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Novel application of *S*-nitrosoglutathione–Sepharose to identify proteins that are potential targets for *S*-nitrosoglutathione-induced mixed-disulphide formation

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Site-specific S-glutathionylation is emerging as a novel mechanism by which *S*-nitrosoglutathione (GSNO) may modify functionally important protein thiols. Here, we show that GSNO–Sepharose mimicks site-specific S-glutathionylation of the transcription factors c-Jun and p50 by free GSNO *in vitro*. Both c-Jun and p50 were found to bind to immobilized GSNO through the formation of a mixed disulphide, involving a conserved cysteine residue located in the DNA-binding domains of these transcription factors. Furthermore, we show that c-Jun, p50, glycogen phosphorylase *b*, glyceraldehyde-3-phosphate dehydrogenase, creatine kinase, glutaredoxin and caspase-3 can be precipitated from a mixture of purified thiol-containing proteins by the formation of a mixed-disulphide bond with GSNO–Sepharose. With few exceptions, protein binding to this matrix correlated well with the susceptibility of the investigated proteins to undergo GSNO- but not diamide-induced mixed-disulphide formation *in vitro*. Finally, it is shown that covalent GSNO–Sepharose chromatography of HeLa cell nuclear extracts results in the enrichment of proteins which incorporate glutathione in response to GSNO treatment. As suggested by DNA-binding assays, this group of nuclear proteins include the transcription factors activator protein-1, nuclear factor-κB and cAMP-response-element-binding protein. In conclusion, we introduce GSNO–Sepharose as a probe for site-specific S-glutathionylation and as a novel and potentially useful tool to isolate and identify proteins which are candidate targets for GSNO-induced mixed-disulphide formation.

Key words: affinity chromatography, cysteine, glutathionylation, nitric oxide, nitrosothiol.

INTRODUCTION

The ubiquitous signalling molecule NO has been implicated in the regulation of key functions in the immune, cardiovascular, and nervous system [1]. The main source of NO in mammalian cells is the enzymic oxidation of L-arginine to L-citrulline and NO by NO synthases [2]. Alternatively, NO may be produced in ischaemic tissues by non-enzymic transformation of nitrite [3]. The reactivity and fate of NO in biological systems is governed by a complex, and as yet not completely elucidated, network of competing reactions with molecular oxygen, superoxide, transition metals and thiols [4–6]. These reactions yield a variety of NO-derived reactive nitrogen species (RNS) such as N_2O_3, peroxynitrite, nitrosyl–metal complexes and S-nitrosothiols. NO and other RNS react rapidly with GSH, the major intracellular low-molecular-mass antioxidant, to yield the S-nitrosothiol *S*-nitrosoglutathione (GSNO). GSNO has been identified in a variety of tissues and is considered to represent a functionally relevant signalling molecule which may act independently of homolytic cleavage to NO or be metabolized to other bioactive nitrogen oxides [6].

The best characterized effect of NO and other NO-derived signalling molecules is the activation of soluble guanylate cyclase by interaction with the haem group of the enzyme [7]. However, accumulating evidence suggests that the diverse biological effects of RNS [8] can be attributed, at least in part, to their potential to modify and regulate the activity of target proteins by tyrosine nitration, carbonyl formation at lysine and arginine residues, methionine oxidation [9], as well as through the oxidation and S-nitrosation of cysteine residues [10]. The modification of protein thiols by RNS has gained considerable attention because of its implication in the regulation of protein function in physiologically relevant signalling as well as in situations of nitrosative stress [11,12]. Depending on the microenvironment of the targeted protein thiol and the chemical reactivity of the NO-derived signalling molecule, protein cysteines may suffer S-nitrosation, or oxidation to sulphenic, sulphinic or sulphonic acid, as well as disulphide-bridge formation [10]. Interestingly, recent work linked the formation of GSNO to the regulation of proteins by mixed-disulphide formation. First suggestions that GSNO may act as an S-glutathionylating agent came from early work on the NO/GSH-dependent modification of yeast alcohol dehydrogenase [13]. A more recent study on the regulation of aldose reductase by GSNO shows that micromolar concentrations of the nitrosothiol inhibited the enzyme through site-specific mixed-disulphide formation at a conserved cysteine residue in the catalytic site [14]. A possible role of NO-induced protein thiolation in intact cells was highlighted by experiments demonstrating that endothelial cells respond to exogenous NO production with the transient thiolation of a number of, as yet unidentified, cellular proteins [15]. The concept of NO-dependent protein thiolation as a regulatory mechanism was further supported by very recent studies on the mechanisms of NO/GSH-dependent modification of H-ras and carbonic anhydrase [16], as

Abbreviations used: GSNO, *S*-nitrosoglutathione; AP-1, activator protein 1; NF-κB, nuclear factor κB; CREB, cAMP-response-element-binding protein; CP-1, CCAAT-binding protein 1; EMSA, electrophoretic mobility-shift assay; RNS, reactive nitrogen species; NP40, Nonidet P40; SOD, Cu,Zn superoxide dismutase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GPDH, glycerol-3-phosphate dehydrogenase.

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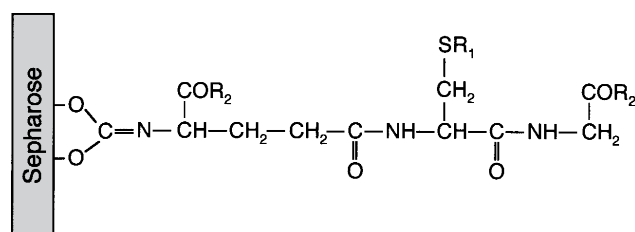
well as glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [17], sarcoplasmic reticulum calcium-ATPase [18], and the cysteine proteases caspase-3 [19] and cathepsin K [20]. A recent report from our laboratory on the regulation of c-Jun DNA binding by NO extends these findings, suggesting a novel role for S-glutathionylation in the regulation of transcription [21]. This study provides evidence that NO inhibits the DNA-binding activity of the activator protein 1 (AP-1) subunit c-Jun *in vitro* in a reversible and redox-independent manner. The underlying mechanism was shown to involve the formation of a mixed disulphide at a conserved cysteine residue in the DNA-binding site of the transcription factor through a reaction which depends on the NO-mediated conversion of GSH into GSNO.

Although the molecular basis of GSNO-induced protein thiolation remains to be established, both previous data (reviewed in [22]) and molecular modelling of S-glutathionylated c-Jun [23] suggest that mixed-disulphide formation could be facilitated by specific interactions between the glutathionyl moiety and basic amino acid side chains which flank the targeted protein thiol. Given that not only c-Jun but a number of other transcription factors, such as members of the Fos, activating transcription factor/cAMP-response-element-binding protein (CREB) and Rel/nuclear factor κB (NF-κB) families, as well as various cytosolic proteins, including glycogen phosphorylase b, GAPDH and creatine kinase, contain potentially redox- and NO-sensitive cysteine residues surrounded by basic amino acids, it is attractive to speculate that GSNO-mediated S-glutathionylation may constitute a general mechanism by which the formation of NO and, consequently, GSNO may be transduced into a functional response at the transcriptional level. As yet, however, there are no methods available which allow a rapid and simple screening for proteins which are candidate targets for GSNO-induced S-glutathionylation. The aim of this study is to provide a novel methodological approach to address this issue. We show that GSNO covalently attached to Sepharose mimicks site-specific S-glutathionylation of the transcription factor c-Jun by free GSNO, and allows the isolation of transcription factors that are potentially modified by GSNO-mediated mixed-disulphide formation from HeLa cell nuclear extracts.

EXPERIMENTAL

Materials

Purified BSA, alcohol dehydrogenase (from baker's yeast), carbonic anhydrase (from bovine erythrocytes), creatine kinase (from rabbit muscle), GAPDH (from rabbit muscle), glycerol-3-phosphate dehydrogenase (GPDH, from rabbit muscle), Hb (from bovine erythrocytes), glycogen phosphorylase *b* (from rabbit muscle), and Cu,Zn superoxide dismutase (SOD, from bovine liver) were purchased from Sigma. S-nitrosated BSA was prepared by incubation of BSA in acidified nitrite, as described in [24]. The recombinant wild-type and mutant DNA-binding domains of human c-Jun (amino acid residues 233–327 of the translated sequence, GenBank accession number J04111) and truncated human p50 (amino acid residues 36–385, GenBank accession number M55643) were expressed *in Escherichia coli* as hexahistidine fusion proteins and purified as described in [25,26] (these constructs are referred to as c-Jun and p50 throughout the text). DNA-binding activities of the recombinant wild-type and mutant proteins were assessed by electrophoretic mobility-shift assay (EMSA). The protease inhibitor Pefabloc was obtained from Roche Molecular Biochemicals. Stock solutions of [^{3}H]GSH were prepared at a final concentration of 20 mM by the addition a freshly prepared solution of 22 mM unlabelled GSH (free acid, SigmaUltra) in water/[^{3}H]GSH [10:1, v/v (40–50 Ci/mmol, approx. 0.02 mM; DuPont New England Nuclear, Boston, MA, U.S.A.)], and stored in small aliquots at −80 °C. [^{3}H]GSNO (1 mM) was prepared by nitrosation of [^{3}H]GSH (1 mM) with sodium nitrite (1 mM) in 10 mM HCl. Unlabelled GSNO was purchased from Alexis Biochemicals. Cell culture products were from Gibco BRL, all other reagents from Sigma–Aldrich.



Scheme 1 Structure of GSH–Sepharose analogues used in this study

GSH–Sepharose (R_1 = H, R_2 = OH) was prepared by reduction of commercially available activated thiol–Sepharose 4B (R_1 = 2-thiopyridyl, R_2 = OH). GSNO Sepharose (R_1 = NO, R_2 = OH) was obtained by subsequent S-nitrosation of GSH–Sepharose with acidified nitrite. At neutral pH, R_2 is deprotonised and adopts a negative charge. The ethyl ester of GSNO–Sepharose (R_1 = NO, R_2 = OC_2H_5) was prepared by esterification of GSH–Sepharose in acidified ethanol prior to S-nitrosation. Where indicated, GSNO–Sepharose was recovered from the GSNO–Sepharose ethyl ester by esterase treatment. Details of the preparation procedures are provided in the Experimental section.

Determinations of free thiol, S-nitrosothiol and protein concentrations

Free thiol and S-nitrosothiol concentrations were determined photometrically by previously described modifications of the Ellman and Saville assay [27] respectively. Protein concentrations were determined by the method of Bradford with BSA as standard protein [28].

Preparation of GSH–Sepharose analogues

GSH– and GSNO–Sepharose (Scheme 1) were prepared from 2,2′-dipyridyldisulphide-activated GSH–Sepharose ('activated thiol–Sepharose 4B', obtained from Amersham Pharmacia Biotech) according to a recently published method [29] with minor modifications. Briefly, activated thiol–Sepharose 4B was suspended in water, and the swollen gel was washed with 10 vol. of water prior to reduction of the activated thiol groups by incubation for 45 min at ambient temperature in 10 vol. of 0.3 M $NaHCO_3$ (pH 8.5) containing 1 mM EDTA and 5% (v/v) 2-mercaptoethanol. This procedure yielded reduced GSH–Sepharose, which was washed with 30 vol. of a 50 mM Tris/HCl buffer (pH 7.4) containing 250 mM NaCl and 1 mM EDTA.

To obtain GSNO–Sepharose, the GSH matrix was washed with 30 vol. of 10 mM HCl and resuspended in 2 vol. of 10 mM HCl prior to addition of 2 vol. of 10 mM $NaNO_2$ in water. The suspension was agitated thoroughly and incubated for approx. 15 min at ambient temperature. The obtained GSNO–Sepharose was washed with 30 vol. of a 50 mM Tris/HCl buffer (pH 7.4), containing 250 mM NaCl and 1 mM EDTA, and stored in this buffer at 4 °C under nitrogen in the dark. Under these conditions, GSNO–Sepharose was relatively stable, and the loss of S-nitrosothiol groups was less than 5% and 15% within 1 and 2 weeks respectively. As judged by the determination of $HgCl_2$-releasable NO (2.4 ± 0.3 μmol/ml of swollen gel, $n = 6$), the conversion of the GSH groups contained in GSH–Sepharose

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(2.5 ± 0.5 μmol/ml of swollen gel, $n = 6$) to S-nitrosothiols was almost quantitative.

The ethyl ester of GSNO–Sepharose (Scheme 1) was prepared in analogy to a previously published procedure described for the preparation of GSH ethyl ester [30]. 2,2′-Dipyridyldisulphide-activated GSH–Sepharose (0.5 g) was suspended in 10 ml of anhydrous ethanol, containing 274 μl of concentrated H_2SO_4, and the incubation was suspended overnight under constant agitation at 30 °C. The Sepharose was rehydrated by subsequent washing steps with 10 vol. of 80, 60, 40, 20 and 0 % ethanol in water (v/v) prior to reduction and S-nitrosation as described above. Finally, the material obtained was washed with 30 vol. of a 50 mM Tris/HCl buffer (pH 7.4) containing 250 mM NaCl and 1 mM EDTA. The S-nitrosothiol content of this Sepharose preparation was determined as 2.1 ± 0.1 μmol/ml of swollen gel ($n = 4$). The content of GSH ethyl ester groups was 2.8 ± 1.1 μmol/ml of swollen gel ($n = 3$), as estimated from enzymic determination of ethanol [31], which was released from the matrix by treatment with porcine liver esterase (100 units/ml of swollen gel). Thus for unknown reasons, esterification was not quantitative, and reached only 56 % of the theoretical content of two ethyl groups per GSH molecule. For some control experiments, GSH–Sepharose was recovered from the GSNO ethyl ester–Sepharose by treatment with 100 units of esterase (purified from porcine liver, purchased from Sigma) per ml of swollen gel for 30 min at ambient temperature.

Precipitation of purified proteins by GSH–Sepharose analogues

To assay purified proteins for covalent binding to one of the GSH–Sepharose counterparts described above, the indicated proteins (10 μg, unless otherwise specified) were incubated at ambient temperature with the Sepharose (0.1 ml, unless otherwise specified) in a final volume of 0.5 ml of buffer A [50 mM Tris/HCl (pH 7.4) containing 250 mM NaCl, 1 mM EDTA and 0.01 % (v/v) Nonidet P40 (NP40)]. After 30 min, the Sepharose matrix was pelleted by short centrifugation (300 ***g*** for 1 min) and washed three times with 1 ml of buffer A. Bound protein was eluted in 0.4 ml of buffer A containing 1 % (v/v) 2-mercaptoethanol. Protein binding to the matrix was quantified by determining protein concentrations in the eluate. For some experiments, elution was performed with 0.1 ml of buffer A containing 1 % (v/v) 2-mercaptoethanol, and samples were analysed by SDS/PAGE on 13 % polyacrylamide gels. Gels were stained for protein with Coomassie Blue and dried.

Preparation of nuclear extracts from HeLa cells

HeLa cells were cultured in RPMI 1640 medium with L-Glutamine (Gibco BRL) containing 9 % (v/v) fetal bovine serum and antibiotics (90 units/ml penicillin and 90 μg/ml streptomycin). Exponentially growing ($\leqslant 75$ % confluence) cells (12 culture dishes, 10 cm diameter) were washed with ice-cold PBS, scraped into PBS, pelleted by centrifugation, and resuspended in a final volume of 10 ml of a 10 mM Hepes buffer (pH 7.9) containing 10 mM NaCl, 1 mM EDTA, 1 % (v/v) glycerol, 0.1 % (v/v) 2-mercaptoethanol and 2 mM Pefabloc. Following incubation for 15 min on ice, NP40 was added at a final concentration of 0.6 %. Subsequently, the cell suspension was agitated vigorously for 10 s and centrifuged for 3 min at 20000 ***g***. The pellet obtained was resuspended in a final volume of 1.5 ml of a 20 mM Hepes buffer (pH 7.9), containing 500 mM NaCl, 1 mM EDTA, 10 % (v/v) glycerol, 0.1 % (v/v) 2-mercaptoethanol and 2 mM Pefabloc, incubated on ice for 30 min, and agitated vigorously several times prior to centrifugation for 6 min at 20000 ***g***. The obtained supernatant, which contained 1–2 mg/ml protein, was stored at −80 °C.

Isolation of nuclear proteins by covalent GSNO chromatography

Pooled nuclear extracts (10–15 mg of total protein) from exponentially growing HeLa cells were diluted 1 : 100 (v/v) in buffer A containing 1 mM of the protease inhibitor Pefabloc and 4–8 ml of thoroughly suspended GSNO–Sepharose. The suspension was incubated for 30 min at ambient temperature with occasional shaking prior to filtration over a fritted column (internal diameter of 1 cm). To the column flow-through containing unbound protein, 2-mercaptoethanol was added at a final concentration of 1 % (v/v), prior to concentration of the solution to a final volume of $\leqslant 5$ ml on Vivapore 20 concentrators (molecular mass cut-off 7500 Da; Vivascience, Binbrook, Lincoln, U.K.). Subsequently, the column was washed with 30 vol. of buffer A and eluted with three vol. of buffer A containing 1 % (v/v) 2-mercaptoethanol. The eluate was concentrated to $\leqslant 1$ ml on Vivapore 20 concentrators. The protein concentrates obtained were stored at −80 °C.

Detection of mixed-disulphide formation with [^{3}H]GSH

Mixed-disulphide formation between the indicated purified proteins and GSH was determined as dithiotreitol-labile, trichloracetic acid-precipitable incorporation of [^{3}H]-labelled GSH as described recently [23]. Briefly, the indicated proteins (2–10 μM) were incubated for 30 min at 37 °C in 0.1 ml of a 20 mM Tris/HCl buffer (pH 7.5), containing 50 mM NaCl, 5 mM $MgCl_2$, 1 mM EDTA, 5 % (v/v) glycerol, 0.01 % (v/v) NP40 and 3 mM [^{3}H]GSH ($6–9 \times 10^5$ c.p.m.) in the absence and presence of either 1 mM [^{3}H]GSNO or 10 mM diamide. Samples that were used to calculate blank values also contained 10 mM dithiotreitol.

To assay the protein fractions which had been obtained by GSNO–Sepharose chromatography of HeLa cell nuclear extracts, 2-mercaptoethanol contained in the protein preparations was replaced by GSH. For this purpose, the proteins were subjected to chromatography on Sephadex G-25 columns (NAP-10; Amersham Pharmacia Biotech) which had been equilibrated with buffer A containing 1 mM GSH. The protein-containing column fractions (0.6–0.8 ml) were pooled and concentrated to a final volume of approx. 0.3 ml on Vivapore 20 concentrators. Subsequently, the sample was assayed for mixed-disulphide formation, which was induced either by the addition of 1 mM [^{3}H]GSH (blank value), 1 mM [^{3}H]GSH and 10 mM diamide (diamide-induced S-glutathionylation), or 1 mM [^{3}H]GSNO (GSNO-induced S-glutathionylation), as described above. S-Glutathionylation reactions were stopped by the addition of 1 ml of 10 % (w/v) ice-cold trichloracetic acid. Samples were incubated on ice for 30 min prior to centrifugation at 4 °C for 10 min at 20000 ***g***. The precipitated protein pellet was washed three times with 1 ml of 10 % (w/v) ice-cold trichloracetic acid prior to incubation with 0.1 ml of 1 N NaOH for 20 min at 70–80 °C. The NaOH-solubilized proteins were added to 14 ml of a standard liquid-scintillation cocktail (ReadySafe; Beckman Instruments, Madrid, Spain) to which 1 ml of 10 % (w/v) trichloroacetic acid was added, and assayed for incorporation of [^{3}H]GSH by liquid-scintillation counting.

Analysis of DNA-binding activity by EMSA

Protein fractions (0.5–2 μg of total protein), which had been obtained by chromatography on GSNO–Sepharose, were incubated in a 20 mM Tris/HCl buffer (pH 7.5) containing 50 mM NaCl, 5 mM $MgCl_2$, 1 mM EDTA, 5 % (v/v) glycerol, 0.01 %

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(v/v) NP40, 0.2 mg/ml BSA and 0.1 mg/ml poly(dI-dC). One of the following ^{32}P-radiolabelled double-stranded oligonucleotides containing the DNA-binding sites for the transcription factors: AP-1 (5′-GGG CTT GAT GAG TCA GCC GGA CC-3′), NF-κB (5′-GGA GAG GGG ATT CCC TGC G-3′), CREB (5′-AGA GAT TGC CTG ACG TCA GAG AGC TAG-3′), CCAAT-binding protein 1 (CP-1; 5′-CCA CAA ACC AGC CAA TGA GTA ACT GCT GG-3′) or Sp1 (5′-ATT CGA TGC GGG CGG GGC GAG C-3′) were also added. Protein samples which were analysed for Sp1 DNA-binding activity were pre-incubated on ice for 30 min in the presence of 3 mM $ZnSO_4$ in order to restore zinc-dependent DNA binding of the transcription factor [32]. Where indicated, incubations additionally contained an approx. 50-fold excess of an unlabelled competitor oligonucleotide. Subsequent to incubation for 30 min on ice, samples were subjected to electrophoresis at 180 V on pre-electrophoresed 6 % non-denaturing polyacrylamide gels with 22 mM Tris/borate/0.5 mM EDTA as running buffer. Gels were dried and visualized by autoradiography.

Data representation

The number of protein cysteines, as well as the quantification of protein S-glutathionylation (mol GSH incorporated/mol protein) and molar protein concentrations given in the text, refer to the native quaternary structure of the respective protein, i.e. monomers (BSA, carbonic anhydrase, glutaredoxin and thioredoxin), homodimers (c-Jun, creatine kinase, GPDH, glycogen phosphorylase *b*, p50 and SOD), heterodimers (Hb), homotetramers (alcohol dehydrogenase and GAPDH) and heterotetramers (caspase-3). Unless otherwise indicated, data are given as means ± S.E.M., with the number of independent experiments (*n*) in parenthesis.

RESULTS

Binding of purified c-Jun DNA-binding domains to GSNO–Sepharose

Recently, we have shown that GSNO induces the formation of a mixed disulphide between the glutathionyl moiety of GSNO and a conserved cysteine residue in the DNA-binding domain of the transcription factor c-Jun [21]. In accordance with a molecular model of thiolated c-Jun [23], which suggested that specific GSNO–protein interactions facilitate mixed-disulphide formation, we speculated that GSNO–Sepharose might serve as a probe of protein thiols which are susceptible to GSNO-induced S-glutathionylation. To address this issue, we studied binding of recombinant c-Jun DNA-binding domains to the GSNO matrix. As shown in Figure 1(A), c-Jun bound to GSNO–Sepharose following saturation kinetics. Binding to 0.1 ml of GSNO–Sepharose, which contained 240 ± 30 nmol ($n = 6$) S-nitrosothiol groups (see also the Experimental section), was virtually quantitative (> 95 % of total protein) when the amounts of c-Jun were < 0.2 nmol, and approached saturation at > 1 nmol c-Jun. In the presence of saturating amounts of protein (2 nmol c-Jun), binding showed a linear dependency on the total amount of GSNO–Sepharose (Figure 1B). From the slope of this graph, the c-Jun-binding capacity of GSNO–Sepharose was calculated as 12 nmol c-Jun monomer/ml of swollen gel (i.e. per 2.4 μmol S-nitrosothiol groups). c-Jun binding to GSNO–Sepharose could not be reverted by high-salt (1 M NaCl) or detergent (5 % SDS), but was completely abolished by millimolar concentrations of thiol-reducing agents, such as 2-mercaptoethanol, GSH, and dithiotreitol (results not shown). These data suggest that c-Jun binds covalently to GSNO–Sepharose through mixed-disulphide bond formation with the thiol groups of the matrix.

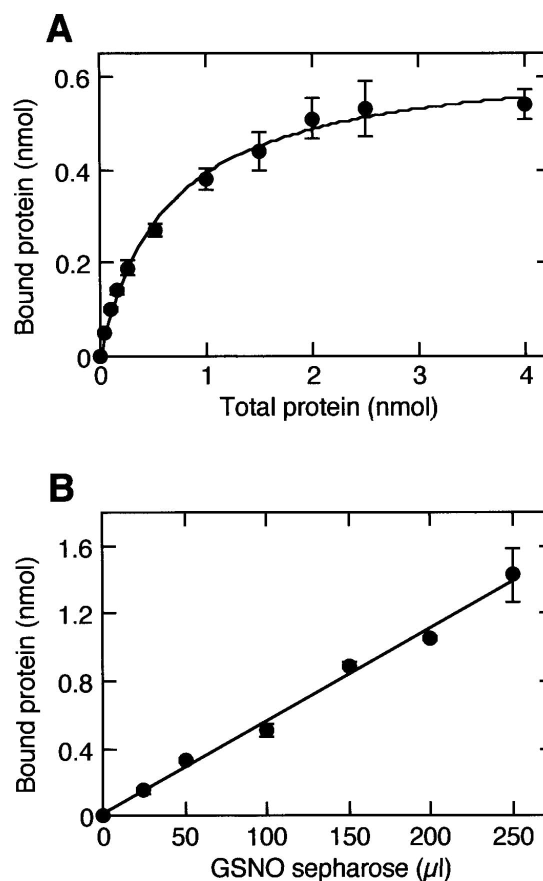

Figure 1 Binding of c-Jun to GSNO–Sepharose

Recombinant c-Jun DNA-binding domains were incubated in a final volume of 0.5 ml with GSNO–Sepharose and assayed for covalent binding to this matrix through mixed-disulphide formation as described in the Experimental section. The dependency of c-Jun binding on the total amount of c-Jun protein (**A**) was assayed with increasing amounts of c-Jun (0.05–4 nmol) and a fixed quantity (0.1 ml) of GSNO–Sepharose. To determine the dependency of c-Jun binding on the amount of GSNO–Sepharose (**B**), a fixed amount of c-Jun (2 nmol) was incubated with increasing amounts of GSNO–Sepharose (25–250 μl). Data are means ± S.E.M. of 4–12 different experiments.

GSNO–Sepharose precipitation of purified proteins susceptible to GSNO-induced S-glutathionylation

The above data, obtained with the cDNA-binding domain of c-Jun as model protein, encourage the view that mixed-disulphide formation between a given protein and GSNO–Sepharose might reflect its susceptibility to GSNO-induced S-glutathionylation. To address this issue, we compared structurally distinct proteins in terms of GSNO–Sepharose binding, as well as GSNO- and diamide-induced S-glutathionylation. In the first set of experiments, we chose p50, Hb and BSA because, similar to c-Jun, they contain critical cysteine residues which have been shown previously to be sensitive to GSNO or other NO donor compounds [24,33–35]. However, the question of whether GSNO may direct mixed-disulphide formation to these proteins has not been addressed so far. We compared GSNO–Sepharose binding

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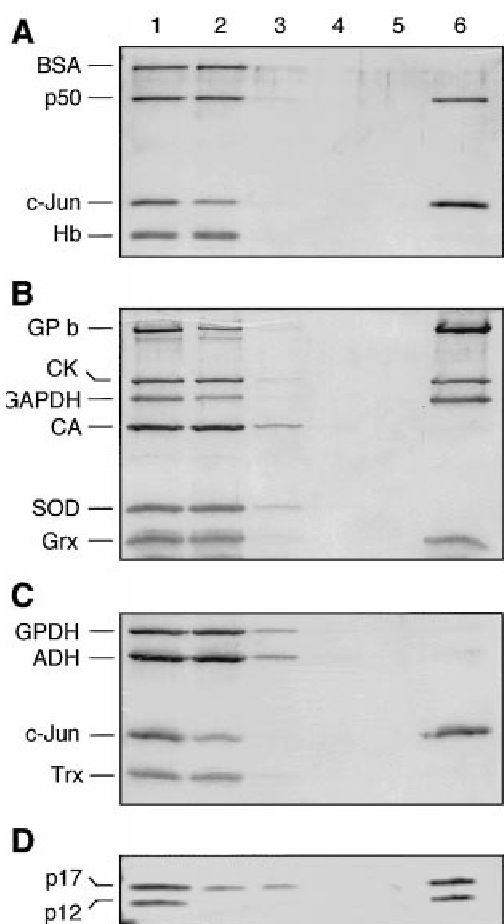

Figure 2 Binding of purified proteins to GSNO–Sepharose

A mixture of various purified proteins, i.e. BSA, p50, c-Jun and Hb (**A**), glycogen phosphorylase *b* (GP b), creatine kinase (CK), GAPDH, carbonic anhydrase (CA), SOD and glutaredoxin (Grx) (**B**), GPDH, alcohol dehydrogenase (ADH), c-Jun and thioredoxin (Trx) (**C**) and caspase-3 composed by the subunits p12 and p17 (**D**) was incubated in a final volume of 0.5 ml with 0.1 ml of GSNO–Sepharose for 30 min at ambient temperature, as described in the Experimental section. The amount of the individual proteins was 10 μg, with the exception of Hb and caspase-3, where the amount was 20 μg. Bound proteins were isolated by short centrifugation of the Sepharose matrix, which was washed three times with 0.5 ml of a 50 mM Tris/HCl buffer containing 250 mM NaCl, 1 mM EDTA and 0.01 % NP40 prior to elution with 0.1 ml of the washing buffer to which 1 % (v/v) 2-mercaptoethanol was added. Aliquots (25 μl) of the complete incubation mixture (lane 1), the supernatant containing unbound proteins (lane 2), the first (lane 3), second (lane 4) and third (lane 5) wash, as well as the Sepharose eluate (lane 6), were subjected to SDS/13 % PAGE, and gels were stained for protein with Coomassie Blue. The gels shown are representative of at least three similar experiments.

of p50, Hb and BSA with that of c-Jun (Figure 2A) by incubating a mixture of the purified proteins with the nitrosothiol matrix (lane 1). The supernatant (Figure 2, lane 2) was discarded, and non-specifically bound proteins were removed by excessive washing of the matrix (lanes 3–5) prior to reductive elution of covalently bound proteins (lane 6). The eluate contained c-Jun and p50, but only barely detectable amounts of Hb and BSA. GSNO–Sepharose binding of these proteins was further quantified in independent experiments by determining their amounts in the eluate with the Bradford assay (see the Experimental section). When 4 nmol of c-Jun and p50 were incubated with 0.1 ml of GSNO–Sepharose, the affinity matrix precipitated 0.54 ± 0.03 ($n = 3$) and 0.38 ± 0.04 ($n = 3$) nmol protein respectively, whereas binding of Hb and BSA was barely detectable [0.09 ± 0.01 ($n = 3$) and 0.07 ± 0.01 ($n = 3$) nmol protein respectively]. Qualitatively similar results were obtained under non-saturating conditions, i.e. when the amount of total protein was reduced to $\leqslant 0.1$ nmol (results not shown). These data correlated well with the GSNO-induced incorporation of [^{3}H]GSH into these proteins, which was considerably lower with Hb and BSA than with p50 and c-Jun (Table 1).

To exclude that lack of BSA and Hb binding to GSNO–Sepharose was due to the inaccessibility of the cysteine residues contained in these proteins to GSH, we used the non-selective thiol-oxidizing agent diamide to induce mixed-disulphide formation. As shown in Table 1, treatment of c-Jun with diamide in the presence of GSH resulted in the stoichiometric incorporation of one GSH molecule into each of the four c-Jun cysteines. In the p50 homodimer, four cysteine residues form intramolecular disulphide bridges, and two further cysteines do not appear to be solvent-accessible [36,37]. Accordingly, only 8 of the 14 cysteine residues were found to be available for mixed-disulphide formation. The Hb tetramer contains two cysteines, which have been shown to be susceptible to diamide-induced mixed-disulphide formation [38]. In fact, we found that both residues incorporated GSH upon diamide treatment. Previous studies on the reactivity of BSA thiols have shown that commercially available BSA contains only one single reactive cysteine residue [39]. In the present study, this is reflected by the diamide-induced GSH incorporation of approx. 1 mol GSH/mol BSA. Thus lack of GSNO–Sepharose binding of Hb and BSA is apparently not due to the lack of cysteine residues that can undergo S-glutathionylation, but rather reflects their low reactivity towards GSNO. Consistent with this hypothesis, activation of the GSH matrix by 2,2′-dipyridyldisulphide bound these four proteins with comparable efficacy (results not shown).

To further support the potential applicability of GSNO–Sepharose to the isolation of proteins that form mixed disulphides with GSNO, we analysed various purified cysteine-containing proteins for GSNO–Sepharose binding (Figure 2B–D) and compared the data obtained with results from [^{3}H]GSH-incorporation experiments (Table 1). To assay these proteins for the total amount of cysteine residues that are accessible to mixed-disulphide formation, [^{3}H]GSH incorporation was induced by diamide. Previous work indicates that glycogen phosphorylase *b* and creatine kinase [40], as well as carbonic anhydrase isoenzymes [41,42], GAPDH [43,44], SOD [45] and glutaredoxin [46], may be modified by redox- or free radical-induced mixed-disulphide formation. Furthermore, in the case of glycogen phosphorylase *b* and an isoform of carbonic anhydrase [16], as well as GAPDH [17], GSNO-induced S-glutathionylation has been reported. Figure 2(B) shows that glycogen phosphorylase *b*, creatine kinase, GAPDH and glutaredoxin bound to GSNO–Sepharose, whereas carbonic anhydrase and SOD apparently did not form mixed disulphides with the NO-activated thiol matrix. These findings fitted well with the differential susceptibility of these proteins to GSNO-induced S-glutathionylation (Table 1). Whereas GSNO-induced GSH incorporation into glycogen phosphorylase *b*, creatine kinase, GAPDH and glutaredoxin was comparable with that found for the GSNO–Sepharose binders c-Jun and p50 (0.9–2 mol GSH/mol protein), S-glutathionylation of carbonic anhydrase and SOD was similar to that found for BSA and Hb (0.1–0.2 mol GSH/mol protein), which, as shown above, could not be efficiently bound by the matrix.

In a previous study, it was speculated that alcohol dehydrogenase may be modified by GSNO-induced S-glutathionylation [13]. A further cysteine-rich enzyme that has been proposed to be regulated by GSH-dependent oxidation of critical cysteine

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Table 1 Diamide- and GSNO-induced protein S-glutathionylation

The total number of protein cysteines was taken from the published amino acid sequences of the investigated wild-type proteins, and refers to their established quaternary structure as given in the Experimental section. Diamide- and GSNO-induced S-glutathionylation of the indicated purified proteins (2–10 μM) were determined in the presence of ^{3}H-labelled GSH (3 mM) and induced by 1 mM [^{3}H]GSNO or 10 mM diamide. Data are means ± S.E.M. for at least three independent experiments. n.d., not determined.

Protein	Number of cysteines	S-Glutathionylation induced by GSH/GSNO (mol/mol protein)	S-Glutathionylation induced by GSH/diamide (mol/mol protein)
c-Jun	4	1.96 ± 0.14	3.83 ± 0.06
Glycogen phosphorylase *b*	18	1.95 ± 0.09	14.34 ± 0.84
GAPDH	16	1.28 ± 0.18	4.17 ± 0.39
Caspase-3	14	1.22 ± 0.12	n.d.
Creatine kinase	8	0.97 ± 0.14	3.08 ± 0.59
Glutaredoxin	4	0.88 ± 0.02	1.02 ± 0.02
p50	14	0.86 ± 0.06	8.06 ± 0.26
Alcohol dehydrogenase	32	0.43 ± 0.01	16.94 ± 0.59
GPDH	22	0.43 ± 0.03	14.49 ± 1.78
Hb	2	0.21 ± 0.01	1.80 ± 0.10
Thioredoxin	3	0.17 ± 0.01	0.64 ± 0.03
Carbonic anhydrase	1	0.11 ± 0.05	0.43 ± 0.03
SOD	6	0.16 ± 0.01	0.34 ± 0.01
BSA	35	0.07 ± 0.01	0.84 ± 0.01

Table 2 Site-specific binding of c-Jun and p50 to GSNO–Sepharose

Wild-type and mutant c-Jun and p50 DNA-binding domains (0.05–0.5 nmol), in which the cysteine located in the DNA-binding site (serine-269) and the adjacent leucine zipper (serine-320) of c-Jun or the cysteine in the DNA-binding site of p50 (serine-62) were substituted by serine, were precipitated by 0.15 ml of GSNO–Sepharose or 2,2′-dipyridyl disulphide-activated GSH–Sepharose, as described in the Experimental section. The amounts of mutant proteins bound to GSNO–Sepharose, which are expressed as a percentage of binding of the corresponding wild-type proteins, are means ± S.E.M. of at least three different experiments.

	Bound protein (% of wild-type)		
	c-Jun (serine-269)	c-Jun (serine-320)	p50 (serine-62)
GSNO–Sepharose	9 ± 6	97 ± 12	15 ± 2
2,2′-Dipyridyl disulphide-activated GSH–Sepharose	84 ± 4	90 ± 5	87 ± 3

residues is GPDH [47,48]. Baker's yeast alcohol dehydrogenase and rabbit muscle GPDH contain 32 and 22 cysteine residues, and incorporate 17 and 14 mole GSH/mol protein upon exposure to diamide respectively (Table 1). Nevertheless, these proteins did not bind to a significant extent to GSNO–Sepharose as compared with the GSNO–Sepharose binder c-Jun (Figure 2C). Both proteins, however, formed low, but significant, amounts (approx. 0.4 mol GSH per mol protein) of mixed disulphides when incubated with GSH/GSNO (Table 1). These data suggest that protein binding to GSNO–Sepharose is not only governed by the overall capacity of a protein to incorporate GSH upon GSNO treatment, but also by other, as yet unknown, factors. A further attempt to identify proteins which contain reactive and GSNO-accessible thiol groups was based on previous work on the thioredoxin system, suggesting that GSNO has free access to, and may react with, one of the active-site cysteines of thioredoxin [49]. As shown in Table 1, purified thioredoxin incorporated $>$ 0.6 mol GSH/mol protein upon diamide treatment but $<$ 0.2 mol GSH/mol protein in the presence of GSNO. In accordance with this relatively poor yield of GSNO-dependent GSH incorporation, we did not detect thioredoxin binding to GSNO–Sepharose (Figure 2C).

Very recent work suggests that the cysteine protease caspase-3 may be modified at various cysteine residues by NO-mediated S-glutathionylation [19]. In the present study, we found that GSNO oxidizes caspase-3 by S-glutathionylation of an overall amount of 1.2 cysteine residues/holoenzyme (Table 1). The susceptibility of the caspase to GSNO mixed-disulphide formation correlated with its capacity to covalently interact with immobilized GSNO (Figure 2D). Furthermore, in accordance with the reported localization of mixed disulphides on both caspase-3 subunits (p12 and p17) [19], we found that both p12 and p17 could be bound by GSNO–Sepharose.

Specificity of protein binding to GSNO–Sepharose

The above data indicate that GSNO-induced S-glutathionylation does not apparently affect each cysteine residue that is accessible to GSH, but may be specifically targeted to a subset of GSNO-sensitive thiols. The c-Jun DNA-binding domain contains two cysteine residues: cysteine-269, located in the basic domain of the protein which makes direct contact with its target DNA sequence; and cysteine-320, in the monomer–monomer interface [50]. However, only cysteine-269 forms a mixed disulphide upon GSNO treatment [21]. Here, we show that such site specificity of GSNO-induced S-glutathionylation is preserved when GSNO–Sepharose is used as the thiolating probe. As shown in Table 2, a cysteine-to-serine mutation at cysteine-269 almost completely abolished c-Jun binding to the immobilized nitrosothiol, whereas binding was virtually unaffected by a cysteine-to-serine mutation at cysteine-320, compared with the wild-type protein. The observed site-specificity was apparently not due to

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steric constraints which inhibit access of Sepharose-coupled GSNO to cysteine-320, since both the serine-320 and serine-269 mutants bound to 2,2′-dipyridyl disulphide activated GSH–Sepharose with comparable efficacy. These data correlated well with recently published results [21] showing that GSNO-induced S-glutathionylation was reduced to 16 ± 6 (5) % of the wild-type protein in the serine-269 mutant and virtually unaffected in the serine-320 mutant [107 ± 11 % ($n = 3$) of the wild-type protein].

Similarly, modification of a single cysteine residue (cysteine-62) has been shown to mediate inhibition of DNA binding of the NF-κB subunit p50 by diamide and NO [26,33]. In accordance with these studies, we found that GSNO inhibited DNA binding of purified p50 with a half-maximal effective concentration of approx. 0.1 mM, and that substitution of cysteine-62 by serine rendered p50 DNA binding insensitive to GSNO (results not shown). It is tempting to speculate, therefore, that cysteine-62 could be the target thiol for GSNO-induced thiolation. [^{3}H]GSH-incorporation experiments showed that GSNO-induced S-glutathionylation of p50 (Table 1) was markedly reduced in the serine-62 mutant [7 ± 4 % ($n = 4$) of the wild-type protein]. Site specificity of GSNO-mediated p50 mixed-disulphide formation was preserved in GSNO–Sepharose binding experiments. As shown in Table 2, point mutation of cysteine-62 to serine markedly reduced the capacity of p50 to bind to GSNO–Sepharose. Similar to c-Jun, site specificity of covalent binding to the thiol–Sepharose was almost completely lost when the GSH moiety of the matrix was activated with 2,2′-dipyridyl disulphide instead of NO (Table 2).

It was suggested that specific GSH–protein interactions may facilitate mixed-disulphide formation [22]. Molecular modelling of c-Jun encourages this view, proposing that electrostatic interactions between basic amino acids surrounding the targeted cysteine residue and negatively charged carboxylates of the GSH moiety may contribute to the susceptibility of protein thiols to S-glutathionylation [23]. To address the question of whether such ionic interactions may play a role in protein binding to GSNO–Sepharose, the negative charges of the matrix were neutralized by converting the carboxylates in the GSNO molecule into the neutral ethyl esters (Scheme 1). Glycogen phosphorylase *b*, creatine kinase, GAPDH and c-Jun were shown above to incorporate $\geqslant 1$ mol GSH/mol protein (Table 1) and to bind well to GSNO–Sepharose (Figure 2). However, as shown in Figure 3(A), binding to GSNO ethyl ester–sepharose was suppressed in the case of glycogen phosphorylase *b* and creatine kinase, and markedly impaired with GAPDH and c-Jun (see also Figure 2). To exclude that such loss of binding was caused by modifications of the matrix other than esterification during the preparation of this GSH Sepharose counterpart, GSNO ethyl ester–Sepharose was treated with esterase to remove the ester groups and, consequently, to recover the negatively charged carboxylate groups. This procedure, in fact, restored the capacity of the matrix to bind glycogen phosphorylase *b* and creatine kinase, and slightly increased c-Jun and GAPDH binding to the immobilized nitrosothiol (Figure 3B). These data indicate that ionic GSH–protein interactions may facilitate protein binding to GSNO–Sepharose. Saturation curves of c-Jun binding to GSNO ethyl ester–Sepharose before and after esterase treatment (results not shown) further corroborated this hypothesis. Maximal binding of c-Jun to these two Sepharose preparations was virtually identical [GSNO ethyl ester–Sepharose, 0.62 ± 0.03 ($n = 3$) nmol protein/0.1 ml of swollen gel; esterase-treated GSNO ethyl ester–Sepharose, 0.59 ± 0.05 ($n = 3$) nmol protein/0.1 ml of swollen gel], whereas the concentration of c-Jun required for half-maximal binding to 0.1 ml of Sepharose was approx. 4-fold higher with the ethyl ester [1.15 ± 0.16 ($n = 3$) nmol protein] as compared with the esterase-treated matrix (0.28 ± 0.07 ($n = 3$) nmol protein).

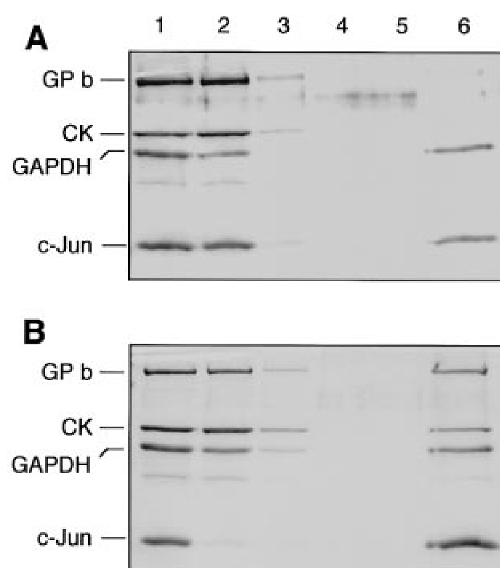

Figure 3 Binding of purified proteins to GSNO ethyl ester–Sepharose

A mixture of purified glycogen phosphorylase *b* (GP b), creatine kinase (CK), GAPDH and c-Jun (10 μg each) was incubated in a final volume of 0.5 ml with 0.1 ml of GSNO ethyl ester–Sepharose for 30 min at ambient temperature, as described in the Experimental section (**A**). The same experiment was repeated with GSNO ethyl ester–Sepharose which had been pretreated with esterase to convert the ethyl ester back into the free carboxylic acid (**B**). Bound proteins were isolated as described in the legend to Figure 2. Aliquots (25 μl) of the complete incubation mixture (lane 1), the supernatant containing unbound proteins (lane 2), the first (lane 3), second (lane 4) and third (lane 5) wash, as well as the Sepharose eluate (lane 6), were subjected to SDS/13% PAGE and gels were stained for protein with Coomassie Blue. The gels shown are representative for three experiments.

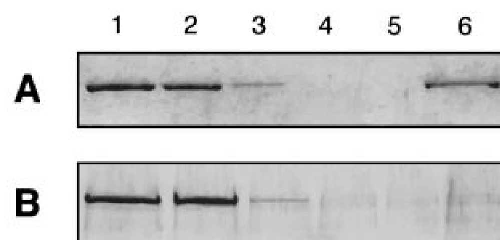

Figure 4 Binding of S-nitrosated BSA to GSH–Sepharose

S-Nitrosated BSA (10 μg) was incubated in a final volume of 0.5 ml with 0.1 ml of GSH–Sepharose for 30 min at ambient temperature, as described in the Experimental section (**A**). Incubations of unmodified BSA with GSNO–Sepharose (**B**) were performed under identical conditions. Bound BSA was isolated as described in the legend to Figure 2. Aliquots (25 μl) of the complete incubation mixture (lane 1), the supernatant containing unbound BSA (lane 2), the first (lane 3), second (lane 4) and third (lane 5) wash, as well as BSA released from GSNO–Sepharose by elution under reducing conditions (lane 6), were subjected to SDS/13% PAGE, and gels were stained for protein with Coomassie Blue. The gels shown are representative for three experiments.

It is currently thought that GSNO-induced S-glutathionylation may involve either a direct nucleophilic attack of a protein thiol on GSNO or a two-step reaction in which the NO group is transferred to the protein thiol prior to reaction of the nitrosated protein with GSH [13,14,16,17,20,21]. Thus it could be argued that protein binding to GSNO–Sepharose depends on the activation of protein thiols via a *trans*-nitrosation reaction, rather

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Table 3 Isolation of nuclear HeLa cell proteins that are susceptible to GSNO-induced S-glutathionylation

HeLa cell nuclear extracts were subjected to chromatography on GSNO–Sepharose, and the total extract, column flow-through and eluate were analysed for protein content and S-glutathionylation. S-Glutathionylation was induced either by the addition of GSNO or diamide, as described in the Experimental section. Data are means ± S.E.M. of three different preparations.

	Total extract	Flow-through	Eluate
Total protein (mg)	12 ± 3	11 ± 3	0.05 ± 0.01
GSNO-induced S-glutathionylation (nmol [^{3}H]GSH/mg of protein)	2 ± 2	3 ± 1	37 ± 14
Recovery of GSNO-reactive thiols (%)	100	138	8
Purification of GSNO-reactive thiols (-fold)	1	1	19
Diamide-induced S-glutathionylation (nmol [^{3}H]GSH/mg of protein)	53 ± 12	67 ± 19	122 ± 12
Recovery of diamide-reactive thiols (%)	100	116	1
Purification of diamide-reactive thiols (-fold)	1	1	2

than on specific GSNO–protein interactions that facilitate a direct reaction between the protein and GSNO. To address this issue, we attempted to study binding of S-nitrosated proteins to GSH–Sepharose. However, almost all of the proteins investigated in this study (listed in Table 1) did not stably incorporate significant amounts of NO (results not shown). One exception was BSA, which formed a relatively stable S-nitrosothiol. Similar to previous studies which reported the incorporation of approx. 0.8 mol NO/mol protein [24], we found that treatment of BSA with acidified nitrite yielded 0.55 ± 0.11 ($n = 6$) mol S-nitrosothiol groups/mol BSA. As shown in Figure 4(A), incubation of S-nitrosated BSA with GSH–Sepharose resulted in the formation of a mixed disulphide with the reduced thiol matrix. Importantly, unmodified BSA does not bind to GSNO–Sepharose (Figure 4B, compare also with Figure 2A) indicating that GSNO–Sepharose rather selects for proteins which react directly with GSNO than for proteins that react with GSH through a GSNO-dependent *trans*-nitrosation reaction.

Isolation of nuclear transcription factors by GSNO–Sepharose chromatography

The above data encourage the view that GSNO–Sepharose might be useful for the isolation and identification of cellular proteins which are potential targets for GSNO-induced S-glutathionylation. When nuclear extracts from exponentially growing HeLa cells were subjected to GSNO–Sepharose chromatography, less than 0.5 % of the total amount of protein loaded on to the nitrosothiol–Sepharose bound to the matrix through mixed-disulphide formation (Table 3). As judged by Coomassie Blue-staining of SDS gels, the protein fraction isolated by this method displayed a substantially distinct protein pattern than the column flow-through (results not shown). Thus to investigate if chromatography on GSNO–Sepharose resulted in a significant enrichment of proteins susceptible to GSNO-induced thiolation, we performed [^{3}H]GSH-incorporation assays comparing unbound (GSNO–Sepharose flow-through) with bound (GSNO–Sepharose eluate) protein fractions. As shown in Table 3, the overall capacity of proteins to undergo GSNO-induced mixed-disulphide formation was 12-fold higher in the GSNO–Sepharose eluate than in the column flow-through. When glutathionylation was induced by diamide, the increase in total GSH incorporation into proteins of the eluate was less than twofold compared with proteins contained in the column flow-through. Thus despite the low yield of this method (8 % recovery of GSNO-sensitive protein thiols), these data suggest that GSNO–Sepharose chromatography may serve to enrich proteins that can be modified by GSNO-mediated incorporation of GSH.

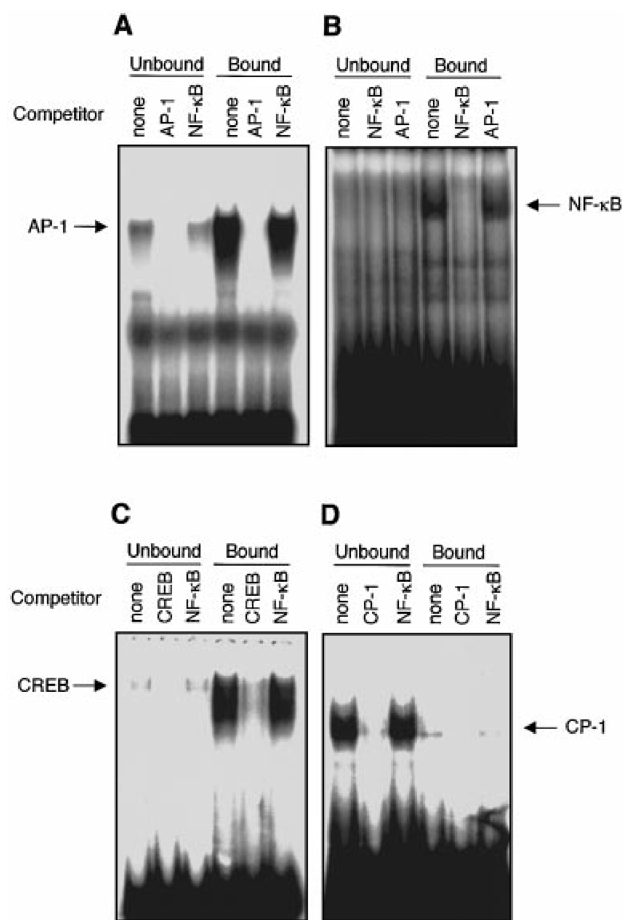

Figure 5 Isolation of AP-1, NF-κB, CREB and CP-1 DNA-binding activities from HeLa cells by GSNO–Sepharose chromatography

HeLa cell nuclear extracts were subjected to chromatography on GSNO–Sepharose. The column flow-through (unbound) and eluate (bound) were analysed for AP-1 (**A**), NF-κB (**B**), CREB (**C**) and CP-1 (**D**) DNA-binding activities by EMSA, as described in the Experimental section. Specificity of protein–DNA complex formation was confirmed by homologous and heterologous competition with a 50-fold excess of the indicated unlabelled oligonucleotides. The gels shown are representative for four similar experiments.

As yet, the only transcriptional activators known to be S-glutathionylated by GSNO *in vitro* are the AP-1 subunit c-Jun [21] and the NF-κB subunit p50 (the present study). Analysis of the GSNO–Sepharose column flow-through and eluate by EMSA

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revealed that both AP-1 (Figure 5A) as well as NF-κB (Figure 5B) DNA-binding activities bound almost quantitatively to the nitrosothiol matrix. Presence of c-Jun and p50 in the GSNO–Sepharose eluate was confirmed by Western blot (results not shown). We further analysed the column fractions obtained for DNA-binding activity of CREB, a transcription factor whose DNA-binding site is homologous to that of c-Jun. As shown in Figure 5(C), the column eluate was enriched in protein(s) which specifically bound to the consensus DNA sequence for CREB. In contrast, DNA-binding activity of the transcription factor CP-1, a cysteine-containing CCAAT-binding protein [51], was almost exclusively detected in the column flow-through (Figure 5D). Similarly, DNA-binding activity of Sp1, which binds to DNA through a Cys_2/His_2 zinc-finger motif [32], was not enriched in the GSNO–Sepharose eluate (results not shown).

DISCUSSION

The S-nitrosothiol GSNO has been implicated in the regulation of cellular functions through the modification of functionally critical cysteine residues in target proteins [10]. Recent data suggest that GSNO may play a role in the regulation of transcription by reversibly transferring its glutathione moiety to a cysteine residue in the DNA-binding site of the transcription factor c-Jun *in vitro* [21]. As yet, it remains unknown whether this modification is unique to c-Jun or if it is shared by other proteins. We addressed this issue using GSNO–Sepharose as a tool which should allow the isolation of proteins through site-specific GSNO–protein interactions, leading to the formation of a mixed disulphide with the immobilized nitrosothiol.

First, to investigate the potential usefulness of GSNO–Sepharose as a probe of GSNO-induced S-glutathionylation, we characterized the interaction between the matrix and the DNA-binding domain of c-Jun. We provide experimental evidence that c-Jun binds to GSNO–Sepharose through the formation of a mixed-disulphide bond. Maximal protein binding to the matrix, however appears rather low comparing the maximal number of c-Jun molecules bound/ml of Sepharose (12 nmol) with the number of S-nitrosothiol groups contained in 1 ml of the matrix (2.4 μmol). This corresponds to a reaction of only 0.5 % of the total S-nitrosothiol groups with protein thiols under saturating concentrations. This low yield can be explained partly by the stringent assay conditions. We performed the binding assays in the presence of 0.25 M NaCl and 0.01 % NP40 to suppress the non-specific formation of mixed disulphides. When the assays were performed in the absence of NP40 and NaCl, binding of c-Jun was increased approx. fivefold (results not shown). Furthermore, the low yield may be due to the low capacity of native proteins to bind to the Sepharose matrix because of steric constraints. In support of this, it was found that 1 ml of activated thiol–Sepharose, the starting material for the preparation of GSNO–Sepharose, bound a maximum of approx. 10–50 nmol of native proteins such as Hb, erythrocyte band III protein and collagen [52–54], whereas up to 200 nmol of denatured proteins and peptides could be retained by 1 ml of the same matrix [55]. Of note is the fact that GSNO is coupled directly, i.e. without the introduction of spacer groups, to the Sepharose carrier through the terminal amino group of its γ-glutamate moiety (Scheme 1). It is conceivable, therefore, that the matrix might impair protein–GSH interactions and, therefore, will not work with proteins whose GSNO-sensitive cysteine residues are buried within the native-folded-protein structure. It remains to be investigated whether the introduction of flexible spacer groups could improve the efficacy of GSNO–Sepharose as a mixed-disulphide-forming matrix.

In accordance with previous data on c-Jun thiolation [21,23], we found that this protein bound to GSNO–Sepharose through the formation of a mixed disulphide with a single cysteine residue which was identified as cysteine-269 by site-directed mutagenesis. It is worth noting that site-specificity of c-Jun S-glutathionylation was not due to lack of accessibility or reactivity of the second pair of cysteine residues (cysteine-320) contained in the c-Jun homodimer. This conclusion is supported by the observation that cysteine-320 was quantitatively converted into a mixed disulphide when diamide was used to induce mixed-disulphide formation. Similarly, when the thiol group of glutathione–Sepharose was activated with 2,2′-dipyridyl disulphide instead of NO, both cysteine-269 and cysteine-320 bound equally to the thiol matrix, indicating that lack of c-Jun binding to GSNO–Sepharose through cysteine-320 was not due to steric constraints. Covalent protein binding to GSNO–Sepharose, therefore, apparently does not just simply detect the presence of reactive and freely accessible cysteine residues but rather reflects the particular reactivity of a protein thiol in terms of its susceptibility to undergo GSNO-induced S-glutathionylation. The basic environment of c-Jun cysteine-269 has been suggested to account for the particular reactivity and redox-sensitivity of this thiol [56]. Thus to investigate whether site-specific binding to GSNO–Sepharose is unique to c-Jun, or is shared by other DNA-binding proteins, we searched for another model transcription factor which contains functionally important cysteine residues but displays marked structural differences to c-Jun. Similar to c-Jun, the NO- and redox-sensitivity of the NF-κB subunit p50 maps to a single cysteine residue (cysteine-62) in its DNA-binding site [26,33]. Moreover, it has been speculated that redox-regulation of NF-κB DNA binding in intact cells could involve S-glutathionylation of cysteine-62 [57]. In the present study, we show that p50 can be precipitated by GSNO–Sepharose and that covalent binding to the immobilized nitrosothiol critically depends on one single cysteine residue. Site-directed mutagenesis revealed that cysteine-62 accounts for covalent binding of the NF-κB subunit to the matrix. [^{3}H]GSH-incorporation assays corroborate the suggestion that cysteine-62 of p50 may represent a novel target for GSNO-induced S-glutathionylation *in vitro*.

The observation that GSNO–Sepharose binds c-Jun and p50 through site-specific disulphide formation with a single thiol suggests that this matrix may permit the isolation of GSNO target proteins from a mixture of cysteine-containing proteins. As a first attempt to address this issue, we investigated the capacity of GSNO–Sepharose to precipitate c-Jun and p50 from a mixture of purified proteins which additionally contained BSA and Hb. It is noteworthy that both BSA and Hb contain reactive cysteine residues which are quantitatively thiolated by GSH/diamide and exhibit some reactivity in terms of mixed-disulphide formation with both solute and immobilized GSNO. When p50 and c-Jun were competing with BSA and Hb for covalent binding to GSNO–Sepharose, the NO-activated thiol matrix precipitated c-Jun and p50 with high specificity (Figure 2). Similarly, a number of other proteins have been shown in the present study to contain reactive cysteine residues that can be S-glutathionylated by GSNO *in vitro*. The data presented in Table 1 and Figure 2 suggest that, in general, GSNO–Sepharose binding reflects the susceptibility of a given protein to react with GSNO, and is not just simply the presence of cysteine residues that can be modified by diamide-induced S-glutathionylation. However, alcohol dehydrogenase and GPDH did not bind to the GSNO matrix despite their capacity to undergo some GSNO-induced S-glutathionylation in solution. As discussed above, this may be explained by steric constraints imposed by the structure of the

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proteins which does not allow access of the Sepharose to cysteine residues, that are not located on the protein surface. Moreover, in some cases, the interpretation of quantitative data as presented in Table 1 is compromised by the lack of information about the contribution of individual cysteine residues to the overall number of GSH molecules incorporated per protein. The targeted cysteine residues in c-Jun and p50 have been identified by site-directed mutagenesis, and it can be concluded that a single cysteine residue accounts for the major part of the observed S-glutathionylation in these proteins. In the case of alcohol dehydrogenase and GPDH, we found that 17 and 15 cysteine residues respectively can be modified by diamide-induced S-glutathionylation. However, only a total of 0.4 of these residues, which are available for mixed-disulphide formation, can be glutathionylated by GSNO. If GSNO-induced S-glutathionylation were not localized to one single protein thiol (i.e. 0.4 mol GSH/cysteine residue), but equally distributed over all of these residues (i.e. 0.03 mol GSH per cysteine residue), the degree of thiolation at the level of individual protein thiols would be even lower than that observed for BSA and Hb (0.07 and 0.21 mol GSH/mol protein respectively) and, therefore, might be too low to result in detectable GSNO–Sepharose binding. Definite conclusions, however, await further studies with the respective cysteine mutants.

So far, little is known about the molecular basis of GSNO-induced protein thiolation and about possible factors that confer site specificity to this modification. Initially, it was proposed that NO induces S-glutathionylation via a two-step mechanism which involves S-nitrosation of the target protein thiol and subsequent replacement of the NO group by the glutathionyl moiety [13]. Our data on the reaction of S-nitrosated BSA with GSH–Sepharose (Figure 4) confirm that this pathway may in fact lead to the formation of a mixed disulphide between a protein and immobilized glutathione. However, the observation that BSA neither incorporated GSNO in solution nor bound to GSNO–Sepharose render it unlikely that such a mechanism accounts for the apparent selectivity and specificity of GSNO-induced mixed-disulphide formation observed here. More recently, it has been suggested that GSNO-induced protein glutathionylation involves a nucleophilic attack of the protein thiol on the sulphur of GSNO [14,17,20,21]. Thus the site-specificity of GSNO-induced thiolation may be explained in part by the basic micro-environment of the target cysteine which lowers its pK_a value and thus increases the nucleophilicity and reactivity of the sulphur atom [58]. A comparison of the sequences and, where available, tertiary structures of the proteins that were found in the present study, both to incorporate GSH upon GSNO treatment and to bind to GSNO Sepharose, confirms that these proteins contain at least one cysteine residue that is surrounded by basic amino acids (results not shown). Finally, previous work on the molecular basis of protein S-glutathionylation suggests that the incorporation of GSH may be facilitated by protein–GSH interactions [22]. In support of this suggestion, kinetic data on the inhibition of aldose reductase by GSNO-mediated thiolation revealed that the reaction of the enzyme with GSNO is in fact preceded by tight-binding of the nitrosothiol to the active site of the enzyme [14]. Furthermore, molecular modelling of S-glutathionylated c-Jun would be compatible with stabilization of the mixed disulphide by electrostatic protein–GSH interactions, involving positively charged arginine and lysine residues in the protein and negatively charged carboxyl groups in the GSH moiety [23]. Similarly, a cluster of positively charged amino acids was proposed to facilitate S-glutathionylation of protein tyrosine phosphatase-1B [59]. We addressed this issue using GSNO ethyl ester–Sepharose as a probe for S-nitrosothiol-induced disulphide formation and show that loss of the positive charges at the carboxyl moieties of the GSH molecule reduces binding of some proteins to the matrix (Figure 4). Although it cannot be excluded that impaired binding of proteins to GSNO ethyl ester–Sepharose is caused by steric constraints imposed by the introduction of the ethyl ester groups, our results are compatible with the view that ionic interactions may contribute to GSNO-dependent S-glutathionylation.

One further step to establish immobilized GSNO as a tool to isolate and identify novel targets of GSNO-induced S-glutathionylation from more complex protein mixtures was to subject HeLa cell nuclear extracts to covalent GSNO–Sepharose chromatography. [^{3}H]GSH-incorporation assays revealed that the proteins isolated by GSNO–Sepharose exhibit a > 10-fold higher capacity to undergo GSNO-induced mixed disulphide formation than the column flow-through (Table 3). Importantly, diamide-induced [^{3}H]GSH incorporation was of the same order of magnitude in both protein fractions, indicating that the observed differences, with regard to GSNO-induced thiolation, were not due to a difference in the number of reactive thiols in the compared protein fractions. In keeping with the idea that diamide-induced [^{3}H]GSH incorporation into a protein reflects the total number of cysteine residues that are accessible to mixed-disulphide formation, and assuming that the cysteine residues that undergo GSNO-induced [^{3}H]GSH incorporation are contained within this group of diamide-sensitive thiols (Table 1), the ratio of GSNO- versus diamide-induced [^{3}H]GSH incorporation should be a measure for the relative number of cysteine residues that can be modified by GSNO-induced S-glutathionylation. Thus from the data presented in Table 3, it can be estimated that in the GSNO–Sepharose flow-through one out of 22 diamide-sensitive cysteine residues (i.e. 3 versus 67 nmol [^{3}H]GSH/mg protein) is modified by GSNO-mediated glutathionylation, whereas in the bound protein fraction one out of three diamide-sensitive cysteine residues forms a mixed disulphide with GSH in response to GSNO treatment (i.e. 37 versus 122 nmol [^{3}H]GSH/mg protein). Thus the nitrosated matrix apparently selects for protein thiols susceptible to GSNO-induced mixed-disulphide formation (i.e. GSNO-induced thiolation) and not just simply for reactive thiol groups (i.e. diamide-induced thiolation). This is also reflected by the purification factors for proteins in the eluate that are susceptible to diamide and GSNO-inducible S-glutathionylation (2 versus 19 respectively). A potential limitation of this technique, however, is its poor yield. As evident from the data presented in Table 3, only 50 μg and 8 % of the total amount of HeLa cell protein thiols that are susceptible to GSNO-dependent S-glutathionylation can be precipitated by the matrix. Saturation of the matrix can be excluded as an explanation because, as estimated from Figure 1, the approx. 5 ml of Sepharose used in these purifications should bind about 1 mg of protein. One possible explication for this low yield may be residual 2-mercaptoethanol contained in the incubation. To inhibit oxidation of cysteine residues during the preparation of the cell extract, the isolation buffers contained 0.1 % 2-mercaptoethanol (see the Experimental section), which could both degrade the GSNO bond and reduce mixed disulphides. Although, the cell extract was diluted 100-fold in the final suspension with the GSNO–Sepharose, it should be taken into account that the final concentration of the thiol in the binding assay is approx. 100 μM. Although addition of 100 μM 2-mercaptoethanol to incubations of purified c-Jun and p50 with GSNO–Sepharose did not significantly lower the yield of bound proteins (results not shown), we cannot exclude the possibility that the same concentration of 2-mercaptoethanol interferes with binding of HeLa cell proteins. Nevertheless, our data indicate

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that nuclear proteins constitute a pool of, as yet, unidentified proteins which are susceptible to GSNO-induced thiolation. We showed that purified c-Jun and p50, which are subunits of the AP-1 and NF-κB transcription factors respectively, bind to immobilized GSNO and can be S-glutathionylated by free GSNO *in vitro*. Consistent with these findings, analysis of the HeLa cell nuclear extracts by covalent GSNO–Sepharose chromatography and EMSA revealed that the DNA-binding activities of AP-1 and NF-κB can be precipitated by GSNO–Sepharose (Figure 5). The specificity of the nitrosothiol matrix was confirmed by the observation that the DNA-binding activity of CREB, which in terms of its DNA-binding site can be considered as a structural homologue of c-Jun, was retained by the matrix, whereas the transcription factors CP-1 and Sp1 did not bind. It is noteworthy that both CP-1 and Sp1 contain reactive cysteine residues in their DNA-binding domains.

In conclusion, these data support the potential applicability of covalent GSNO–Sepharose chromatography to the isolation of cysteine-containing cellular proteins, including nuclear transcription factors, that can be modified by GSNO-induced S-glutathionylation.

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