of the polyol pathway. It has been suggested that hyperglycemic tissue injury and dysfunction during diabetes are due to an enhanced rate of glucose reduction via AR (*1*, *4*). Inhibition of the enzyme has been shown to ameliorate microvascular and other complications of long-term diabetes such as cataractogenesis, nephropathy, and neuropathy (*1*, *4*, *5*). The expression of AR is enhanced during diabetes (*6*), and overexpression of the enzyme in transgenic mice has been shown to accelerate diabetic cataractogenesis (*7*) and other pathological changes associated with diabetes (*8*).

Recent studies suggest that AR or its isoforms may also be involved in cell growth. It has been shown that AR is the most upregulated protein in astrocytes treated with basic fibroblast growth factor (FGF) (*9*), and FGF enhances the expression of the delayed-early product (FR-1) in murine NIH 3T3 cells, which is 70% homologous to AR (*10*, *11*). The expression of AR is also enhanced during hepatocarcinogenesis (*12*), and may be related to the relative resistance of hepatomas to cytotoxic aldehydes (*13*). Inhibition of AR has been shown to prevent glucose-induced hyperproliferation and hypertrophy of vascular smooth muscle cells (*14*). Interestingly, AR also bears structural similarity to the β-subunit of the Shaker K^+ channels (*15*, *16*). AR also appears to be related to several tissue-specific physiological functions, such as osmoregulation in kidney medulla (*17*), synthesis of fructose in seminal vesicles (*18*), biosynthesis of tetrahydrobiopterin (*19*), and the metabolism of corticosteroids (*20*). However, the most general role of the enzyme may relate to detoxification of cytotoxic aldehydes. The enzyme displays remarkably high affinities for unsaturated aldehydes such as 4-hydroxy-2-*trans* alkenals (*3*, *21*), generated during lipid peroxidation, and for 3-deoxyglucosone, an active intermediate in nonenzymatic glycation (*22*), and toxic carbohydrate−2-oxoaldehyde "osones" formed by the cleavage of carbohydrate residues from glycated proteins (*23*), suggesting that this enzyme may be an integral component of secondary antioxidative defenses. In support of this view, it has been recently reported that expression of AR is enhanced upon exposure of vascular smooth muscle cells to hydrogen peroxide and the lipid peroxidation product HNE (*24*).

AR has been isolated and purified from several tissues (*1*). The isolated protein is a single polypeptide chain with

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* To whom correspondence should be addressed: Satish K. Srivastava, Ph.D., Department of Human Biological Chemistry & Genetics, University of Texas Medical Branch, 619 Basic Science Bldg., Galveston, TX 77555-0647. Telephone: (409) 772-3926. Fax: (409) 772-9679. E-mail: ssrivast@utmb.edu.

‡ Department of Human Biological Chemistry & Genetics, University of Texas Medical Branch.

§ Washington University.

|| Department of Physiology & Biophysics, University of Texas Medical Branch.

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a molecular mass of 36 kDa, which adopts a $(\beta/\alpha)_8$-barrel motif (*25*, *26*). The enzyme contains active site thiols (*26*) and is highly sensitive to oxidation, which changes its catalytic properties and sensitivity to inhibitors and activators (*1*, *27*). It has been suggested that enzyme oxidation occurs *in vivo*, and may represent an important mode of post-translational regulation of the enzyme activity (*28*, *29*). While nonspecific thiol oxidation, mixed disulfide formation, and thiol−disulfide exchange may be important means of regulation (*30*), recent studies suggest that nitrosylation by the free radical second messenger NO and its derivatives may be a more specific mode of redox control, and may serve signaling functions similar to phosphorylation (*31*, *32*).

In this paper, we report that modification by the *S*-nitrosoglutathione (GSNO) results in the inactivation of AR. The nitrosothiol GSNO is formed by aerobic modification of GSH by NO, and has been suggested to mediate some of the biological effects of NO, such as vasodilation (*33*), prevention of platelet aggregation (*34*), and inhibition of the hexose monophosphate shunt (*35*). Because high concentrations of GSH are present in most cells, GSNO most likely supports the NO transfer reactions to sulfhydryl centers of cytosolic proteins (*32*, *34*, *35*). Our studies show that GSNO causes selective S-thiolation of AR at the active site, and suggest that such modification may represent a redox mechanism for the regulation of AR and its associated functions by cellular nitrosothiols.

MATERIALS AND METHODS

GSNO was obtained from Molecular Probes Inc. The Sephadex G-25 column (PD-10) was purchased from Pharmacia Fine Chemicals (Uppsala, Sweden). All other reagents used were of analytical grade. The recombinant human placenta AR and site-directed mutant forms of the enzyme were prepared as described before (*36*).

Chemical Synthesis. 4-Hydroxy-2-*trans* nonenal (HNE) was synthesized as its dimethyl acetal (HNE-DEA) starting from the dimethyl acetal of fumaraldehyde as described before (*37*) and was stored in chloroform. To prepare free HNE, chloroform was removed by bubbling nitrogen through 0.5 mL of solution. The oily residue was suspended in 0.5 mL of 10^{-3} N HCl and stirred until the solution was clear (in approximately 60 min), indicating complete saponification. HNE thus prepared eluted as a single peak, monitored at 224 nm on high-pressure liquid chromatography (HPLC) with a retention time of 42 min using a Rainin reverse phase ODS C_{18} column (1 × 30 cm). The column was pre-equilibrated with 3% methanol (solvent A) at a flow rate of 1 mL/min. Elution was performed with a gradient of solvent A going to 100% solvent B (30% methanol in 30 min) and maintaining at 100% B for another 20 min. The purity of the synthesized HNE was determined by NMR (*37*). For use in enzyme activity determinations, the HNE solution was diluted in 0.1 M potassium phosphate (pH 7.0), and the concentration of the HNE solution was determined by measuring its absorbance at 224 nm using an extinction coefficient of 13 750 M^{-1} cm^{-1}.

Reduction of the Recombinant Enzyme. Before each experiment, stored AR was reduced by incubating with 0.1 M DTT at 37 °C for 1 h in 0.1 M potassium phosphate (pH 7.0). Excess DTT was removed by gel filtration through the Sephadex G-25 column (PD-10), pre-equilibrated with nitrogen-saturated 0.1 M potassium phosphate (pH 7.0) containing 1 mM EDTA. All operations were carried out at 4 °C to prevent oxidation of the enzyme, and the DTT-free, reduced enzyme was stored under nitrogen and was used within 1 h.

Enzyme Assay. The activity of the enzyme in the inactivation experiments was determined at 25 °C in a 1 mL system containing 50 mM potassium phosphate (pH 6.0), 0.4 M lithium sulfate, 10 mM DL-glyceraldehyde, and 0.1 mM NADPH. Steady-state kinetics as well as the sensitivity to sorbinil and sulfate were measured in 0.1 M potassium phosphate (pH 7.0). The reaction was monitored by measuring the rate of disappearance of NADPH at 340 nm using a Gilford Response II spectrophotometer. One unit of enzyme is defined as the amount of the enzyme required to oxidize 1 μmol of NADPH/min. The control cuvette (blank) contained all the components of the reaction mixture except the enzyme.

Modification of AR by GSNO. Reduced AR (0.5 mg/mL) was incubated with varying concentrations of freshly prepared GSNO in 0.1 M potassium phosphate (pH 7.0) at room temperature, and aliquots were withdrawn at the indicated time intervals to measure the enzyme activity.

Carboxymethylation of AR. AR was carboxymethylated by incubating the reduced enzyme with 1 mM iodoacetic acid (IAA) in 100 mM Tris-HCl (pH 8.0) containing 1 mM EDTA as described before (*38*). At various time points, aliquots of the incubation mixture were withdrawn and the catalytic activity of the enzyme and its sensitivity to the AR inhibitor, sorbinil, were measured. When no further decrease in the catalytic activity and sorbinil sensitivity of the enzyme was observed, the reaction was terminated by the addition of 5 mM DTT. Excess DTT and IAA were removed by gel filtration as described above.

Electrospray Ionization Mass Spectrometry (ESI-MS). The electrospray ionization mass spectra were obtained on a Finnigan-TSQ70 (upgraded to TSQ700) triple-quadropole instrument with a Vestec electrospray ionization source with a tapered fused silica capillary needle (50 μm inside diameter). Species added to the enzyme sample (DTT or GSNO) were removed prior to electrospray by gel filtration on a PD-10 column. The enzyme samples were electrosprayed from solution of a 1:1 mixture of the protein in 10 mM ammonium acetate and a 10/1 methanol/acetic acid mixture (v/v). The concentration of protein in the samples was approximately 50 mg/mL. The analyte solutions were infused into the mass spectrometer source using a Harvard syringe pump at a rate of 0.82 μL/min. The spray voltage was set at 3.5 kV and the nozzle voltage at 250 V, with a repeller voltage of 9−11 V. A source temperature of 249−252 °C was used for most experiments; however, as pointed out in the text, for some experiments, the source temperature was lowered to 229 or 157 °C. Source bath nitrogen was turned off or set to 3.2 psi as needed to achieve good spectra for standard proteins and left at the same setting for the samples analyzed on that day. The Quad 3 was scanned from 600 to 200 amu/3 s, and 128 scans were averaged before data were acquired. Spectra were deconvoluted using an input mass range (InMR) of 600−200 Da, an output mass range of 10000−40000 Da, a mass step of 0.1, and a peak width of 2 amu, using a deconvolution algorithm (BioMass, FinniganMAT).

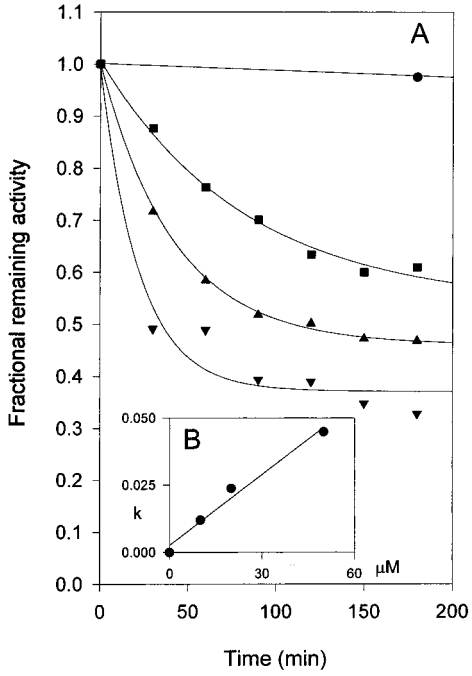

FIGURE 1: Kinetics of modification of human placental recombinant AR by GSNO. Exhaustively reduced enzyme (*ca.* 20 mg) was incubated with N_2-saturated 0.1 M potassium phosphate (pH 7.0) containing 1 mM EDTA, with 0 (●), 10 (■), 20 (▲), and 50 (▼) μM GSNO at room temperature. The total volume of the incubation mixture was 0.2 mL. Aliquots (25 μL) were withdrawn at the indicated time intervals and assayed for enzyme activity in 0.1 M potassium phosphate (pH 6.0) containing 0.4 M Li_2SO_4 with 0.1 mM NADPH and 10 mM DL-glyceraldehyde. In panel A, data are plotted as discrete points, whereas the curves are the best fit of eq 1 to the data. Panel B shows the dependence of the pseudo-first-order rate constant for modification on the concentration of GSNO in the incubation mixture. The calculated values of the rate constants are given in Table 1.

Statistical Analysis. The observed rate constants for inactivation of the enzyme were calculated using the following single-exponential equation:

$$y = a \exp^{-t/\tau} + b \quad (1)$$

For analysis of some experimental data, a single-exponential equation with no constant term was used:

$$y = a \exp^{-t/\tau} \quad (2)$$

where y is the fractional remaining activity, a is the pre-exponential factor, τ is the first-order time constant for the inactivation, and b is the unmodified fraction of the enzyme activity. Best fits of the parameters were estimated by nonlinear regression using NFIT (Island Products, Galveston, TX). Data are presented as ± standard error of the mean (SEM). The Student's t test was used for comparing two groups. For multiple comparisons of the control mean with other group means, the Dunnett t test was performed following one-way analysis of variance (ANOVA). Data were considered statistically significant when the p value was <0.05.

RESULTS

Inactivation of the Enzyme by GSNO. Incubation of the reduced AR with GSNO led to a time-dependent loss of catalytic activity (Figure 1). The maximal inhibition of the enzyme was approximately 60%. Increasing the concentration of GSNO and/or the time of exposure did not result in further loss of activity. Addition of fresh GSNO to the incubation mixture after 90 min of incubation with 50 μM GSNO (corresponding to a 60% loss of activity) did not lead to additional inactivation. The rate of inactivation could be well described as a single-exponential process. The calculated values of the rate constants for modification are shown in Table 1. The pseudo-first-order rate constant for inactivation displayed a linear dependence on the concentration of GSNO (Figure 1 inset), indicating that GSNO does not form a kinetically significant dissociable complex before inactivation. The second-order rate constant for inactivation, determined from the slope of the linear relationship between the GSNO concentration and the pseudo-first-order inactivation rates, is 0.087 ± 0.009 M^{-1} min^{-1}. Neither the rate nor the extent of modification was affected when the enzyme was incubated with GSNO under anaerobic conditions as compared to the enzyme samples treated with GSNO in the presence of air.

Table 1: Kinetic Parameters for the Modification of Recombinant Aldose Reductase by GSNO[a]

treatment	a	b	τ (min)	R^2
WT + GSNO at 10 μM	0.46 ± 0.028	0.54 ± 0.030	83.0 ± 12	0.999
WT + GSNO at 20 μM	0.53 ± 0.006	0.46 ± 0.004	40.0 ± 1.3	0.999
WT + GSNO at 50 μM	0.62 ± 0.057	0.37 ± 0.027	22.3 ± 5.6	0.995
WT + GSNO + NADPH at 35 μM	0.13 ± 0.009	0.86 ± 0.009	23.3 ± 4.6	0.999
C303S + GSNO at 50 μM	0.65 ± 0.05	0.34 ± 0.02	3.53 ± 0.82	0.998
C80S + GSNO at 50 μM	0.64 ± 0.04	0.32 ± 0.03	25.0 ± 5.2	0.997
C298S + GSNO at 50 μM	−	>0.99	>120	
WT + IAA at 1 mM	0.60 ± 0.006	0.39 ± 0.008	20.5 ± 1.2	0.998
CM-AR	−	>0.98	>120	
WT + GSSG at 1 mM	0.918 ± 0.008	0.083 ± 0.005	10.5 ± 0.22	0.999
WT + GSNO at 50 μM + GSSG at 1 mM	0.993 ± 0.008	−	317.4 ± 26.8	0.998

[a] Values of the time constant (τ) were calculated using eq 1 for all cases and eq 2 for WT + GSNO + GSSG. a and b are the sensitive and the insensitive fractions of the enzyme, respectively. τ is the time constant for inactivation. R^2 is the regression coefficient of the fit. WT is the wild-type enzyme. C80S, C298S, and C303S are mutant forms of AR in which the corresponding cysteine residue was replaced with serine. All enzymes were reduced before inactivation. CM-AR is carboxymethylated aldose reductase.

To identify whether the GSNO-induced modification of the enzyme was due to thiol oxidation, the modified enzyme was separated from unbound GSNO by rapid gel filtration using a PD-10 column or Matrix orange affinity column equilibrated with 100 mM phosphate (pH 7.0). Upon incubation of the enzyme obtained from the PD-10 or Matrix orange columns with 5 or 10 mM DTT, for 30 min at room temperature, approximately 75% of the enzyme activity was recovered, indicating that the GSNO-induced inactivation of the enzyme is mainly due to oxidation of the thiol(s) residue(s) of the protein.

Since dismutation of GSNO results in the generation of GSSG, we examined whether inactivation of the enzyme could be due to the GSSG generated. Incubation of reduced AR with 50 μM GSSG did not result in significant loss of

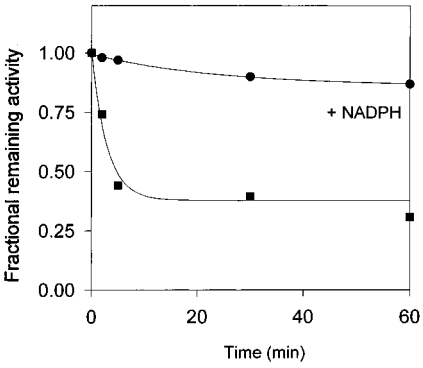

FIGURE 2: Protection of AR by NADPH against modification by GSNO. The reduced enzyme was incubated with either 50 μM GSNO (■) or 50 μM GSNO and 35 μM NADPH (●) in 0.1 M potassium phosphate (pH 7.0) containing 1 mM EDTA. Aliquots were withdrawn at the indicated time intervals, and the enzyme activity was determined at pH 6.0. The data are shown as discrete points, and the curves are best fits of eq 1 to the data.

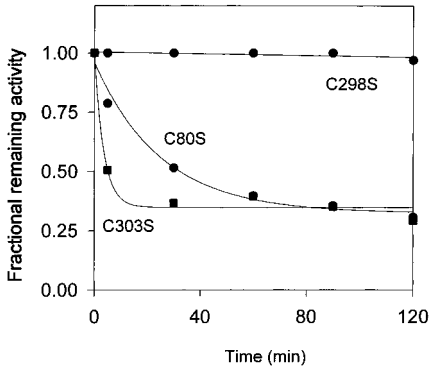

FIGURE 3: Inactivation of mutant forms of AR by GSNO. Mutant forms of AR were prepared in which Cys-80 (AR−C80S), Cys-298 (AR−C298S), and Cys-303 (AR−C303S) were replaced with serine, as described in the text. The enzymes were reduced with 0.1 M DTT, and excess DTT was removed by gel filtration. The enzymes were then incubated with 50 μM GSNO in 0.1 M potassium phosphate (pH 7.0) at room temperature. Aliquots of the reaction mixture were withdrawn at the indicated time points, and the enzyme activity was determined at pH 6.0. Data are shown as discrete points, and the curves are best fits of eq 1 to the data.

enzyme activity. However, incubation with much higher concentrations (1 mM) of GSSG led to almost complete inactivation of the enzyme (see below). From these experiments, we conclude that under the reaction conditions used, inactivation of the enzyme caused by incubating with 10−50 μM GSNO is not likely to be due to GSSG that may result from spontaneous dismutation of GSNO in solution.

To determine if GSNO-induced enzyme inactivation was due to modification of one or more active site residues, the enzyme was modified by GSNO in the presence and absence of NADPH. As shown in Figure 2, the E:NADPH binary complex was much more resistant to inactivation by GSNO. The difference in reactivity is reflected mainly by the large fraction of the enzyme activity (approximately 86%) not sensitive to GSNO (Table 1), indicating that the residue(s) modified by GSNO is at or close to the NADPH binding site, or becomes inaccessible to GSNO as a result of the conformational change that occurs on coenzyme binding.

Identity of the Modification Site. Since nitrosation of thiols proceeds faster than that of amines or tyrosine, we examined whether the cysteine residues of the enzyme were modified by GSNO. Completely reduced AR contains three solvent-exposed cysteine residues (*39*) located at positions 80, 298, and 303 (*26*). To identify the specific residue(s) modified by GSNO, three mutant forms of the enzyme were generated in which Cys-80, -298, and -303 were individually replaced by serine. As shown in Figure 3, the C303S and C80S mutants were inactivated upon incubation with GSNO. The rate of inactivation of C80S was comparable to that of the wild-type enzyme (cf. Figure 1), whereas the inactivation of the C303S mutant occurred more rapidly. The time constants for the inactivation of the WT enzyme and mutants C80S and C303S are shown in Table 1. After 60 min of exposure, the activities of WT, C80S, and C303S were reduced to about 40% of the control values. No inactivation of AR−C298S was observed upon incubation with GSNO under identical conditions. These data indicate that inactivation of AR by GSNO is due to selective modification of Cys-298. While these results do not rule out the possibility that other cysteine residues are also modified by GSNO, the lack of inactivation of C298S by GSNO suggests that modification of only Cys-298 leads to enzyme inactivation.

Table 2: Steady-State Kinetic Parameters of Reduced and GSNO-Modified Aldose Reductase[a]

parameter	reduced AR	modified AR
K_m for DL-glyceraldehyde	24.6 ± 8.0	57.4 ± 18.9[b]
K_m for NADPH	37.5 ± 5.1	10.7 ± 1.4[b]
K_m for HNE	19.9 ± 6.1	23.6 ± 3.9
V_{max} (glyceraldehyde and NADPH)	0.029 ± 0.001	0.015 ± 0.004[b]
V_{max} (HNE and NADPH)	0.041 ± 0.004	0.025 ± 0.001[b]

[a] The enzyme was modified by incubating with 50 μM GSNO for 60 min, until no further loss of activity was observed. The kinetic constants were determined at pH 7.0 in 0.1 M potassium phosphate. The values of K_m are in micromolar and of V_{max} in micromoles per milliliter per minute. [b] $p < 0.05$.

Kinetic Characterization of the Residual Enzyme Activity. To determine the kinetic properties of the GSNO-modified enzyme, the reduced WT−AR was incubated with 50 μM GSNO for 60 min (time required for maximal enzyme inactivation). As shown in Table 2, the K_m for NADPH of the enzyme was reduced almost 4-fold following modification with GSNO. However, modification of the enzyme did not cause significant alterations in its K_m for the lipid peroxidation product HNE, although the K_m of the enzyme for DL-glyceraldehyde was elevated. The maximal enzyme activity was reduced regardless of whether DL-glyceraldehyde or HNE was used as the substrate, although the observed decrease in the enzyme activity was slightly less with HNE.

Modification of the WT enzyme by GSNO reduced the sensitivity of the enzyme to sorbinil. The residual activity of the reduced enzyme observed in the presence of 10 μM sorbinil was 9.8 ± 0.2% ($n = 3$). However, when the enzyme was modified by incubation with 50 μM GSNO for 60 min, the residual activity with 10 μM sorbinil was 25.2 ± 2.0% ($n = 3$), which is significantly ($p < 0.005$) higher than that observed with the untreated enzyme.

One of the characteristic features of AR is its sensitivity to divalent anions such as sulfate (*1*). In agreement with previous investigations, we found that addition of 0.4 M lithium sulfate led to a 2.1 ± 0.2-fold ($n = 3$) increase in the catalytic activity of the reduced WT enzyme when measured with 10 mM glyceraldehyde and 100 μM NADPH. Upon modification of the enzyme with 50 μM GSNO for

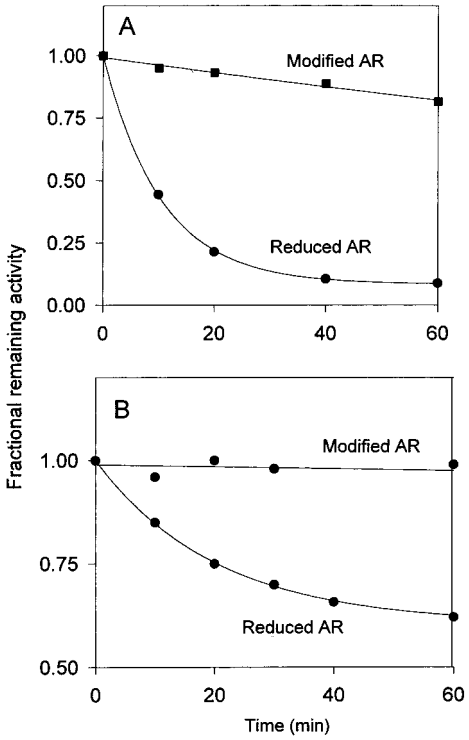

FIGURE 4: Inactivation of reduced and GSNO-modified AR by GSSG and IAA. The reduced enzyme was incubated with 50 μM GSNO for 60 min in 0.1 M potassium phosphate (pH 7.0), after which the enzyme was re-incubated with 1 mM GSSG (A) or IAA (B); aliquots of the incubation mixture were withdrawn, and the activity was determined at pH 6.0 at the indicated time points. $t = 0$ is the time of addition of GSSG or IAA. Also shown is the time course for the loss of activity of the reduced enzyme (without GSNO modification) upon incubation with 1 mM GSSG or IAA, under identical conditions. The data are shown as discrete points, and the curves are best fits of eq 1 to the data for reduced AR and eq 2 for the modified enzyme incubated with GSSG.

60 min, the increase in activity with 0.4 M lithium sulfate was 1.5 ± 0.03-fold ($n = 3$). Thus, the modified enzyme appears to be significantly ($p < 0.05$) less sensitive to sulfate than the reduced enzyme.

The enzyme modified by GSNO was found to be insensitive to oxidative modification by GSSG. As shown in Figure 4A, incubation of reduced WT AR with 1 mM GSSG led to a more than 90% loss of catalytic activity in 60 min. In contrast, the GSNO-modified enzyme was relatively insensitive to GSSG-induced inactivation. The pseudo-first-order time constant for inactivation by GSSG of the reduced WT AR was 10.5 ± 0.2 min, compared to 317 ± 26 min for the GSNO-modified enzyme (Table 1). When C80S and C303S mutants were incubated with GSNO and then re-incubated with 1 mM GSSG, no significant loss of catalytic activity was observed, indicating that GSSG S-thiolates the enzyme selectively at Cys-298. However, if Cys-298 is modified by prior exposure to GSNO, the enzyme is not inactivated by GSSG.

We also measured the effect of GSNO-induced modification on inactivation of the enzyme due to iodoacetate (IAA), since we have shown previously that IAA causes selective carboxymethylation of the enzyme at Cys-298 (*40*). As shown in Figure 4B, when reduced WT AR and GSNO-modified WT AR were incubated under identical conditions with 1 mM IAA in 0.1 M potassium phosphate (pH 7.0), the reduced enzyme was inactivated by IAA, whereas the

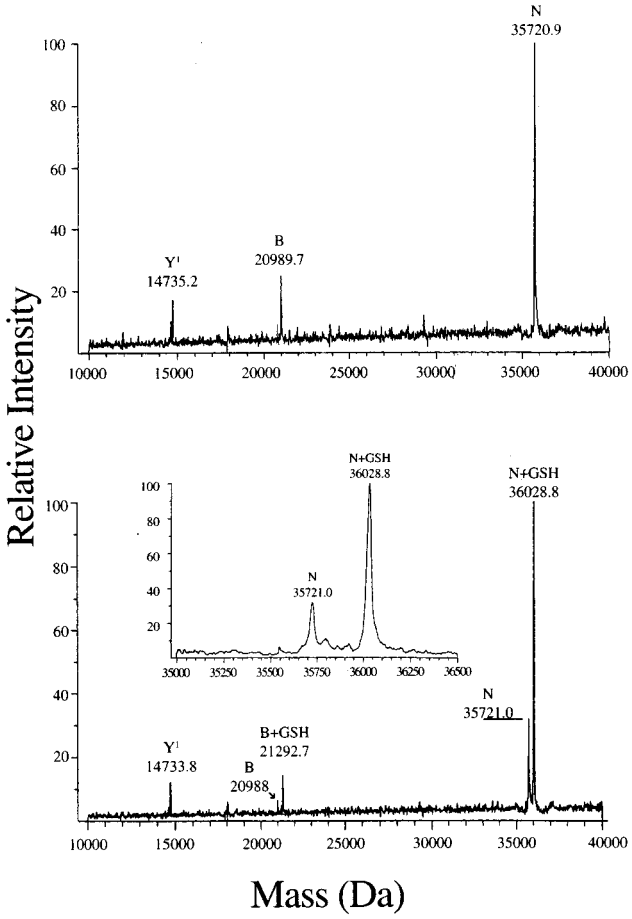

FIGURE 5: ESI-M spectra of reduced (upper panel) and GSNO-treated (lower panel) human recombinant AR. GSNO (50 μM) was incubated with the enzyme for 1 h, after which the unreacted GSNO was removed by rapid gel filtration on a PD-10 column equilibrated with nitrogen-saturated ammonium acetate. The upper panel shows the spectrum with a major species, N, corresponding to the native enzyme (35 720.8 Da) and two minor peaks Y′ and B (14 734.8 and 20 987 Da) corresponding to the protein fragments from Met-1 to Val-131 and Pro-132 to Phe-312, respectively. The lower panel shows the spectrum with two major species N plus GSH corresponding to a molecular mass of 36 028.8 (N plus GSH) and 35 721 Da (N). Note that the peptide fragment Y′ is unaffected by the treatment, whereas the molecular mass of B increased by 305.7 Da (B plus GSH), consistent with the addition of a GSH molecule to the C-terminal part of the protein. Block temperature = 251 °C; repeller voltage = 9 V. The inset shows the two major peaks at higher resolution.

GSNO-modified enzyme was insensitive to IAA ($\tau > 120$ min, Table 1). Also, the catalytic activity of the carboxymethylated enzyme was not affected upon incubation with 50 μM GSNO (Table 1).

ESI-MS of GSNO-Modified AR. As shown in Figure 5 (upper panel), the ESI-M spectra of the untreated, reduced enzyme displayed a major peak corresponding to a molecular mass of 35 723.0 ± 3.0 Da ($n = 7$), which is close to the expected molecular mass of 35 722.3 Da. The error in the determined molecular mass of AR was similar to that observed for the standard proteins apomyoglobin and bovine serum albumin that were used to calibrate the instrument. Significant amounts of two other species corresponding to 20 987.7 ± 1.5 ($n = 4$) Da and 14 735.0 ± 0.9 ($n = 9$) Da were also observed in the untreated enzyme sample. Examination of the amino acid sequence of AR indicated a possible source for the 20 988 and 14 735 Da species as the respective Y′ and B fragments obtained from the cleavage

of the protein at the Val-131−Pro-132 amide bond. The observation that the relative amounts of these two species increased with the use of more energetic ionization conditions, i.e. a higher repeller voltage and a higher source block temperature, adds support to this hypothesis.

To examine chemical changes accompanying modification, the enzyme was incubated with 50 μM GSNO for 1 h, after which the reaction was terminated by rapid gel filteration on a nitrogen-saturated PD-10 column as described in Materials and Methods. The ESI-M spectra of the GSNO-modified enzyme (at a source block temperature of *ca*. 250 °C) displayed a prominent species which accounted for 70% of the signal and conformed to a molecular mass of 36 028.8 ± 0.3 Da (Figure 5, lower panel). Upon subtraction of the average molecular mass for the untreated enzyme (obtained under identical conditions), a measured net increase of 306.8 Da was calculated, which corresponds to the addition of a single glutathione molecule to the protein, resulting in the formation of AR−SSG. The expected increase in the molecular mass due to the formation of such an adduct is 305.3 Da. A deviation of 1.5 Da between the predicted and the expected molecular mass is well within the error limits of these analyses. Furthermore, the peptide Y′ was not affected by GSNO, whereas the molecular mass of the peptide B was increased by 305.7 Da upon treatment with GSNO, indicating the formation of a glutathione adduct with the C-terminal part of the protein. However, no evidence of the generation of a nitrosated protein (E−NO) was obtained. Some normal enzyme, corresponding to a molecular mass of 35 721.0 Da, was also found.

Since nitrosothiol bonds are labile and the amount of the nitrosated protein varies with the amount of the energy applied to the peptide ions (*41*), it is possible that the failure to observe a nitrosated form of the protein may be due to disruption of the nitrosothiol bond under more energetic ionization conditions. To test for this possibility, the GSNO-modified enzyme was examined by ESI-MS at lower block temperatures. For these experiments, the enzyme was only partially modified by GSNO to allow detection of nitrosated forms even if formed as an intermediate. Lowering the source block temperature during ESI-MS resulted in a shift of the protein charge state profiles to the lower-mass higher charge states. The most abundant charge state for the GSNO-treated enzyme at a block temperature of 248 °C was the +29 ion (1232.8 Da), while at 158 °C, the +32 to +34 ions were the most abundant charge states. Less resolution of the ions for proteins of similar masses at these lower-mass charge states was observed, and the signal/noise ratio was much lower in the portion of the spectrum in which the resolution was better. Furthermore, at the lower block temperature, the ions resulting from the association of counterions other than a proton, e.g. ammonium (+17 Da), sodium (+22 Da), and potassium (+38 Da), were also more abundant. Also, less in-source fragmentation was observed at the lower block temperature, and the protein fragments resulting from putative cleavage of the Val-131−Pro-132 amide bond (14 735 and 20 988 Da species) were either absent or detected at much lower levels at 157 °C than at 248 °C. The GSNO-modified enzyme, however, appeared to survive the ESI-MS process when the block temperature was set at 157 °C. In concurrent runs, at 157 °C, three major species were detected corresponding to 35 722.2 ± 2.1, 35 747.5 ± 2.2, and 36 024.6 ± 2.4 Da, whereas at 248 °C, only two species corresponding to 35 717.6 ± 2.8 and 36 023.2 ± 2.2 Da were observed. While the 36 025 Da species was ascribed to AR−SSG, and the 35 720 Da species appears to be due to the native enzyme, the identity of the 35 747 Da species could not be rigorously established. However, the observation that this species disappears at 258 °C suggests that it is probably due to association of a counterion such as sodium.

DISCUSSION

Nitrosothiols such as GSNO display several biological effects that include relaxation of the vascular smooth muscle cells (*42*), platelet deactivation (*34*), immunosuppression (*43*), and neurotransmission (*44*). Exposure of neutrophils to NO results in the conversion of intracellular GSH to GSNO which activates the hexose monophosphate shunt (*35*). Micromolar concentrations of nitrosothiols have been detected in the plasma and in bronchial lavage fluid (*45*). On the basis of these observations, it has been suggested that nitrosothiols may be the bioreactive intermediates that mediate some of the observed effects of NO. Nonetheless, the mechanisms by which nitrosothiols modulate cell function and alter protein structure are not well-understood. It has been suggested that nitrosothiols which spontaneously degrade to liberate free thiols and NO may represent a mechanism for the storage and transport of NO, and that the biological effects of nitrosothiols may be due to their tendency to liberate free NO. However, recent evidence suggests that spontaneous release of NO from nitrosothiols, particularly GSNO, is a relatively inefficient process (*46*); instead, GSNO forms adducts with low-molecular mass thiols which may cause modifications distinct from those due to nitrosation (*47*).

In this paper, we report that modification of human AR by GSNO results predominantly in the formation of a mixed disulfide, leading to enzyme inactivation. Exposure to GSNO led to a rapid decrease in the enzyme activity, and the second-order rate constant for enzyme inactivation was calculated to be 0.08 M^{-1} min^{-1}, suggesting a high affinity of the enzyme for GSNO. Although due to different experimental conditions used it is difficult to compare the GSNO sensitivity of AR to that observed with other proteins, AR appears to be more sensitive to GSNO than other enzymes. Maximal inhibition of glutathione reductase for example requires incubation of the enzyme with 2 mM GSNO for 2 h (*48*), whereas 0.04 mM GSNO is required for half-maximal inactivation of glyceraldehyde 3-phosphate dehydrogenase (*49*). Moreover, the reported IC_{50} of GSNO for the inhibition of ribonucleotide reductase was reported to be 1 mM (*50*). In this regard, the sensitivity of AR is similar to that of creatine kinase (*51*) which is also inactivated at comparable concentrations of GSNO.

Our results suggest that GSNO-induced inactivation of AR appears to be due to the modification of the active site of the enzyme, because the E:NADPH binary complex was more resistant to inactivation by GSNO than the apoenzyme. In view of the high reactivity of nitrosothiols with protein thiols, and the high susceptibility of AR thiols to oxidation (*1*), we examined whether modification due to GSNO was due to critical thiol residues of the enzyme. The reduced AR contains three solvent-exposed cysteine residues located at positions 80, 298, and 303 (*26*). To determine whether

one or more of these residues are modified by GSNO, we prepared three mutant forms of AR in which each of the solvent-exposed cysteines was replaced with serine. We found that of these the C298S was completely insensitive to GSNO-induced inactivation. In contrast, the extent of inactivation of the C80S and C303S mutants was comparable to that of the wild-type enzyme. These data suggest that modification of Cys-298 is the main cause of GSNO-induced inactivation of the enzyme. Analysis of the crystals of the E:NADPH binary complex shows that Cys-298 is located within 4.1 Å of the nicotinamide C4-position of NADPH (*26*). Consequently, the formation of the E:NAPDH binary complex which prevents access of Cys-298 to the solvent is much more resistant to thiol-oxidizing agents, which also appears to be the case with GSNO. Therefore, both the NADPH protection studies and the site-directed mutagenesis studies are in agreement and indicate that the loss of the catalytic activity of the enzyme results from GSNO-induced modification of Cys-298.

That the GSNO-induced inactivation of AR is due to the modification of the active site thiol is also suggested by the ESI-MS results which show that the molecular mass of the GSNO-modified enzyme was enhanced by 306 Da compared to that of the untreated enzyme. This increase in molecular mass is consistent with the addition of a single glutathione molecule to the enzyme, leading to the formation of AR−SSG. Since C298S was not inactivated by GSNO, we infer that the inactivation of the enzyme by GSNO is due to the formation of a single mixed disulfide specifically at Cys-298. This residue is highly susceptible to oxidation, and its avid oxidizability is the major reason for the high variability in the kinetic properties and the inhibitor sensitivity of AR (*1*). Inactivation of the enzyme by several oxidants has been found to be due to modification of Cys-298, and it has been recently shown that GSSG-induced inactivation of the enzyme is due to the formation of a mixed disulfide between Cys-298 and the glutathione (*52*). Selective modification of Cys-298 by thiol reagents has been suggested to be due to the high (6.1 $Å^2$) solvent accessibility of this residue. While Cys-303 also has high solvent accessibility, the preferential thiolation of Cys-298 may be due to a specific interaction of the glycyl carboxylate of GSH and His-110 (*52*), in a manner reminiscent of the interaction between His-110 and the carboxylate oxygen of zopolrestat (*53*), which is a potent AR inhibitor. As a result of such noncovalent "tethering" and energy stabilization, AR displays selective reactivity with glutathione. Nonspecific oxidation of other thiol residues of the enzyme is minimal since these have poor solvent accessibility or are completely sequestered from the solvent (*52*).

The GSNO-induced S-thiolation of AR could be due to several mechanisms. The formation of AR−SSG could be due to GS liberated in solution by the homolytic degradation of GSNO in solution, or due to a nucleophilic attack of ARS^- on GSNO, leading to the formation of AR−SSG and the release of NO^-. Alternatively, a mixed disulfide could arise from transnitrosation of Cys-298 by GSNO followed by a sequential reaction between AR−SNO and GSH, generating AR−SSG and hyponitrous acid. In addition, GSNO could form a *N*-hydroxysulfenamide, due to a nucleophilic attack of ARS^- on the nitrogen rather than the sulfur of GSNO (*47*). Several products could arise from the homolytic scission and reduction of the hydroxysulfenamide, including AR−SSG (*47*). While our data do not permit direct identification of the reaction pathway, some possibilities could be discounted. First, it appears unlikely that modification of the enzyme is due to either GS or NO release by homolysis of GSNO in solution. As pointed out by several investigators, GSNO is stable in solution ($t_{1/2}$ = 7.8 h; *54*), and its rate of decomposition is too slow to account for the modification reaction observed. Moreover, an equimolar concentration of GSSG did not inactivate the enzyme, and no nitrosated enzyme was observed, indicating that modification of the enzyme by GSNO is preceded by selective binding of the GSNO to the active site of the enzyme, leading to the formation of a nondissociable complex (as indicated by our kinetic data; Figure 1B). Thus, reactions leading to the formation of AR−SSG could involve a nucleophilic attack at either the nitrogen or the sulfur of GSNO. However, since no GSNO adducts corresponding to the sulfonamide, *N*-hydroxysulfinamide, or sulfonamide complex between AR and GSNO were observed, the simplest mechanism may be a direct nucleophilic attack of the enzyme thiol on the sulfur of GSNO, resulting in the formation of AR−SSG and NO^-.

Despite the high sensitivity of the enzyme to GSNO, the nitrosothiol did not lead to complete inactivation of the enzyme. The persistent catalytic activity of the enzyme, even after prolonged incubations, however, does not appear to be due to exhaustion of GSNO. On the basis of the reported half-life of GSNO, we estimate that in 60 min of incubation, not more than 12% GSNO could have dissociated. Moreover, addition of fresh GSNO did not lead to further modification, indicating that GSNO exhaustion is unlikely to account for the residual enzyme activity. The ESI-MS results show that upon incubation with GSNO for 1 h, about 70% of the enzyme was in the AR−SSG form while the molecular mass of the rest of the protein was indistinguishable from that of the untreated enzyme. In view of our previous experiments which show complete inactivation of the enzyme by GSSG (*27*) and the molecular modeling studies which suggest that binding of His-110 to the glycyl carboxylate of glutathione prevents access of the aldehyde to the active site defined by His-110, Tyr-48, and C4 of the nicotinamide ring (*52*), we conclude that AR−SSG has no significant catalytic activity, and ascribe the residual activity observed even after exhaustive modification by GSNO to an intramolecular disulfide. The observed resistance of the residual enzyme to inhibition by GSSG and IAA (Figure 4) and the observed changes in its kinetic constants, inhibitor, and sulfate sensitivity are consistent with the idea that the non-AR−SSG form of the GSNO-exposed enzyme is due to the formation of intramolecular disulfides. Our previous investigations show that oxidation of enzyme thiols generates several forms of the enzyme with kinetic properties (*1*) similar to those observed with the GSNO-treated AR. However, the changes in substrate specificity of the GSNO-exposed AR are different from those observed in our previous site-directed mutagenesis studies with the enzyme thiols (cf. ref 36). Thus, the structural and kinetic changes induced by thiol oxidation seem to depend upon the nature of the modification introduced.

The specificity and the selectivity with which AR is modified by GSNO suggest that this may represent an endogenous mechanism for the regulation of AR and its associated functions. Moreover, since this residue is conserved in several aldo−keto reductases, e.g. fibroblast growth

factor-regulated protein (FR-1; *10*), mouse vas deferens protein (MVDP; *55*), and the expected gene product of auxin-induced mRNA from *Nicotiana tabacum* (*56*), these proteins may also be subject to similar modification. Other related proteins such as aldehyde reductase (*57*), chlordecone reductase (*58*), the β-subunit of the Shaker K^+ channels (*15*, *16*), aflatoxin-metabolizing aldehyde reductase (*59*), prostaglandin F synthase (*60*), 3-α-hydroxysteroid reductase (*61*), and proteins of unknown function from nonmammalian organisms [YCR107w (*62*), BBOAKR (*63*), and PORF (*64*)] which do not have a cysteine at position 298 may not be sensitive to similar redox modifications. In fact, the C-terminal region of the aldo−keto reductases is the most variable part of these proteins, suggesting divergence of function and specific regulation may relate to the C-terminal end. It is therefore tempting to speculate that the presence of Cys-298 at the active site of AR and related proteins allows for selective redox regulation of these enzymes as opposed to other aldo−keto reductases.

In conclusion, our results show that GSNO inactivates AR, and this inactivation is due to the formation of a single mixed disulfide between GSH and Cys-298 of the enzyme. The resultant enzyme has no catalytic activity. Since equimolar concentrations of GSSG were not effective, these results suggest that at least for AR GSNO may be a more potent inducer of mixed disulfide formation than GSSG. While several investigators have suggested that nitrosothiols can generate disulfides with low-molecular mass thiols such as GSH (e.g. ref 47), to the best of our knowledge, this paper is the first report directly demonstrating GSNO-induced mixed disulfide formation in a protein. While the general applicability of this phenomenon remains to be established, our results do raise the possibility that, in addition to their well-studied role as nitrosating agents, nitrosothiols could also modify protein structure and function by inducing mixed disulfides and intramolecular disulfides, which may represent a novel means of NO-mediated redox signaling.

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