Glutathionylation of the p50 Subunit of NF-κB: a Mechanism for Redox-Induced Inhibition of DNA Binding†

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ABSTRACT: The cellular redox status can modify the function of NF-κB, whose DNA-binding activity can be inhibited by oxidative, nitrosative, and nonphysiological agents such as diamide, iodoacetamide, or *N*-ethylmaleimide. This inhibitory effect has been proposed to be mediated by the oxidation of a conserved cysteine in its DNA-binding domain (Cys62) through unknown biochemical mechanisms. The aim of this work was to identify new oxidative modifications in Cys62 involved in the redox regulation of the NF-κB subunit p50. To address this problem, we exposed p50, both the native form (p50WT) and its corresponding mutant in Cys62 (C62S), to changes in the redox pair glutathione/glutathione disulfide (GSH/GSSG) ratio ranging from 100 to 0.1, which may correspond to intracellular (patho)physiological states. A ratio between 1 and 0.1 resulted in a 40−70% inhibition of the DNA binding of p50WT, having no effect on the C62S mutant. Mass spectrometry studies, molecular modeling, and incorporation of ^{3}H-glutathione assays were consistent with an S-glutathionylation of p50WT in Cys62. Maximal incorporation of ^{3}H-glutathione to the p50WT and C62S was of 0.4 and 0.1 mol of ^{3}H-GSH/mol of protein, respectively. Because this covalent glutathione incorporation did not show a perfect correlation with the observed inhibition in the DNA-binding activity of p50WT, we searched for other modifications contributing to the maximal inhibition. MALDI-TOF and nanospray-QIT studies revealed the formation of sulfenic acid as an alternative or concomitant oxidative modification of p50. In summary, these data are consistent with new oxidative modifications in p50 that could be involved in redox regulatory mechanisms for NF-κB. These postranslational modifications could represent a molecular basis for the coupling of pro-oxidative stimuli to gene expression.

NF-κB is an inducible transcription factor that belongs to the NF-κB/Rel family of transcription factors, characterized by an N-terminal region of approximately 300 amino acids known as the Rel homology domain. This domain exhibits a characteristic sequence motif with one cysteine and three arginine residues in the DNA-binding region (*1*−*4*). NF-κB may be constituted by heterodimers or homodimers of the Rel family of proteins. In many cells, the most abundant complex is a heterodimer formed by the proteins p50 and p65, although p50/p50 homodimers are also capable of binding to DNA (*5*−*7*).

This factor is regulated in part by its cellular localization. In the cytosol, it is found as an inactive complex bound to the inhibitory molecule IκB. The degradation of IκB is, by itself, a highly regulated phenomenon in which a set of specific kinases participate in the phosphorylation of IκB (*8*). This ultimately leads to the degradation of IκB by the proteasome and the eventual translocation of NF-κB to the nucleus. NF-κB is involved in the expression of a wide variety of cellular and viral genes, such as interleukin 2, nitric oxide synthase 2, or tumor necrosis factor α, which participate in several crucial biological pathways including inflammation and protection from apoptosis (*9*). Although the activation of NF-κB through the release and degradation of IκB-α has been reported to occur under oxidative conditions in some cells (*10*, *11*), the binding of NF-κB to its cognate cis regulatory element has been shown to be dependent on the integrity of a cysteine residue (Cys62) which must be in a reduced state (*12*, *13*). This residue is surrounded by a cationic environment that renders the thiol group highly reactive and particularly susceptible to oxidation. The formation of an inter- or intramolecular disulfide involving Cys62 has been suggested as one mechanism that could mediate the observed DNA-binding inhibition. Nevertheless, only about 10% of the molecule is in a dimer state

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when the protein is incubated in the presence of oxidants (*12*). This implies that the remaining 90% is probably in a different oxidative state and, hence, that other modifications, accounting for the inhibition in DNA binding, are very likely to occur in such conditions.

The intracellular redox status appears to be a critical determinant of NF-κB activation (*14*, *15*). Within the cellular context, the redox status depends on the relative amounts of the oxidized and reduced partners of major redox regulators such as glutathione (i.e., the redox pair glutathione/glutathione disulfide (GSH/GSSG)).[11] This molecule is an ubiquitous cellular sulfhydryl tripeptide, which plays an important role in maintaining cellular defenses under oxidative stress. In normal conditions, GSH prevails over GSSG in mammalian cells (*16*). Thus, the oxidation of a limited amount of GSH to GSSG can dramatically change this ratio and affect the redox status within the cell. In these conditions of moderate oxidative stress, thiol groups of intracellular proteins can be modified by the reversible formation of mixed disulfides between protein thiols and low-molecular-mass thiols such as glutathione, a process known as S-glutathionylation (*16*). Furthermore, under moderate conditions of oxidative stress, thiol groups (−SH) can gain oxygen atoms to yield sulfenic (−SOH), sulfinic ($-SO_2H$), or sulfonic ($-SO_3H$) moieties. Only the oxidation to sulfenic acid is reversible but represents an unstable modification that requires the absence of vicinal Cys−SH groups for its stabilization (*17*). This reaction could represent another mechanism by which oxidative stress may regulate the function of a protein.

In this work, we demonstrate for the first time, using an in vitro model, that the p50 subunit of NF-κB undergoes S-glutathionylation in the Cys62 residue of its DNA-binding domain and that this modification can reversibly inhibit its DNA-binding activity. We also show that the formation of a protein sulfenate in the same residue is another alternative modification whose contribution to the p50 DNA-binding inhibition seems to be less important. These results provide evidence for new modifications that could operate in the NF-κB-mediated regulation of gene expression under oxidative stress.

MATERIALS AND METHODS

Materials. Tritium-labeled glutathione (^{3}H-GSH; 45−50 Ci/mmol, ∼0.02 mM) was from DuPont NEN and adjusted to a final concentration of 10 mM by the addition of 10 volumes of an 11 mM solution of unlabeled GSH (Sigma, St. Louis, MO). ^{3}H-GSSG was prepared by the oxidation of ^{3}H-GSH as described (*18*). GSH (free acid, SigmaUltra), GSSG (free acid, SigmaUltra), and dimedone were from Sigma-Aldrich (Milwaukee, WI).

Expression and Purification of Wild-Type and Mutant p50 DNA Recombinant Proteins. The fragment corresponding to residues 36−385 of the protein KBF1, corresponding to the binding domain of the p50 subunit of NF-κB (EMBL accession number M55643), was obtained by a polymerase chain reaction (PCR) using primers with additional restriction enzyme cleavage sites. The primers were 5′-CGG GGA TCC GCA CTG CCA ACA GCA GAT-3′ and antisense 5′-CTC CCT AAG CTT CCA GCT CCG GCA CCA CTA-3′. PCR products were digested with *Bam*HI and *Hind*III and ligated into the expression vector pQE-30 (Qiagen). The plasmid with the insert was transformed into *Escherichia coli* (M15-[pREP], Qiagen) and the product obtained was verified by dideoxynucleotide sequencing. The cysteine of this fragment (Cys62) was mutated to serine by PCR mutagenesis using the wild-type fragment as the template and the same primers described above with an additional primer (5′-CTC AAG CTT GTA CCC ATG GGA TGG GCC TTC CGA-3′) which contained the mutation and introduced a new and unique restriction site *Nco*I. The mutation was confirmed by automatic sequencing.

For the expression of the fragment, *E. coli* transformed with the recombinant plasmid was grown and processed as described (*19*) and was frozen at −80 °C. The frozen-cell suspension was thawed rapidly at 37 °C and stirred for 1 h at room temperature. Subsequently, 2−3 mL (bed volume) of a nickel−chelate resin (Ni−NTA, Qiagen) was equilibrated with buffer A (50 mM phosphate buffer, pH = 8.0, containing 350 mM NaCl and 0.1% (v/v) 2-mercaptoethanol) with a supplement of 10 mM imidazole, and the suspension was stirred for 2 h at room temperature. The mixture was poured into a chromatography column (inner diameter of 1.5 cm) and washed with 15 bed volumes of buffer A (10 mM imidazole), followed by 5 bed volumes of buffer A with 50 mM imidazole. Finally, the protein was eluted with 5 volumes of buffer A with 250 mM imidazole. The eluted protein was preserved with a buffer containing 0.01% NP-40 and 5% glycerol and was frozen at −80 °C. The purity of the obtained protein preparations was estimated at >95% as judged by Coomassie-stained SDS gels. Concentrations of the purified protein, determined by amino acid analysis and absorbance at 280 nm, were in the range of 0.4−0.5 mM.

Electrophoresis Mobility Shift Assay (EMSA). DNA-binding domains of wild-type or C62S mutant p50 at 10 μM were preincubated in buffer B (20 mM Tris/HCl buffer, pH = 7.5, containing 50 mM NaCl, 5 mM $MgCl_2$, 1 mM EDTA, 5% glycerol, and 0.01% NP-40) in the presence of different GSH/2GSSG ratios ranging from 100 to 0. Two microliters of the preincubation mixture was diluted with 15 μL of buffer B plus 0.2 mg/mL of bovine serum albumin, 0.1 mg/mL of poly(dI-dC), and GSH/GSSG at the same ratio which was established during preincubation. After that, 3 μL of the ^{32}P-radiolabeled double-stranded NF-κB oligonucleotide 5′-GGA GAG GGG ATT CCC TGC G-3′ from the cyclooxygenase-2 promoter, comprising bases −452 to −433 from the transcriptional start site in the reported sequence (*20*), was added, and the resulting sample was incubated for 30 min at room temperature to allow for the binding of DNA prior to electrophoresis. Electrophoresis, gel processing, and reversibility studies of S-glutathionylation were carried out as previously described (*19*).

Quantitative Determination of p50 S-Glutathionylation. S-Thiolation of p50 was determined as previously described (*19*). The DNA-binding domains of the wild-type and mutant proteins were incubated at 10 μM for 1 h at 37 °C in buffer B and in the presence of different ratios of ^{3}H-GSH and ^{3}H-

[11] Abbreviations: GSH, glutathione; GSSG, glutathione disulfide; DTT, dithiotreitol; NEM, *N*-ethylmaleimide; HPLC, high-pressure liquid chromatography; EMSA, electrophoretic mobility shift assay; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; C62S, human p50 DNA-binding domain with a Cys62−Ser mutation.

GSSG. The total concentration of the GSH equivalents was maintained at 3 mM in the incubation mix. The glutathionylated protein was precipitated with 1 mL of 10% ice-cold trichloroacetic acid (*18*). The incorporation of ^{3}H-GSH to the protein was quantified by liquid-scintillation counting.

Detection of S-Glutathionylated p50 by Mass Spectrometry (MS). The DNA-binding domains of wild-type and mutant p50 were incubated with 1.5 mM GSSG or 1 mM DTT for 30 min at 37 °C and subsequently diluted in a 20 mM sodium phosphate buffer, pH = 7.5, in the presence of 4 mM urea and *N*-ethylmaleimide (NEM) in 10 times the excess over the total number of thiols. After 30 min at 37 °C, trypsin (2.3 mg/mL) was added to get a final protein/trypsin ratio of 1:20. The mixture was incubated overnight at 37 °C. The tryptic fragments were separated by reverse-phase HPLC on a Vydac 218TP5415 column (4.6 × 150 mm) using an AKTÄ prime system (Pharmacia Biotech). The initial mobile-phase composition was held at 100% water containing 0.1% trifluoroacetic acid (TFA) for 10 min followed by a gradient elution (from 0.1% TFA to 0.1% TFA−90% acetonitrile) for 100 min. The eluted fractions were dried and resuspended in 10 μL of methanol/water (1:1) containing 0.1% formic acid. Aliquots of 0.5 μL were analyzed by MALDI-TOF, and the rest were reserved for nanospray-QIT.

Analysis by MALDI-TOF MS was performed using either a Kompact Probe instrument (Kratos-Shimazdu, Manchester, U.K.), equipped with an extended flight tube of 1.7 m, delayed extraction, and operating in linear mode or a Reflex II instrument (Bruker, Bremen, Germany), also operating in linear mode. A total of 0.5 μL of the fractions to be analyzed was applied onto the target and dried out. Then, 0.5 μL of a saturated α-cyano-4-hydroxycinnamic acid matrix in water/acetonitrile (1:1) containing 0.1% TFA was added and dried out. The calibration was performed externally by the use of a set of synthetic peptides.

Analysis by nanospray-ion-trap (nESI-IT) MS was performed using an ion-trap mass spectrometer model LCQ (Finnigan, ThermoQuest, San Jose, CA) equipped with a nanospray interface, exactly as described previously (*21*).

Detection of Sulfenic Acid in Cys62. Wild-type and mutant p50 domains were incubated in the presence of 1.5 mM GSSG and 1 mM dimedone for 1 h at 37 °C and subsequently diluted in a 20 mM sodium phosphate buffer, pH = 7.5, in the presence of 4 mM urea and NEM in 10 times the excess over the total number of thiols. p50 was then subjected to tryptic digestion, HPLC, and MS analysis, as described above.

Molecular Modeling. The model of the three-dimensional (3D) structure of S-glutathionylated p50 was built from the 2.3-Å resolution X-ray cocrystal structure of the NF-κB homodimer *(22)*. The Cartesian coordinates used were from the Brookhaven Protein Data Bank (PDB code 1NFK). For the simulation of p50 S-glutathionylation, the glutathione adduct was arbitrarily positioned into one of the equivalent protein monomers via a disulfide bridge to the target Cys62. The flexible loops L1−5 of the p50 protein and the glutathione adduct of such a modified structure were subjected to energy minimization until convergence, using a combination of steepest-descent and conjugate-gradient algorithms. The energy calculations were carried out under the AMBER force field (*23*). Computations were performed on a Power Challenge R10000 by using the BIOSYM software package, Release 95.0 (Molecular Simulations, Inc., San Diego, CA).

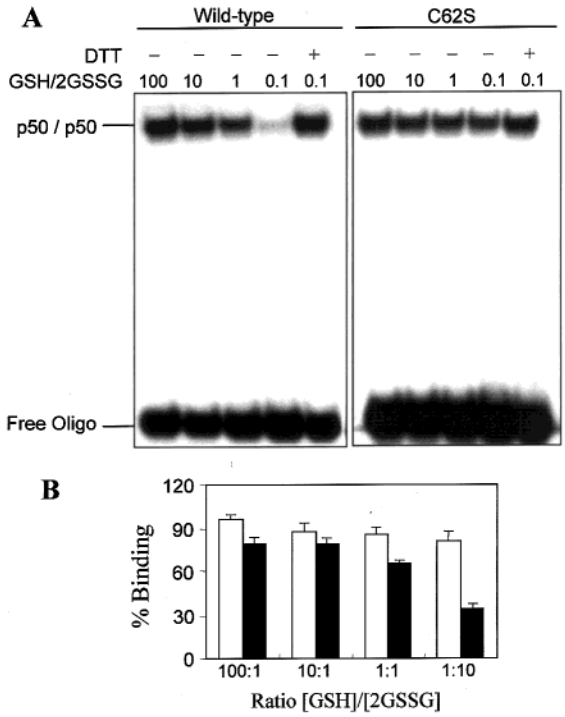

FIGURE 1: DNA binding of p50 is regulated by redox conditions: (A) Purified DNA-binding domains of wild-type and C62S mutant p50 (10 μM) were preincubated in the presence of different ratios of reduced and oxidized glutathione (expressed as GSH/2GSSG ratios of 100, 10, 1, and 0.1). The concentration of glutathione equivalents was maintained at 3 mM, and the DNA-binding activity of p50 was assayed by EMSA as described in the Materials and Methods section. The samples were incubated in the presence or absence of 1 mM DTT where indicated. The autoradiography is representative of six different experiments. (B) Densitometry values of the DNA-binding experiments: (filled bars) wild-type p50 and (empty bars) C62S mutant.

RESULTS

p50 DNA-Binding Activity Inhibited by Changes in the Redox Potential. Given its physiological relevance, we chose the GSH/2GSSG redox pair to establish a gradient of oxidative stress, which would modify the DNA-binding activity of p50. The DNA-binding domain of p50 was preincubated in the presence of different GSH/2GSSG ratios (100−0.1) but maintaining the total amount of glutathione equivalents at 3 mM. The DNA-binding activity was subsequently analyzed by EMSA (*19*). As shown in Figure 1A (left panel), the DNA-binding activity of p50 was significantly inhibited when the ratio between reduced and oxidized glutathione was diminished. The inhibition values were 26 ± 4%, 34.5 ± 2.5%, and 66 ± 2.5% (n = 7) at the ratios of 10, 1, and 0.1, respectively (Figure 1B). The reducing agent DTT was able to prevent the observed inhibition, suggesting that a sulfhydryl modification is involved in the effect observed on the DNA-binding activity of p50 by the increase in oxidative experimental conditions.

The wild-type protein contains seven cysteine residues located in its DNA-binding domain. Cys62 is believed to be the redox "sensor" of the p50 molecule. To find out if this cysteine was also responsible for the inhibition observed in our experimental conditions, we performed EMSA studies

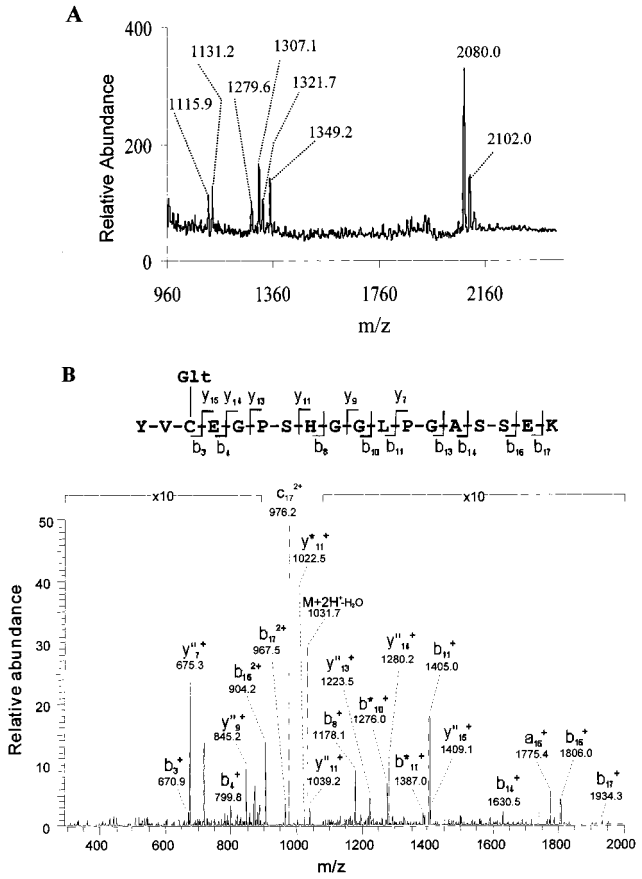

FIGURE 2: Detection of S-glutathionylation in Cys62 by matrix-assisted laser-desorption ionization time-of-flight (MALDI-TOF) and nanospray quadrupole ion-trap (nanoES-QIT) MS. p50 was incubated under oxidative stress and digested with trypsin, and the generated peptides were separated by HPLC. (A) MALDI-TOF mass spectrum of an HPLC fraction corresponding to 29−31 min. Peptides with 2080 and 2102 Da corresponded to glutathione-modified forms of the tryptic peptide YVCEGPSHGGLPGASSE and its sodium adduct, respectively. (B) Nanospray-QIT MS/MS spectrum of the modified peptide. The ion chosen as the precursor was the double-charged species of the peptide. The peptide sequence and the assignment of the fragmentation series are also indicated, according to the nomenclature of Roepstorff and Fohlman (*43*). "Glt" represents the glutathione moiety covalently attached to the cysteine.

with the mutant p50 (C62S) protein in the same experimental conditions as the wild-type protein (Figure 1A, right panel). In these conditions (Figure 1B), a certain inhibition in the DNA-binding activity was observed (inhibition values of 12 ± 5.5%, 14 ± 4.5%, and 19 ± 6.4% ($n = 7$) at the GSH/GSSG ratios of 10, 1, and 0.1, respectively). This result strongly suggests that the reversible inhibition of p50 DNA-binding activity associated to changes in the redox potential can be assigned preferentially to Cys62 but that other modified residues could also contribute.

Detection of S-Glutathionylation in the Cys62 of the p50 Protein by MS. Studies by our group with a sepharose matrix modified with S-nitrosoglutathione (GSNO) had previously suggested the formation of a mixed disulfide between glutathione and the SH− group of Cys62 induced by GSNO (*24*). To provide biochemical evidence for S-glutathionylation in Cys62, we used MS. Figure 2A shows the mass spectra of an HPLC fraction obtained by the tryptic digestion of S-glutathionylated p50. The eluted fractions were dried and analyzed by MALDI-TOF MS. One fraction (29−31 min) contained a peak whose mass could correspond to the theoretical value of a tryptic fragment plus the mass of glutathione. This fraction included a peptide with an average mass of 2080 Da, as well as its sodium adduct at 2102 Da, which was in agreement with the theoretical mass of the peptide YVCEGPSHGGLPGASSEK (monoisotopic mass of 1774.8) bearing a glutathione adduct (monoisotopic mass of 2079.9). To confirm the identity of this modification, this peptide was analyzed by nanospray-QIT. A high-resolution scan ("ZoomScan", not shown) allowed for the identification of a double-charged peptide with a mass of 1040.4 Da, which corresponded to a peptide with a monoisotopic mass of 2079.8 Da ($M + H^+$) in very good agreement with the theoretical value. This species was also subjected to MS/MS fragmentation (Figure 2B). In the partial peptide fragmentation obtained, the fragment spectrum was entirely consistent not only with the expected peptide sequence but also with the addition of a glutathione moiety at the cysteine located in position 3 in the peptide. Thus, all of the fragments corresponding to the *b* series, starting from b_3, showed an increment in the mass of glutathione, while all of the fragments corresponding to the *y* series, including y_{15}, were unmodified. The mass corresponding to the modified peptide was not observed in the controls with the mutant protein of the DTT-treated samples (not shown). Hence, these data demonstrated the formation of a mixed disulfide with glutathione within Cys62 of p50.

Quantitative Determination of p50 S-Glutathionylation. To study the extent of p50 S-glutathionylation induced by different ratios of the glutathione redox couple, the DNA-binding domain was incubated with different GSH/2GSSG ratios. As shown in Figure 3A, p50 is able to incorporate 0.4 ± 0.003 mol of GSH/mol of protein ($n = 8$) at a ratio of 1 of ^{3}H-GSH/^{3}H-GSSG. At most pro-oxidative conditions (0.1 of ^{3}H-GSH/^{3}H-GSSG), the degree of incorporation decreased to 0.3 ± 0.02. These data correlate well with our previous results on S-thiolation induced by GSNO (*24*). Moreover, this incorporation is time-dependent (not shown) and completely reversible in the presence of DTT.

To estimate the specific S-glutathionylation which can be assigned to Cys62, we compared the ^{3}H-GSH incorporation into the wild-type and mutant forms of p50. As shown in Figure 3B, the p50 S-glutathionylation in Cys62 is approximately 0.4 ± 0.03 mol of GSH/mol of protein, whereas in the C62S mutant, the incorporation was about 0.13 ± 0.01 mol of GSH/mol of protein, indicating that glutathione is essentially incorporated into Cys62. However, a minor proportion may be incorporated into other residue(s), because S-glutathionylation of Cys135 was also detected by MS assays (data not shown). The preferential incorporation of glutathione into Cys62 is supported by the fact that the difference between incorporations of ^{3}H-GSH to the wild-type and C62S p50 is approximately 0.3 (0.26 ± 0.003 mol of GSH/mol of protein). These data are consistent with the notion that p50 undergoes S-glutathionylation in Cys 62 with nonequimolecular stoichiometry and that this modification is related in part with the previously observed inhibition of its DNA-binding activity.

Additional Biochemical Modifications Detected in the p50 DNA-Binding Domain under Oxidative Conditions. The formation of an inter- or intramolecular disulfide between

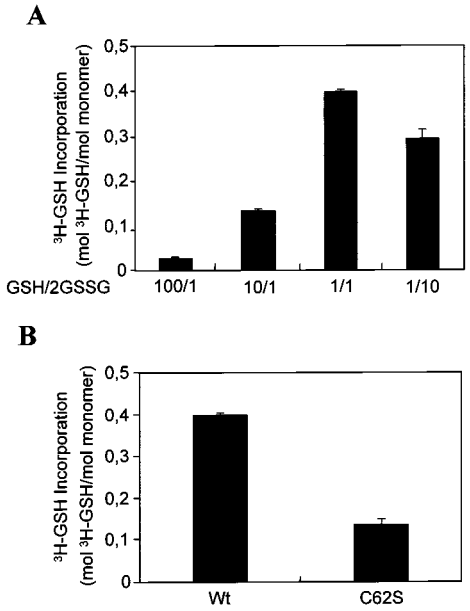

FIGURE 3: S-Glutathionylation of p50 depends on oxidative conditions. (A) The DNA-binding domain of p50 (10 μM) was incubated in a Tris/HCl buffer for 30 min at 37 °C in the presence of different ratios of reduced and oxidized ^{3}H-labeled glutathione, and the amount of ^{3}H-GSH that was incorporated into the protein was determined (see Materials and Methods). Values are expressed as mol of glutathione/mol of protein monomer. (B) Wild-type and C62S mutant p50 (10 μM) were incubated for 30 min at 37 °C with 1.5 mM ^{3}H-GSH and 0.75 mM ^{3}H-GSSG and then assayed for the incorporation of ^{3}H-GSH into the protein, as previously described. Values are expressed as mol of glutathione/mol of protein (monomer). Data are mean values ± SEM from at least six independent experiments.

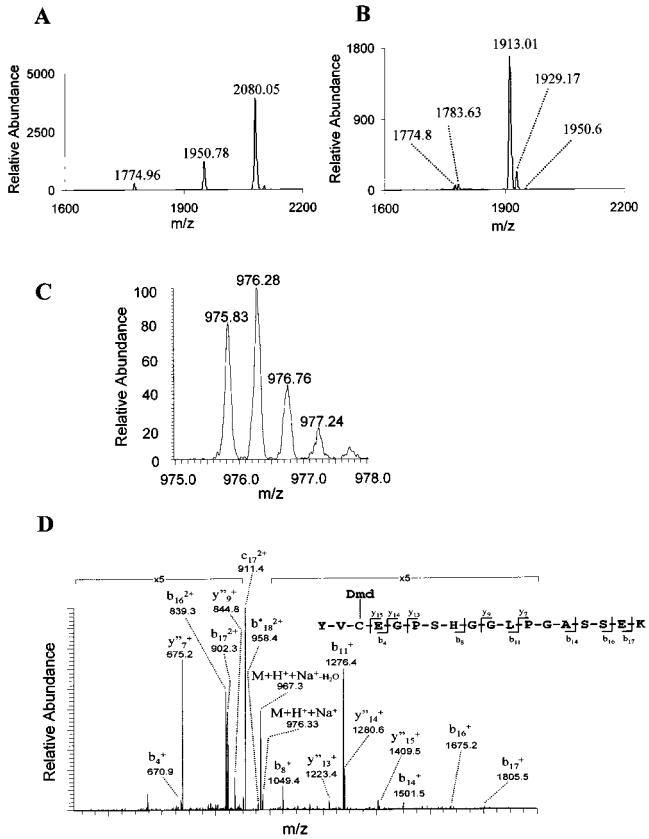

FIGURE 4: Detection of a sulfenic acid in Cys62 under oxidative conditions by MS. The DNA-binding domain of p50 was incubated under intense oxidative conditions, in the presence of the sulfenic-specific reactive dimedone and digested with trypsin, and the generated peptides were separated by HPLC. (A) MALDI-TOF mass spectrum of the HPLC fractions corresponding to 41 min after treatment of the protein with 1.5 mM GSSG. A peptide with 1951 Da was observed, which corresponded to the sodium adduct of the oxidized form of the dimedone derivative of the tryptic peptide containing Cys62. (B) MALDI-TOF mass spectrum of the same fraction after treatment of the protein with 1 mM H_2O_2. Peptides at 1913, 1929, and 1951 Da corresponded to the dimedone derivative, its oxidized form, and the sodium adduct of the tryptic peptide containing Cys62, respectively. (C) Nanospray-QIT high-resolution analysis (ZoomScan mode) of the double-charged species corresponding to the peptide identified in A. The isotopic envelope corresponded to a peptide with a monoisotopic mass of 1950.6 Da ($M + H^+$). (D) Nanospray-QIT MS/MS spectrum of the double-charged species analyzed in C. The peptide sequence and the assignment of the fragmentation series are also indicated, according to the nomenclature of Roepstorff and Fohlman (*43*). "Dmd" represents the sodium adduct of the dimedone moiety covalently attached to oxidized cysteine.

Cys62 and another protein thiol has been suggested as the main modification that could interfere in the binding of p50 to DNA. Here, we show that other biochemical changes aside from S-glutathionylation must be considered (*25*). The stoichiometry of the glutathione incorporation is not 1:1, and under oxidative conditions, only about 10% of the protein is in a dimer state because of a disulfide in which Cys62 is implicated (not shown; *12*). Besides and most important, although a maximal degree of thiolation is already reached at the GSH/2GSSG ratio of 1:1 (Figure 3A), a further inhibition in the DNA-binding capacity is observed with more intense pro-oxidative experimental conditions (^{3}H-GSH/2^{3}H-GSSG ratio of 0.1, Figure 1). To account for this difference, we considered that other types of biochemical modifications, such as sulfenic, sulfinic, or sulfonic acids, could be contributing to the maximal inhibition. These oxidation states could impede a major level of glutathione incorporation in those conditions. The concomitant or alternative formation of cysteine sulfenic acids has been described in different studies (*26*−*28*). To explore this possibility, wild-type p50 was preincubated in the presence of 1.5 mM GSSG and 1 mM dimedone, an agent which has been described to react specifically with the sulfenate sulfur displacing a water molecule and forming an irreversible thioether derivative (*28*, *29*). MS studies revealed the formation of a sulfenic acid in Cys62. Figure 4A shows the MALDI-TOF mass spectrum of the HPLC fraction from 41 min, obtained by the separation of tryptic peptides generated from p50 treated with GSSG and dimedone. One peptide in this fraction had an average mass of 1951 Da. This peptide was further analyzed by nanospray-QIT MS, using the ZoomScan mode (Figure 4C); this analysis identified a double-charged species with 975.8 Da, which corresponded to a peptide with a monoisotopic mass of 1950.68 Da ($M + H^+$). Although this mass could not be assigned to the expected dimedone derivative of any tryptic peptide generated from p50, MS/MS fragment analysis of the double-charged species allowed its identification as a further modified dimedone derivative. As shown in Figure 4D, the fragment spectrum unambiguously identified the sequence of the tryptic peptide containing Cys62, assuming that this residue had a chemical modification producing a mass increment of 176.0 Da. Consistently, the main backbone peptide fragmentation was essentially identical to that

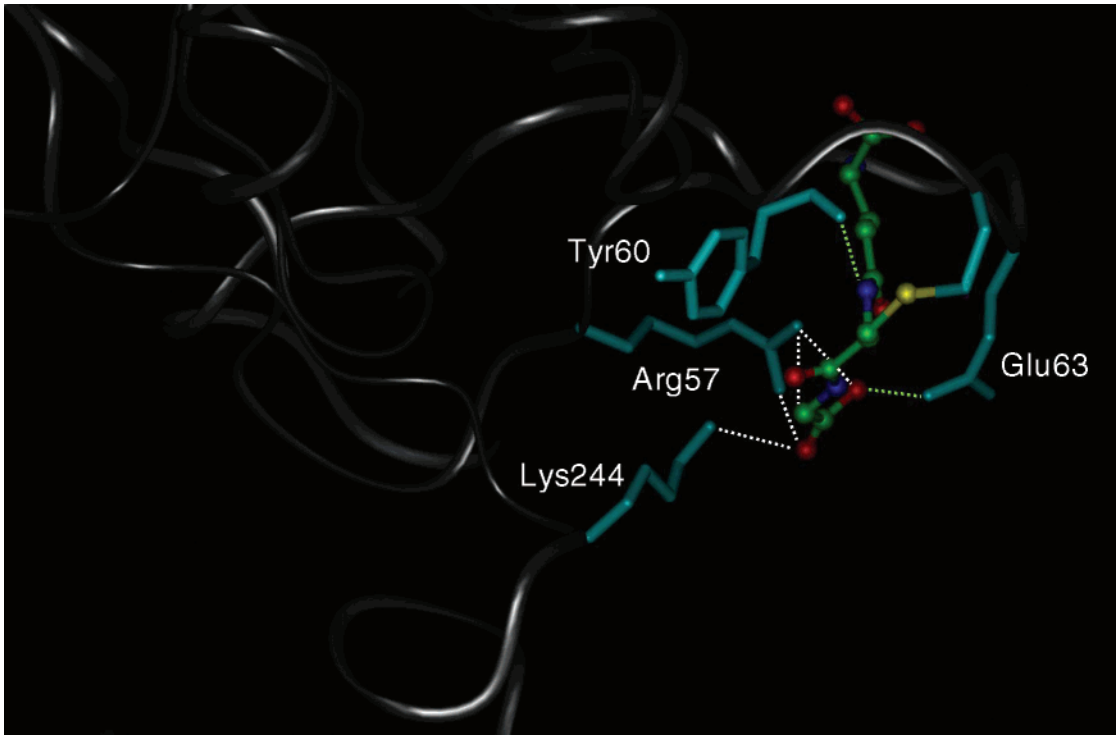

FIGURE 5: Model of the mixed disulfide between GSH and Cys62 of the p50 monomer. An energy-minimized model of the S-glutathionylated p50 monomer was built as described in Materials and Methods. A schematic drawing of glutathione bound to Cys62 of p50 through a disulfide bond is shown. The protein backbone of p50 is displayed in a partial ribbon representation (dark purple). The glutathione molecule is a ball-and-stick representation with the carbon atoms in green and all of the other atoms in standard colors for the atom type: (red) oxygen, (blue) nitrogen, (yellow) sulfur. The disulfide bridge between glutathione and the corresponding cysteine residue (Cys62) of p50 is represented by a solid stick. Amino acid side chains which make contacts with glutathione are stick representations in cyan. Electrostatic interactions (<3.5 Å) between the arginine (Arg57) and lysine (Lys244) residues of the protein and the glutathione moiety are indicated by white dotted lines. Additional van der Waals contacts ($\geq 80\%$ overlap) are indicated by green dotted lines.

observed with the glutathione-modified peptide previously identified (compare with Figure 2B). The increment in 176.0 Da was adequately explained by assuming a sulfur oxidation of the dimedone-derivatized cysteine, followed by the formation of a sodium adduct (theoretical monoisotopic mass of 1950.8 Da).

Treatment of p50 with H_2O_2 in the presence of dimedone also produced a set of peptides whose masses were consistent with the presence of dimedone-modified sulfenate moieties at Cys62. In this experiment, MALDI-TOF analysis of the corresponding HPLC fraction showed the presence of a major peptide with 1913 Da. In addition, two minor peptides with masses of 1929 and 1951 Da (Figure 4B) were detected. The masses of these three peptides agreed very well with the dimedone derivative of the Cys62-containing tryptic peptide (theoretical mass of 1912.9 Da), the sulfur-oxidized form of this derivative (1928.9 Da), and the sodium adduct (1950.9 Da). These findings are in agreement with the data described previously and also suggest that other oxidative treatments were capable of introducing the formation of a sulfenic moiety at Cys62. No modification in Cys62 was observed in a sample incubated with dimedone alone (not shown).

Estimation of the Contribution of the Formation of the Sulfenic Moiety to DNA-Binding Inhibition. To ascertain the functional relevance of sulfenate formation to the inhibition of the binding of p50 to DNA, we performed EMSA with p50 in the presence of GSSG with and without dimedone or DTT. We compared the degree of DNA binding, after DTT treatment, between two samples: one treated with GSSG followed by DTT and another incubated with GSSG plus dimedone followed by DTT. In the presence of dimedone, we assumed that DTT would only prevent the formation of the mixed disulfide but not of the sulfenate irreversibly complexed with dimedone. In the first sample, only irreversible modifications should be responsible for DNA-binding inhibition. However, in the second sample, the dimedone−sulfenate adduct formed would contribute to the irreversible states previously described generating a lesser degree of reversibility. The difference in their DNA-binding activity after DTT treatment gave us an estimation of the amount of sulfenate formation (measured as a dimedone−sulfenate adduct). In these conditions, only about 12% ($n = 5$) of the total inhibitory effect may be attributed to the formation of the sulfenate moiety (data not shown).

Molecular Model of the Binding of Glutathione to the Residue Cys62 of p50. To gain some insight into the structural basis of p50 S-glutathionylation, we built a structural model of the mixed disulfide between GSH and Cys62 of the p50 monomer (Figure 5), which was derived from the previously determined crystal structure of the unmodified NF-κB protein (*22*). With the exception of the five highly flexible loops, which are involved in DNA binding and contain Cys62, the incorporation of glutathione did not induce any significant conformational changes in the 3D arrangement of the protein. A previous model of S-glutathionylated *c-Jun* (*19*) suggested that the DNA-binding structural motif of *c-Jun* specifically recruits GSH as a redox reagent by stabilizing the protein−GSH adduct

via specific intermolecular interactions. In keeping with this model, p50-bound glutathione was stabilized by specific interactions with positively charged amino acid side chains located in the DNA-binding domain of the NF-κB p50 subunit. The two oxygen atoms of the terminal glycyl α-carboxylate group of GSH appear as good candidates to participate in electrostatic interactions with the amino groups of the guanidinium moiety of Arg57 (2.8 and 3.3 Å) and the ϵ-amino group of Lys244 (1.9 Å). In addition, the model suggests that the glycine and cysteine moieties of the glutathione molecule make nonpolar van der Waals contacts with Arg57, Tyr60, glutamate 63, and Lys244 of the p50 monomer.

DISCUSSION

The sensitivity of the transcription factor NF-κB to oxidative stress was reported about a decade ago (*32*). Furthermore, it has been shown that nitrosative stress as well as exposure to nonphysiological agents, such as NEM, iodoacetamide, diamide, or hydrogen peroxide, results in the inhibition of NF-κB DNA-binding activity (*25*, *33*−*35*). All of these effects are believed to occur after the disruption of the reduced state in the thiol group of Cys62. The S-nitrosylation of p50 in Cys62 has been demonstrated in vitro (*25*). Although this modification was shown after an oxidative treatment with a nonphysiological agent, another group has recently demonstrated that the S-nitrosylation of p50 can be induced by other more physiological conditions, suggesting that it could represent a new mechanism for p50 regulation (*35*).

We now report data consistent with the existence of previously unidentified oxidative modifications within the DNA-binding domain of NF-κB. We demonstrated the incorporation of glutathione to Cys62 of this domain using radioactive GSH and MS. S-Glutathionylation has been described for a large amount of proteins, including glyceraldehyde-3-phosphate dehydrogenase (*36*), creatine kinase (*37*), and the transcription factor AP-1 (*19*, *24*). Even when the inhibition can be preferentially attributed to the modification of Cys62, the fact that a slight degree of inhibition can also be observed with the C62S mutant suggests that other cysteine residues might suffer modifications, which may account for a small amount of the DNA-binding inhibition observed. It has been suggested that this mutant has a high dissociation constant with respect to DNA, but it has also been proposed that the formation of the complex depends on the specific oligonucleotide and construction used (*38*). In our hands, the C62S mutant seems to have a similar affinity for DNA as the wild-type protein with the specific oligonucleotide employed. This suggests that the inhibition observed with the mutant protein is not related with a lower affinity for DNA. Another important point is that the correlation between the formation of the mixed disulfide and inhibition of DNA binding is not complete. At a ratio of GSH/GSSG equal to 1, the degree of inhibition is about 35%, and the incorporation of the tripeptide has a value of approximately 0.4 mol of GSH/mol of protein. This suggests that, in such conditions, S-glutathionylation may be responsible for the inhibition that we observe. However, when higher pro-oxidative conditions (GSH/GSSG ratio equal to 0.1) are applied, the level of inhibition is around 66%, but the amount of labeled glutathione incorporation remained similar, indicating that, in these conditions, S-glutathionylation only accounts for 40% of the observed inhibition (compare Figures 1 and 2). A possible explanation for this low incorporation could be obtained from the molecular-modeling studies (Figure 5). From these data, we concluded that the weaker stabilization of GSH by p50 might be the reason for the minor degree of p50 S-glutathionylation. The p50 DNA-binding domain does not exhibit any evident structural homologies to the DNA-binding domain of *c-Jun*, where electrostatic interactions could help to stabilize the mixed disulfide as previously suggested by us (*19*). The target cysteine in *c-Jun* (Cys269) forms part of an α-helical DNA-binding domain (*22*) and is flanked by a cluster of lysine and arginine residues (-Lys-Cys-Arg-Lys-Arg-Lys). In contrast to *c-Jun*, the target thiol in p50 (Cys62) is embedded neither in a cluster of basic amino acids (-Phe-Arg-Tyr-Val-Cys-Glu-Gly-Ser-Pro-) nor in a rigid α-helical structure but resides in a flexible DNA-contacting loop, which forms part of a multiloop/β-strand DNA-binding motif (*39*). Thus, despite evident structural differences, both *c-Jun* (*19*) and p50 appear to stabilize the mixed disulfide through electrostatic protein−GSH interactions. However, whereas the stabilization of GSH by *c-Jun* involves symmetric electrostatic interactions with both of the GSH carboxylate groups (i.e., with the carboxylates of glycine and γ-glutamate (*19*)), according to this model, p50 may form salt bridges only with the glycine half-site of the GSH molecule (this study). This difference might explain why GSH is bound to *c-Jun* in an extended conformation, whereas in p50, the tripeptide adopts an energetically less favorable bent conformation, as has been reported previously for S-glutathionylated hemoglobin (*40*).

Alternative explanations which may account for this incomplete incorporation include the partial glutathionylation of the protein because (a) a proportion of the accessible cysteines susceptible to thiolation possibly maintain their reduced state and, thus, are subsequently irreversibly modified before the S-glutathionylation occurs or (b) in such pro-oxidative conditions, alternative or concomitant oxidative modifications could occur that compete with glutathione incorporation. Among the possible reactions that could impede a major degree of S-glutathionylation are the irreversible oxidations to sulfinic or sulfonic moieties or the reversible formation of a sulfenic acid. We searched for the sulfenic acid with the aid of the sulfenate specific probe dimedone. This modification has been proposed to act as an intermediary molecule which may subsequently react with GSH to the corresponding mixed disulfide (*41*). However, it also could give way to more oxidative states in the presence of oxygen, such as sulfinic and sulfonic acids. We observed additional irreversible oxidative modifications by MS studies (MALDI-TOF), including the formation of sulfinic and sulfonic moieties as well as the S-glutathionylation of Cys135 (data not shown), which could explain the slight degree of glutathione incorporation in the C62S mutant. Incubation with 1.5 mM GSSG and dimedone yielded a 1950 Da fragment corresponding to the sodium adduct of a peptide with a dimedone complex, indicating the existence of a sulfenate. The dimedone adduct is only reversible in very acidic conditions (*29*); hence, it is licit to presume that, in our experimental system, the covalent bond remains stable. The results of MS experiments were compatible with the

addition of dimedone to Cys62. The increment in 176.0 Da was adequately explained by assuming a sulfur oxidation of the dimedone-derivatized cysteine followed by the formation of a sodium adduct (theoretical monoisotopic mass of 1950.8 Da). Although the reaction of a sulfenate with dimedone was expected to produce a water loss (*29*), it is also expected that the S-alkylation induced by dimedone would make the sulfur more sensitive to oxidation. This has been shown to occur with the sulfur atom of methionine in proteins subjected to SDS−PAGE or to the acrylamide-modified forms of cysteine (*30, 31*); this finding is not surprising given the more intense pro-oxidative conditions employed. Also, sodium and potassium adducts are often encountered when separating peptides by HPLC.

The formation of a sulfenic acid has been suggested for different transcription factors such as OxyR, NF1, or AP-1 (*17*). Specifically, for OxyR, it was proposed that a sulfenic acid form occurred prior to an intramolecular disulfide between two cysteine residues essential for transcriptional activation (*17*). In this regard, our data do not exclude the possibility that the formation of a sulfenate may precede S-glutathionylation. We tried to quantify the amount of sulfenate moiety using the sulfhydryl/sulfenate probe 7-chloro-4-nitro-2-oxa-1,3-diazole (NBD-Cl; not shown). Although we detected an intermediary compound with a maximum absorbance at 347 nm (the absorbance of a sulfenate−NBD-Cl complex), the impossibility of eliminating completely the free probe, which absorbs at 343 nm (*42*), significantly overestimated the amount of sulfenate formed. For that reason, we used EMSA to estimate the degree of reversible oxidation to sulfenic acid. Although we could infer that about 12% of the protein is in this state (measured by the remaining inhibition after a treatment with DTT of samples incubated with GSSG or dimedone; see also sections on Materials and Methods and Results), a more exhaustive study would be required to confirm this estimation. Taken together, our results suggest that the Cys62 residues within the p50 homodimer may be in a state of reduction (the remaining 34% which still binds DNA) plus other states which include reversible (S-glutathionylation and sulfenic acid) and irreversible modifications (sulfinic and sulfonic acids). The herein described S-glutathionylation may account for the observations from different laboratories reporting that NF-κB can be negatively regulated by changes in the relation GSH/GSSG toward a pro-oxidative state (*14*−*16*). It is possible to speculate that, when the redox equilibrium could be reestablished by the cell, thioredoxin (*12*) or reduced GSH could revert the modification.

Overall, our results suggest a complex scenario in the redox regulation of NF-kB binding to DNA, suggesting that more than one modification of Cys62 is feasible and that S-glutathionylation and sulfenic acid formation are occurring in different degrees. The translation of these concepts into the cellular environment will require complementary approaches and techniques, which will hopefully render information regarding the relevance of these mechanisms in vivo.

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