Structural and kinetic modifications of aldose reductase by *S*-nitrosothiols

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Modification of aldose reductase (AR) by the nitrosothiols *S*-nitroso-*N*-acetyl penicillamine (SNAP) and *N*-(β-glucopyranosyl)-N^2-acetyl-*S*-nitrosopenicillamide (glyco-SNAP) resulted in a 3–7-fold increase in its k_{cat} and a 25–40-fold increase in its K_m for glyceraldehyde. In comparison with the native protein, the modified enzyme was less sensitive to inhibition by sorbinil and was not activated by SO_4^{2-} anions. The active-site residue, Cys-298, was identified as the main site of modification, because the site-directed mutant in which Cys-298 was replaced by serine was insensitive to glyco-SNAP. The extent of modification was not affected by P_i or O_2, indicating that it was not due to spontaneous release of nitric oxide (NO) by the nitrosothiols. Electrospray ionization MS revealed that the modification reaction proceeds via the formation of an *N*-hydroxysulphenamide-like adduct between glyco-SNAP and AR. In time, the adduct dissociates into either nitrosated AR (AR-NO) or a mixed disulphide between AR and glyco-*N*-acetylpenicillamine (AR-S-S-X). Removal of the mixed-disulphide form of the protein by lectin-column chromatography enriched the preparation in the high-K_m–high-k_{cat} form of the enzyme, suggesting that the kinetic changes are due to the formation of AR-NO, and that the AR-S-S-X form of the enzyme is catalytically inactive. Modification of AR by the non-thiol NO donor diethylamine NONOate (DEANO) increased enzyme activity and resulted in the formation of AR-NO. However, no adducts between AR and DEANO were formed. These results show that nitrosothiols cause multiple structural and functional changes in AR. Our observations also suggest the general possibility that transnitrosation reactions can generate both nitrosated and thiolated products, leading to non-unique changes in protein structure and function.

Key words: diethylamine NONOate, *N*-(β-glucopyranosyl)-N^2-acetyl-*S*-nitropenicillamide, nitric oxide, nitrosation, *S*-nitroso-*N*-acetylpenicillamine.

INTRODUCTION

Aldose reductase (AR) is a member of the aldo–keto reductase superfamily, which catalyses the NADPH-dependent reduction of a wide variety of substrates, varying from aldo-sugars to aromatic and aliphatic aldehydes [1,2]. This enzyme constitutes the first and rate-limiting step of the polyol pathway and has been suggested to be responsible for tissue injury and dysfunction associated with hyperglycaemic states such as diabetes mellitus and galactosaemia by catalysing the reduction of glucose and galactose to their corresponding alcohols [1,2]. In several experimental models, inhibitors of AR have been shown to ameliorate or prevent secondary complications. However, the clinical efficiency of these drugs remains to be established [1,3]. Recent investigations suggest that the physiological functions of AR might extend well beyond its role in the polyol pathway. In several cell types, AR is up-regulated in response to growth factors and cytokines [4–6], suggesting that the enzyme might be involved in cell growth and/or inflammation. In addition, aldehydes derived from lipid peroxidation, as well as their glutathione conjugates, are reduced more efficiently by AR than glucose [7], indicating that AR might be a component of the antioxidant defences against oxidative stress. Nevertheless, the mechanisms by which AR participates in its various cellular functions and those that regulate its activity and expression remain unclear. However, the elucidation of the mechanisms regulating AR is essential for understanding its cellular role and for assessing its contribution to hyperglycaemic injury.

Previous studies show that AR is susceptible to multiple modifications. Homogeneous preparations of AR undergo spurious oxidation on storage and handling [8–12]; 'activated' and 'unactivated' forms of the enzyme have been described in normal and diabetic tissues [13,14]. In addition, our studies suggest that redox changes regulate AR catalysis and ligand binding [11,12,15]. We have identified Cys-298, located at the active site, as the main site of these changes, and have shown that modifications specific to this residue enhance the catalytic rate of the enzyme and increase its K_m for aldehydes [11,12,16].

Although non-specific oxidation of thiols has been suggested as an important mode of redox regulation [17], recent studies suggest that thiol modifications by nitric oxide (NO) are specific post-translational changes that regulate the structure and function of several cellular proteins [17,18]. We have previously shown that AR is inactivated by *S*-nitrosoglutathione (GSNO) and that this inactivation is due to the formation of a single adduct between glutathione and Cys-298 of AR [19]. On the basis of these observations we suggested that, in addition to S-nitrosation, S-thiolation is also an important form of protein modification induced by nitrosothiols (compare [20]). This view was confirmed by subsequent studies showing transthiolation of low-pK_a thiols of proteins, such as glyceraldehyde-3-phosphate dehydrogenase and cathepsin K, by nitrosothiols [21,22]. Nevertheless, the possibility that AR is directly nitrosated by NO or its metabolites has not been examined and the conditions leading to S-nitrosation or S-thiolation have not been identified. Here we show that, in contrast with S-thiolation by GSNO, modifications

Abbreviations used: AR, aldose reductase; DEANO, diethylamine NONOate; DTT, dithiothreitol; ESI^+–MS, electrospray ionization MS; glyco-SNAP, *N*-(β-glucopyranosyl)-N^2-acetyl-*S*-nitrosopenicillamide; GSNO, *S*-nitrosoglutathione; SNAP, *S*-nitroso-*N*-acetyl-D,L-penicillamine; glyco-NAP, glyco-SNAP without NO.

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by other nitrosothiols, although specific to Cys-298, generate a thiolated as well as a nitrosated protein, with markedly different effects on enzyme activity. The reaction pathway identified for the modification of AR by nitrosothiols might be of general significance in understanding the mechanisms by which nitrosothiols modify protein thiols.

MATERIALS AND METHODS

Materials

N-(β-glucopyranosyl)-*N*²-acetyl-*S*-nitrosopenicillamide (glyco-SNAP) and diethylamine NONOate (DEANO) were purchased from Calbiochem. *S*-Nitroso-*N*-acetyl-D,L-penicillamine (SNAP) was from Molecular Probes. Concanavalin A–Sepharose, NADPH, D,L-glyceraldehyde, GSSG and dithiothreitol (DTT) were obtained from Sigma. Sephadex G-25 columns (PD-10) were purchased from Pharmacia. Sorbinil was a gift from Pfizer. All other reagents were of the highest grade available. Recombinant human AR and its mutant forms were prepared as described previously [16].

Reduction of recombinant AR

Before each experiment, the stored enzyme was reduced by incubation 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 on a Sephadex G-25 column (PD-10), pre-equilibrated with nitrogen-saturated 0.1 M potassium phosphate (pH 7.0) containing 1 mM EDTA. To prevent oxidation of the enzyme, all operations were performed at 4 °C. The reduced enzyme, separated from DTT, was stored under nitrogen and used within 2–4 h.

Enzyme assay

Enzyme activity was measured at 25 °C in a 1 ml reaction system containing 10 mM Hepes, pH 7.4, with 10 mM glyceraldehyde and 0.15 mM NADPH as substrates. The reaction was monitored by measuring the disappearance of NADPH at 340 nm with a Varian spectrophotometer (Cary Bio 100). One unit of enzyme activity is defined as the amount of enzyme required to oxidize 1 μmol of NADPH/min. For measurement of steady-state kinetic parameters, initial velocity was measured at seven to nine different concentrations of substrate; the parameters were calculated as described previously [11,15,16].

Modification of AR by NO donors

For the modification experiments, AR was reduced and separated from DTT as described above. Unless indicated otherwise, the freshly reduced DTT-free enzyme (0.3 mg/ml) was incubated at 25 °C with various concentrations of freshly prepared NO donors in 0.1 M potassium phosphate, pH 7.0, and aliquots of the reaction mixture were withdrawn at the indicated time intervals to measure the enzyme activity. In some experiments, the modified enzyme was applied to a concanavalin A–Sepharose column (0.4 ml packed volume), pre-equilibrated with 0.1 M potassium phosphate, pH 6.0. The unadsorbed protein was collected separately and the column was washed with at least 10 bed vols of equilibration buffer. The enzyme bound to the column was eluted with 20 mM glucose.

Electrospray ionization MS (ESI^+–MS)

The modified forms of AR were identified by ESI^+–MS with a Micromass LCZ mass spectrometer. Before electrospray, the enzyme was desalted and separated from the modifying reagents by gel filtration on a PD-10 column equilibrated with 10 mM ammonium acetate. The purified enzyme was diluted with the flow injection solvent, 10 mM ammonium acetate/acetonitrile/formic acid (50:50:1, by vol.). The solution was introduced into the mass spectrometer with a Harvard syringe pump at a rate of 10 μl/min. The operating parameters were as follows: capillary voltage, 3.1 kV; cone voltage, 27 V; extractor voltage, 4 V; source block temperature, 100 °C; desolvation temperature, 200 °C. Spectra were acquired at the rate of 200 atomic mass units/s over the range 20–2000 atomic mass units. The instrument was calibrated with myoglobin.

RESULTS

Modification of AR by SNAP

In the first series of experiments we examined the modification of AR by SNAP and determined the kinetic properties of the modified enzyme. The reduced enzyme was incubated with different concentrations of SNAP in 0.1 M potassium phosphate, pH 7.0. After different durations of incubation, aliquots of the reaction mixture were withdrawn and the enzyme activity was measured in 10 mM Hepes to exclude the confounding effects of high ionic strength on AR activity. The concentration of glyceraldehyde in the assay (10 mM) was kept much higher than its K_m (approx. 0.06 mM; see below) to ensure saturation. Incubation of the purified enzyme with different concentrations of SNAP led to a time-dependent increase in the enzyme activity, which reached steady-state at durations above 150 min (Figure 1A). The increase in enzyme activity was related to the concentration of SNAP in the incubation mixture; at steady-state, a maximum of 3–4-fold increase in enzyme activity was observed.

To analyse the kinetics of AR modification by SNAP, the following general scheme was considered:

$$E + A \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} EA \underset{k_{32}}{\overset{k_{23}}{\rightleftharpoons}} EA^* \quad (1)$$

The first-order solution of this equation is a sum of exponentials with decay rates λ_i, where $i = 1$ to n, and n is the number of first-order processes.

The solution of eqn (1) with one intermediate complex EA is [23]:

$$[EA] = Cb(-e^{-\lambda_2 t} + e^{-\lambda_3 t}) \quad (2)$$

where:

$$[EA^*] = C(d + f e^{-\lambda_2 t} - g e^{-\lambda_3 t}) \quad (3)$$

$$b = k_{12}/(\lambda_2 - \lambda_3)$$

$$d = k_{12}k_{23}/(\lambda_2\lambda_3)$$

$$f = k_{12}k_{23}/[\lambda_2(\lambda_2 - \lambda_3)]$$

$$g = k_{12}k_{23}/[\lambda_3(\lambda_2 - \lambda_3)]$$

$$\lambda_2 = \tfrac{1}{2}\{k_{12} + k_{21} + k_{23} + [(k_{12} + k_{21} + k_{23})^2 - 4k_{12}k_{23}]^{1/2}\} \quad (4)$$

$$\lambda_3 = \tfrac{1}{2}\{k_{12} + k_{21} + k_{23} - [(k_{12} + k_{21} + k_{23})^2 - 4k_{12}k_{23}]^{1/2}\} \quad (5)$$

When the enzyme concentration is in excess, i.e. when $[E] \gg [A]$, the modified enzyme has a saturating value of $C = [A]$ and the second-order rate constant $k_{12} = k'_{12}[E]$. In the converse, the saturating value of $C = [E]$ and the second-order rate constant $k_{12} = k'_{12}[A]$. In each case the prime denotes a pseudo-second-order rate constant. During the modification process, the total activity ($v[E]_t$), measured at any time, is the sum of the activities due to the modified and the unmodified enzyme forms, E, EA and EA*, with the activities α, β and χ, respectively:

$$v[E]_t = \alpha\{[E]_t - [EA] - [EA^*]\} + \beta[EA] + \chi[EA^*] \quad (6)$$

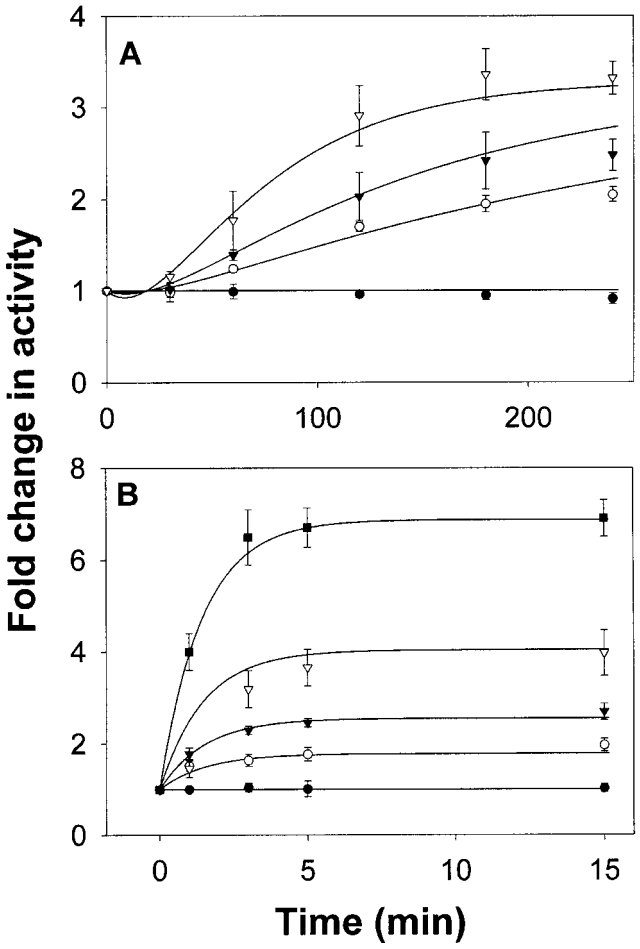


Figure 1 Activation of AR by glyco-SNAP

Reduced AR was incubated with nitrogen-saturated 0.1 M potassium phosphate, pH 7.0, containing 1 mM EDTA at 25 °C, with 0 (●), 0.1 (○), 0.2 (▼) and 0.5 (▽) mM SNAP (**A**) or 0 (●), 0.25 (○), 0.5 (▼), 1.0 (▽) and 2 (■) mM glyco-SNAP (**B**) at 25 °C. Aliquots were withdrawn at the indicated times and assayed for enzyme activity as described in the text. The data are shown as discrete points (means ± S.D.; $n = 3$) and the curve is drawn as the best fits of eqn (8) for SNAP and eqn (11) for glyco-SNAP ($R^2 = 0.97$–0.98).

Table 1 Kinetic properties of the native and glyco-SNAP-modified aldose reductase

The reduced enzyme was incubated in the presence or absence of 200 μM SNAP (for 90 min) or 2 mM glyco-SNAP (for 25 min) at 25 °C. The glyco-SNAP-modified enzyme was passed through a concanavalin A–Sepharose column. The kinetic constants of the native and unadsorbed enzymes were determined at pH 7.4 in 10 mM Hepes buffer as described in the Materials and methods section. The glyceraldehyde concentration was varied form 0.01 to 1 mM for the native enzyme and from 0.05 to 10 mM for the modified enzyme. Initial velocity was measured at seven to nine different concentrations of glyceraldehyde. Individual saturation curves used to obtain k_{cat} and K_m were fitted to a general Michaelis–Menton equation by using a non-linear iterative fitting procedure. R^2 values were between 0.998 and 0.995. Values shown are means ± S.E.M.

Enzyme form	$K_m^{glyceraldehyde}$ (mM)	k_{cat} (min^{-1})	k_{cat}/K_m ($min^{-1} \cdot mM^{-1}$)
Native AR	0.06 ± 0.01	36 ± 6	590 ± 97
SNAP-modified AR	1.46 ± 0.18	76 ± 9	52 ± 6
Glyco-SNAP-modified AR	1.99 ± 0.35	82 ± 14	41 ± 7
Glyco-SNAP-modified AR (unadsorbed on lectin column)	2.6 ± 0.43	131 ± 16	52 ± 12

or

$$= (\chi-\alpha)[EA^*]+(\beta-\alpha)[EA]+\alpha[E]_t$$

Dividing through by $\alpha[E]_t$ we obtain:

$$\frac{v}{\alpha} = \left\{\frac{\chi-\alpha}{\alpha}\right\}\frac{[EA^*]}{[E]_t} + \left\{\frac{\beta-\alpha}{\alpha}\right\}\frac{[EA]}{[E]} + 1 \quad (7)$$

When normalized to the activity of the unmodified form, the fold change in activity (v/α) with time is a linear function of $[EA]/[E]_t$ with a slope $\Delta = [(\beta-\alpha)/\alpha]$, and of $[EA^*]/[E]_t$ with a slope $\Gamma = [(\chi-\alpha)/\alpha]$, where the dimensionless parameters Δ and Γ are the contributions of the modified enzyme forms.

The modification of AR by SNAP followed a bi-exponential time course (Figure 1A). The following equation was used to fit the data, with the use of the solutions of [EA] and [EA*] from eqns (2–5):

$$v/\alpha = \Gamma(d+fe^{-\lambda_2 t}-ge^{-\lambda_3 t})-b(-e^{-\lambda_2 t}+e^{-\lambda_3 t})+1 \quad (8)$$

The simplest model consistent with these data is given by the condition $[E] \ll [A]$, where $C = [E]$, $k_{12} = k'_{12}[A]$ and $k_{21} = k_{32} = 0$. A significantly better fit was obtained with $\Delta = -1$ (i.e. $\beta = 0$) than with $\Delta = 0$ (i.e. $\alpha = \beta$), indicating that the kinetic behaviour of modification is best described by the assumption that intermediate EA is catalytically inactive. Γ was calculated from eqn (8) to be 2.28 ± 0.16, with $k_{12} = 37.7 \pm 4.8\ M^{-1} \cdot min^{-1}$ and $k_{23} = 0.39 \pm 0.004\ min^{-1}$ (values are means ± S.E.M.). The best fit of eqn (8) to the data is shown in Figure 1(A). This analysis indicates that the modification of AR by SNAP involves the formation of an intermediate complex EA, which has no catalytic activity. In addition to EA, other partly modified or inactive forms of the enzyme might be generated during the modification process (see below); however, within the limits of our experimental error and statistical curve fitting, only one such form seemed to contribute to the overall kinetics of modification.

To characterize the SNAP-induced modification of AR, the kinetic properties of the maximally modified enzyme were determined in 10 mM Hepes buffer, with different concentrations of glyceraldehyde. Although the enzyme activity displayed a hyperbolic dependence on glyceraldehyde concentration, at concentrations above 3 mM, glyceraldehyde displayed substrate inhibition, which at 10 mM led to a 6–8 % decrease in the enzyme activity compared with the activity with 1 mM. When the upper limit of glyceraldehyde concentration was restricted to 1 mM, the K_m of the native enzyme was 0.06 mM (Table 1). In contrast, the SNAP-modified enzyme displayed a K_m of approx. 1.5 mM with a range of glyceraldehyde concentrations from 0.01 to 10 mM. The k_{cat} of the modified enzyme was approximately twice that of the native enzyme (Table 1). Moreover, in comparison with the unmodified enzyme, the SNAP-modified AR was relatively insensitive to inhibition by sorbinil or activation by 0.4 M SO_4^{2-} ions. Thus the steady-state kinetic properties of the SNAP-modified enzyme are similar to those of 'activated' AR described previously [13,14]. This form of AR is generated upon modification of the enzyme by oxidants and thiol-modifying reagents. To ascertain that the activation of AR by SNAP was also due to thiol modification, the native and SNAP-modified enzymes were incubated separately with 1 mM GSSG for 1 h. The activity of the SNAP-modified enzyme was not affected by GSSG, whereas the native enzyme lost more than 85 % of its activity. Because the formation of a mixed disulphide with GSSG inactivates native AR [19], insensitivity of the SNAP-modified enzyme to GSSG suggests that the thiol(s) of the SNAP-modified AR were unavailable to GSSG.

To identify the chemical nature of AR modification by SNAP, the native and modified forms of AR were examined by ESI^+–MS.

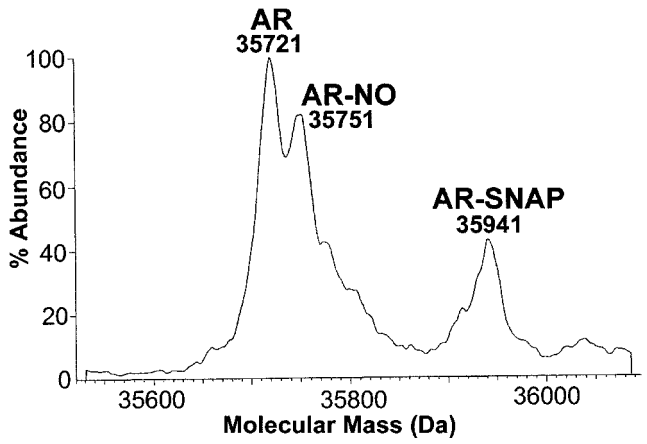


Figure 2 ESI$^+$–MS spectrum of SNAP-treated AR

The reduced enzyme was treated with 0.2 mM SNAP in 0.1 M phosphate, pH 7.0, for 90 min at 25 °C. Unreacted SNAP was removed by rapid gel filtration on a PD-10 (G-25) column, with nitrogen-saturated 10 mM ammonium acetate. The ESI$^+$–MS spectrum of the desalted incubation mixture shows native (unmodified) AR (corresponding to a molecular mass of 35721 Da), AR bound to a single NO molecule (molecular mass 35751 Da) and a 1:1 adduct between AR and SNAP (molecular mass 35941 Da).

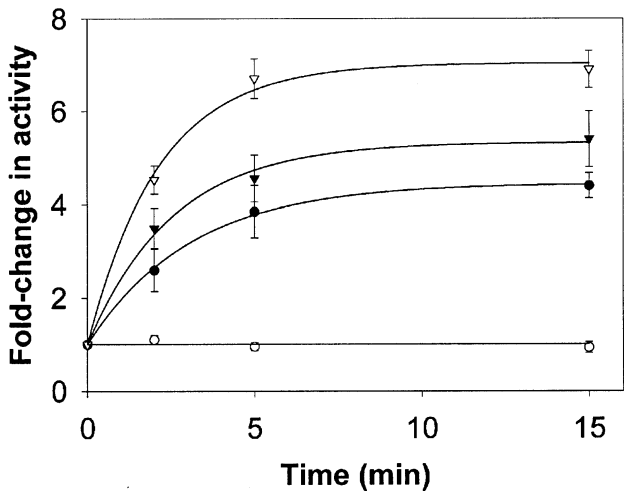


Figure 3 Modification of wild-type AR and the site-directed mutants AR_{C303S}, AR_{C298S} and AR_{C80S} by glyco-SNAP

Reduced wild-type AR (▽), AR_{C80S} (●), AR_{C298S} (○) or AR_{C303S} (▼) proteins were incubated with 2 mM glyco-SNAP in nitrogen-saturated 0.1 M potassium phosphate, pH 7.0, at 25 °C. Aliquots were withdrawn at the indicated times and assayed for enzyme activity as described in the text. Data are shown as discrete points (means ± S.D., $n = 3$) and the curves are drawn as best fits of eqn (11) to the data ($R^2 = 0.98$).

The native enzyme formed several charge states corresponding to a well-defined envelope. The deconvoluted spectra of the charge states corresponded to a unique molecular mass of 35722 ± 3 Da ($n = 7$). No other contaminating species of more than 10% abundance were evident (results not shown). In contrast, the SNAP-modified AR displayed additional charge states, corresponding to molecular masses of 35721, 35751 and 35941 Da. The relative abundances of these species were approx. 100%, 80% and 40% respectively (Figure 2). The species with a molecular mass of 35721 Da was assigned to the native (unmodified) protein. Because nitrosation leads to an increase in the molecular mass by 30 Da, the 35751 Da species seems to be due to the nitrosation of a single thiol residue of AR. The difference in the molecular mass of the third species of 35941 Da and that of the native protein is 220 Da. Because this is not a multiple of 30, this species could not be due to multiple nitrosations of AR but instead seems to be due to the direct binding of SNAP (molecular mass 220.2 Da) to AR. Thus, from the ESI$^+$–MS data, we infer that the modification of AR by SNAP results in the formation of two distinct species, one in which AR is nitrosated (AR-NO) and another in which the entire SNAP molecule is bound to AR (AR-SNAP). However, the presence of these multiple enzyme forms in the incubation mixture confounds kinetic analysis because it is difficult to conclude whether the changes in the kinetic properties are due to the formation of AR-NO or to that of AR-SNAP. Thus, to distinguish between these possibilities, we examined the modification of AR by glyco-SNAP, which we expected to be similar to that observed with SNAP. However, because the enzyme bound to glyco-SNAP would resemble a glycosylated protein, it might be possible to separate this form of the protein from AR-NO by lectin affinity chromatography and to determine the kinetic properties of these two forms individually.

Modification by glyco-SNAP

As with SNAP, incubation of AR with glyco-SNAP resulted in a time-dependent increase in enzyme activity (Figure 1B). This increase in activity was more rapid than with SNAP, reaching a steady state within 5 min of exposure. The extent of modification (fold change in activity over more than 10 min) was proportional to the concentration of glyco-SNAP. The results can be described by a first-order process. Thus eqn (1) simplifies to the reaction scheme:

$$E + A \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} EA^* \tag{9}$$

with the solution:

$$[EA^*] = C(1 - e^{-\lambda t}) \tag{10}$$

where $\lambda = k_{12} + k_{21}$. Under the condition that $C = [A]$ and $k_{12} = k'_{12}[E]$, eqn (7) simplifies to:

$$\frac{v}{\alpha} = \Gamma \frac{[A]}{[E]_t}(1 - e^{-\lambda t}) + 1 \tag{11}$$

From eqn (11), the calculated value of $\Gamma \{= [(\chi - \alpha)/\alpha]$, see eqn (7)$\}$ is 6.0 ± 0.12 with $k_{12} = 0.69 \pm 0.058\ M^{-1} \cdot min^{-1}$ and $k_{21} = 0$. The inclusion of an intermediate complex (EA) did not significantly improve the goodness-of-fit. Therefore, within the limits of our experimental error, the reaction does not seem to involve the formation of a kinetically significant intermediate complex and only one modified state seems to exist, which we ascribe to EA*.

Neither the rate nor the extent of modification of the enzyme by glyco-SNAP was altered when, instead of 0.1 M potassium phosphate buffer, the modifications were performed in 0.1 M Hepes (results not shown); this indicated the absence of buffer-specific effects. The glyco-SNAP-induced modification of AR also seemed to be due to thiol modification because the modified enzyme was relatively insensitive to inhibition by GSSG and sorbinil and to activation by sulphate (results not shown). In addition, the modification was specific for the active-site residue, Cys-298. The site-directed mutant forms of the enzyme in which Cys-80 and Cys-303 were replaced by serine were activated by glyco-SNAP, whereas AR_{C298S} was unaffected (Figure 3).

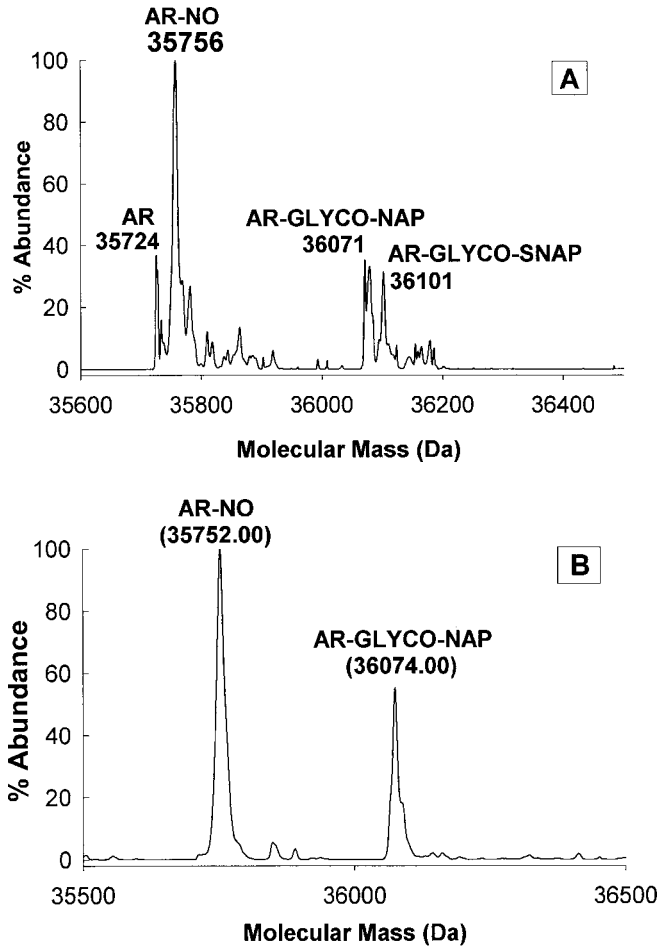


Figure 4 ESI$^+$–MS spectra of glyco-SNAP-treated AR

The reduced enzyme was incubated with glyco-SNAP in 0.1 M phosphate, pH 7.0, for 10 min (**A**) or 25 min (**B**) at 25 °C. Excess glyco-SNAP was removed and the ESI$^+$–MS spectra of the desalted incubation mixture were determined as described in the text. After 10 min of treatment, the incubation mixture displayed unmodified AR (molecular mass 35724 Da) as well as AR bound to NO (35756 Da), AR-glyco-SNAP (36101 Da) and AR-glyco-NAP (36071 Da). After 25 min the two major protein species observed were assigned to AR bound to a single NO molecule (AR-NO; molecular mass 35752 Da) and a mixed disulphide between AR and glyco-NAP (36074 Da).

On the basis of these observations we infer that the modifications of AR by glyco-SNAP are specific for Cys-298 located at the active site of the enzyme.

ESI$^+$–MS of AR incubated with glyco-SNAP for 5 min revealed four major species with molecular masses of 35724, 35756, 36071 and 36101 Da (Figure 4A). The species with a molecular mass of 35724 Da corresponds to the native (unmodified) AR. The error in the measured (35724 Da) and expected (35721 Da) molecular mass of AR was similar to that observed for the standard protein (myoglobin) that was used to calibrate the instrument. The species with a mass of 35756 Da, which was the most abundant form of the enzyme, was assigned to AR nitrosated by a single NO molecule (AR-NO). As with SNAP, the formation of a complex between AR and glyco-SNAP was also observed, giving rise to the species corresponding to a molecular mass of 36101 Da (expected 36103 Da). Moreover, the species with a molecular mass of 36071 Da, which is 30 Da less than AR-glyco-SNAP, was assigned to a mixed disulphide between AR and glyco-NAP (glyco-SNAP without NO). This complex is expected to be formed after the release of NO from the AR-glyco-SNAP complex. To examine whether the distribution of the enzyme forms changes in time, the modified enzyme was examined after 25 min of incubation with glyco-SNAP. In the incubation mixture, no unmodified AR or the AR-glyco-SNAP complex was observed. The modified enzyme was distributed between two species of 35752 and 36074 Da, which were assigned to AR-NO and AR-NAP respectively. Taken together, these results show that, as with SNAP, modification by glyco-SNAP results in the formation of multiple enzyme forms. However, some of these forms seem to be transient; after long periods of incubation, the major species recovered were a singly nitrosated AR and a mixed disulphide between AR and glyco-NAP.

Table 2 Properties of glyco-SNAP-treated AR

The reduced enzyme was incubated in the absence or presence of 2 mM glyco-SNAP for 25 min at 25 °C and the modified enzyme was passed through the lectin (concanavalin A) column. The enzyme activity was determined at pH 7.4 in 10 mM HEPES as described in the Materials and methods section. In the measurement of inhibition by GSSG, the native or modified enzyme was incubated with 1 mM GSSG for 60 min at 37 °C before measurement of the enzyme activity. Values are expressed as means ± S.D. ($n = 3$).

		Modified enzyme	
Parameter	Native enzyme	Before lectin column	After lectin column
Specific activity (units/mg of protein)	0.61 ± 0.2	2.39 ± 0.16	3.12 ± 0.24
Inhibition by 10 μM sorbinil (%)	87.7 ± 2.52	8.8 ± 1.3	13.9 ± 3.5
Inhibition by 1 mM GSSG (%)	86.0 ± 1.4	20.0 ± 1.4	12.0 ± 5.7

The nitrosated and mixed disulphide forms of AR, generated by glyco-SNAP, were separated by lectin-affinity chromatography. For this, the enzyme was incubated with 2 mM glyco-SNAP; when no further change in enzyme activity was observed, the incubation mixture was applied to a concanavalin A–Sepharose column equilibrated with 0.1 M potassium phosphate, pH 6.0, containing 1 mM EDTA. A large fraction (70–80 %) of the protein was eluted in the unadsorbed fraction (Table 2). The specific activity of the enzyme recovered in this fraction was 3.1 ± 0.2 units per mg of protein, which was 25–30 % higher than the specific activity (2.4 ± 0.1 units/mg of protein) of the modified enzyme applied to the column (0.2 mg/ml). Moreover, unlike the native enzyme, the unadsorbed enzyme was not inhibited by 0.01 mM sorbinil and was insensitive to 1 mM GSSG (Table 1). In addition, the unadsorbed protein displayed a 44-fold higher K_m for glyceraldehyde in comparison with the native enzyme, with a more than 3-fold increase in k_{cat}. The protein bound to the column was eluted with 20 mM glucose. No detectable enzyme activity was associated with the eluted protein fractions, indicating that the AR-NAP was enzymically inactive.

Modification of AR by DEANO

To examine whether AR is directly nitrosated by non-thiol NO donors, which rapidly release NO to the medium, the reduced enzyme was incubated with 0.5 mM DEANO, in 0.1 M Hepes. Incubation with DEANO led to a rapid increase in AR activity, which achieved steady-state within 30 min. In three separate experiments, incubation with DEANO led to a 6–7-fold increase in enzyme activity. However, when oxygen was rigorously removed from the medium, no increase in enzyme activity was observed, indicating that modification of AR by DEANO

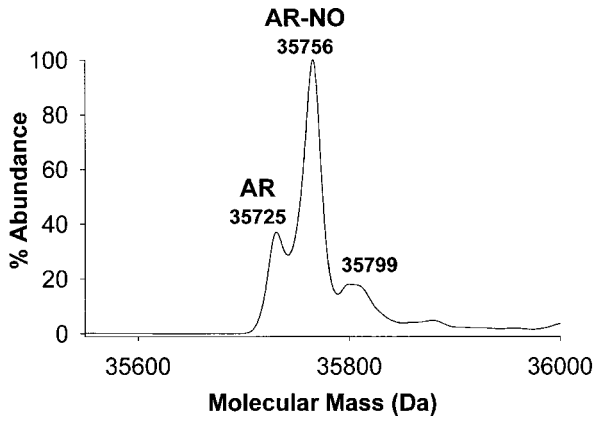


Figure 5 ESI$^+$–MS spectrum of DEANO-treated AR

The enzyme was reduced and incubated with 0.5 mM DEANO in 0.1 M Hepes, pH 7.0. After 30 min of incubation, excess DEANO and the salts in the incubation mixture were removed by gel filtration on a PD-10 column; the protein was suspended in the flow-injection solvent. The two major species, corresponding to molecular masses of 35725 and 35756 Da, were ascribed to unmodified AR and AR-NO respectively. A minor species with a molecular mass of 35799 Da remains unidentified.

was oxygen-dependent. To examine the structural changes caused by DEANO, the modified enzyme was desalted over a PD-10 column, diluted in the flow-injection solvent and injected into the electrospray. The DEANO-modified enzyme displayed two major species of molecular masses 35725 and 35756 Da (Figure 5), of which the latter was more abundant and was ascribed to AR modified by a single NO molecule (AR-NO). The 35725 Da species seems to be due to native (unmodified) AR. Although a minor unidentified species with a molecular mass of 35799 Da was observed, no species corresponding to an adduct between AR and DEANO was observed (expected mass 35982 Da). These observations suggest that NO, released in the medium by rapidly dissociating non-thiol donors, nitrosates AR in an oxygen-dependent manner without directly forming an adduct with the protein.

DISCUSSION

AR is a member of the aldo–keto reductase superfamily, which catalyses the reduction of a wide variety of substrates such as aldo-sugars, steroids and saturated and unsaturated aldehydes [1,2]. Owing to its ability to catalyse the reduction of glucose to sorbitol, the enzyme has been implicated in the pathogenesis of secondary complications associated with diabetes. Previous studies have shown that two forms of AR ('activated' and 'unactivated') are present in diabetic tissues [13,14]. The 'activated' form of the enzyme was found to be insensitive to sorbinil and related hydantoin inhibitors of AR. Because these inhibitors bind to the active site of the enzyme, changes in sensitivity to sorbinil suggest active-site modification. The enzyme is also modified by oxidation, glycosylation and glutathiolation; these modifications alter its catalytic and ligand-binding properties [8–12]. Here we report that the NO donors glyco-SNAP and DEANO modify AR so that the kinetic properties of the modified enzyme seem to be similar to 'activated' AR described previously [13,14]. Because NO is a ubiquitous second messenger, it seems likely that modification by NO might represent an endogenous, post-translational mechanism regulating AR.

Our previous studies show that GSNO inactivates AR by forming a single glutathione adduct with Cys-298 present at the active site of the enzyme [19]. No nitrosated forms of AR were observed. The formation of a mixed disulphide, rather than a nitrosated protein, suggests that the reaction between nitrosothiols with protein thiols might be complex, involving the formation of several transient intermediates and yielding multiple end products. These intermediates and end products might have different effects on protein function. Hence, to understand the mechanism of nitrosothiol-induced modification, we examined the effects of thiol and non-thiol NO donors on AR structure and catalysis in detail.

Several lines of evidence suggest that, as with GSNO, Cys-298 is the main residue modified by glyco-SNAP. The glyco-SNAP-modified AR was insensitive to sorbinil and sulphate. These features are characteristic of AR oxidized at Cys-298 [11,12,15]. Moreover, the glyco-SNAP-modified enzyme was unaffected by GSSG at concentrations that specifically lead to the glutathiolation of Cys-298 of the native enzyme [19], suggesting that Cys-298 is not available for glutathiolation in AR modified by glyco-SNAP. In addition, glyco-SNAP generated a high-K_m–high-k_{cat} form of the enzyme, which is similar to that generated by the modification of Cys-298 by iodoacetate [12], or to that in which Cys-298 is replaced by serine [16]. Finally, in contrast with the wild-type enzyme and its site-directed mutants AR_{C80S} and AR_{C303S}, AR_{C298S} was not affected by glyco-SNAP, indicating that the changes in enzyme activity caused by glyco-SNAP are specifically due to the modification of Cys-298. The reasons for the selective modification of Cys-298 by NO donors are not readily apparent. Like Cys-298, the other two cysteine residues, Cys-80 and Cys-303, are also solvent-exposed [24]. However, Cys-80 and Cys-303 are not modified by thiol-modifying reagents such as GSSG and iodoacetate, which selectively modify Cys-298 [11,18]. Cys-298 is located at the active site of the enzyme, in a relatively basic environment, which might be responsible in part for imparting a lower pK and a higher intrinsic reactivity to this residue in comparison with other solvent-exposed cysteine residues. However, further studies will be required to confirm this speculation.

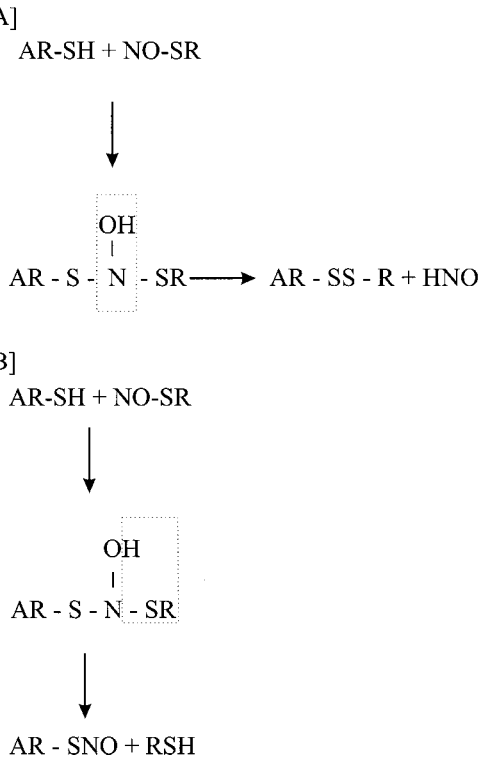
Protein thiols could be modified by NO donors by several different mechanisms. The simplest possibility is that the NO donor could dissociate spontaneously in solution by homolytic cleavage, liberating free NO. That NO liberated in the medium could directly nitrosate AR is evident from our observation that AR is rapidly nitrosated by the non-thiol NO donor DEANO, which rapidly liberates free NO in solution [25]. However, the mechanism by which AR is modified by nitrosothiols, which release NO slowly to the medium, seems to be more complex. Our kinetic analysis indicates that the modification of AR by SNAP proceeds via the formation of an intermediate complex between AR and SNAP. This is consistent with the structural analysis, which shows that AR forms a 1:1 adduct with the entire SNAP molecule. It has been reported previously [26] that the first step in the transnitrosation reaction of GSH and GSNO is the direct conjugation of the two thiols via an NO bridge leading to the formation of *N*-hydroxysulphenamide (R_1S-N[OH]-SR_2). As the reaction proceeds, the complex decomposes into GSSG, GSNO, $GSNO_2$ or $GSNH_2$. The formation of these products involves multiple reductions by GSH and oxidation by molecular oxygen [26]. Our results suggest that similar chemical steps might be involved in the reaction between protein thiols and nitrosothiols. As with the GSH/GSNO couple, the *N*-hydroxysulphenamide-like complex is also formed between AR and SNAP, because this complex was directly detected in our ESI$^+$–MS experiments. Apparently, this complex (ARS-N[OH]-SNAP) is relatively stable and persists even after 90 min of incubation of AR with SNAP. However, in addition to this

complex, a significant fraction of AR in the medium was in the nitrosated form (AR-NO). This species could be formed either by dissociation of the AR-SNAP complex or by direct nitrosation of AR by free NO liberated spontaneously in the medium by SNAP.

Although on the basis of the current data we cannot rule out the possibility that a portion of AR is nitrosated by free NO in the medium, our results support the view that most of the nitrosated protein is generated from the dissociation of the parent *N*-hydroxysulphenamide-like complex. Both kinetic and structural results are consistent with this possibility. The kinetic analysis of the rate of modification of AR by SNAP and glyco-SNAP shows that the rate of modification of AR by glyco-SNAP is approx. 30-fold faster than by SNAP. This is the reverse of what would be expected if nitrosation were due to free NO in the medium. The spontaneous time of release of NO by SNAP is 10 h [27], whereas that by glyco-SNAP is 27 h [28]; thus modification by SNAP would be expected to be more rapid. The release of NO from nitrosothiols depends on several factors, such as pH, ionic strength, metal contamination and light; thus minor changes in the reaction mixture could have contributed to the difference in the kinetics of modification by SNAP and glyco-SNAP. However, this seems unlikely because the enzyme was modified by the two reagents under identical conditions and the difference in the modification rates was too large to be accounted for by minor variation in the reaction conditions. Moreover, the modification of AR by glyco-SNAP was not affected by the availability of molecular oxygen and was not prevented by the high concentration of phosphate in the medium. Previous studies have shown that NO liberated in solution does not react directly with thiols; instead, it is first converted into N_2O_3 by molecular oxygen [29], which then nitrosates thiols. The formation of N_2O_3 in the medium is limited by the availability of molecular oxygen; the N_2O_3 is avidly scavenged by P_i ions [26]. However, the modification of AR with glyco-SNAP was not affected by excluding molecular oxygen from the medium or by using Hepes instead of phosphate as the incubation buffer, suggesting that there was little, if any, free NO in the medium. In contrast, the modification of AR by the non-thiol NO donor DEANO was prevented by excluding oxygen and was quenched by P_i, indicating that in this case the nitrosation of AR was due to free NO in the medium. Thus, taken together, these results suggest that the modification of AR by glyco-SNAP involves the formation of an intermediate complex, which on dissociation yields AR-NO. Our structural analyses also support this possibility and show that the AR-glyco-SNAP adduct (corresponding to a molecular mass of 36101 Da) is formed during the early phase of the reaction. However, in contrast with AR-SNAP, the AR-glyco-SNAP complex seems to be less stable because the formation, or the dissociation, of this complex did not appear as a kinetically significant term in the rate equation describing the modification process. That the formation of this complex was transient is also suggested by our mass analyses, which show that the complex disappears after 10 min of incubation. We therefore suggest that the modification of AR by both SNAP and glyco-SNAP involves the formation of a similar *N*-hydroxysulphenamide-like complex. However, the AR-glyco-SNAP complex dissociates more rapidly than AR-SNAP and the more rapid dissociation of AR-glyco-SNAP accounts for the greater rate of modification of AR by glyco-SNAP in comparison with SNAP.

The predominant end product generated by the dissociation of the AR-SNAP complex was AR-NO, whereas the AR-glyco-SNAP complex seems to dissociate in equal proportions into AR-NO and the AR-glyco-NAP mixed disulphide. The separation of these forms by lectin chromatography revealed that the inactive form of the protein was retained on the column, whereas the protein unadsorbed by the column displayed a higher K_m and a higher k_{cat} than the unmodified enzyme. We assign the high-K_m–high-k_{cat} form of the enzyme to AR-NO and suggest that the inactive form of the enzyme is an AR-glyco-NAP mixed disulphide. This assignment is consistent with our previous observation that AR-SSG is catalytically inactive, presumably because the bulky mixed disulphide occludes the active site. Moreover, the modification of AR by DEANO, which exclusively generates the nitrosated protein, leads to an increase in enzyme activity, indicating that the high-K_m–high-k_{cat} form of the enzyme is the nitrosated protein and that nitrosation does not inhibit catalysis but alters ligand binding at the active site. The kinetic properties of the high-K_m–high-k_{cat} nitrosated AR, and its insensitivity to sulphate anions and sorbinil, are similar to those of the previously characterized 'activated' form of the enzyme [13,14]. However, it remains to be seen whether the 'activated' AR, isolated previously from euglycaemic and hyperglycaemic tissues, is an intrinsically nitrosated form of the protein.

Our results suggest that protein modifications by nitrosothiols might be more complex than previously expected. Although free NO nitrosates protein thiols in an oxygen-dependent manner, leading to a unique modification, the reaction of proteins with nitrosothiols might be less predictable. We suggest that, in general, the first step in the reaction of nitrosothiols with protein thiols might be the formation of an *N*-hydroxysulphenamide-like complex (demonstrated here for SNAP and glyco-SNAP, and in [26] for GSH). As shown in Scheme 1, this complex can dissociate into two distinct end products, a nitrosated protein or a mixed



Scheme 1 Reaction of AR with nitrosothiols

Incubation of AR with nitrosothiols leads to the formation of a *N*-hydroxysulphenamide-like complex, which dissociates into either a mixed disulphide (**A**) or nitrosated AR (**B**).

disulphide, the relative proportions of which depend on cellular conditions such as pH, P_i or oxygen. Because nitrosation and thiolation are chemically different modifications, it is likely that, as reported here for AR, these modifications cause different changes in protein function. Additional modifications are also possible: sulphenic and sulphonic acid derivatives can also be formed by the reaction of protein thiols with nitrosothiol [22]. Thus, unlike the singular changes caused by phosphorylation or prenylation, modifications by nitrosothiols are likely to be non-unique and could induce multiple changes in protein structure and function. Alternatively, the specificity of this modification might be regulated further, either by intracellular conditions or by the local environment of the protein thiol, which might stabilize one form of the modification over the other.

In summary, we report here that the modification of AR by glyco-SNAP leads to specific changes in the protein at Cys-298. Our results show for the first time that the modification of a protein by nitrosothiols results in the formation of a stable intermediate complex between the nitrosothiol and the protein. The disproportionation of this complex generates diverse structural alterations (nitrosation or mixed disulphide formation), leading to different kinetic changes resulting from the same initial modification.

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