In Vitro and in Vivo Protein-bound Tyrosine Nitration Characterized by Diagonal Chromatography*

Bart Ghesquière†§¶, Niklaas Colaert‡§, Kenny Helsens‡§, Lien Dejager||**, Caroline Vanhaute‡‡, Katleen Verleysen‡‡, Koen Kas‡‡, Ewy Timmerman‡§, Marc Goethals‡§, Claude Libert||**, Joël Vandekerckhove‡§, and Kris Gevaert‡§ §§

A new proteomics technique for analyzing 3-nitrotyrosine-containing peptides is presented here. This technique is based on the combined fractional diagonal chromatography peptide isolation procedures by which specific classes of peptides are isolated following a series of identical reverse-phase HPLC separation steps. Here, dithionite is used to reduce 3-nitrotyrosine to 3-aminotyrosine peptides, which thereby become more hydrophilic. Our combined fractional diagonal chromatography technique was first applied to characterize tyrosine nitration in tetrinitromethane-modified BSA and further led to a high quality list of 335 tyrosine nitration sites in 267 proteins in a peroxynitrite-treated lysate of human Jurkat cells. We then analyzed a serum sample of a C57BL6/J mouse in which septic shock was induced by intravenous Salmonella infection and identified six in vivo nitration events in four serum proteins, thereby illustrating that our technique is sufficiently sensitive to identify rare in vivo tyrosine nitration sites in a very complex background. Molecular & Cellular Proteomics 8:2642–2652, 2009.

Nitration of protein-bound tyrosines can have important implications on the structure and activity of proteins (4–6) and is linked to a variety of pathological conditions such as Alzheimer disease (7) and atherosclerosis (8). Proteins containing 3-nitrotyrosine residues have mainly been identified by one- or two-dimensional PAGE followed by Western blotting using 3-nitrotyrosine-specific antibodies (9) or following affinity enrichment (10, 11). However, rather few in vitro tyrosine nitration sites have thus far been mapped onto proteins, and hence, the exact sites of in vivo nitration remain elusive. This is highly likely due to the overall low abundance of this modification as was recently illustrated by the identification of only 31 nitration sites in 29 proteins in a comprehensive analysis of mouse brain tissue covering 7,792 proteins (12). Furthermore, it was estimated that in diseased cells or organs the number of nitrated tyrosines should be as low as 0.00001–0.001% of all tyrosines (5), numbers that clearly indicate the need to enrich for 3-nitrotyrosine peptides prior to mass spectrometric analysis. In addition, several MS and MS/MS detection problems for 3-nitrotyrosine peptides were reported (13, 14) that also influence identification of such peptides.

Recently, a procedure for enriching 3-nitrotyrosine peptides was described (10). In brief, reduced and alkylated proteins were digested after which primary amino groups were blocked by acetylation. Nitrotyrosines were then reduced to aminotyrosine using dithionite (15), unveiling novel primary amino groups on which sulfhydryl groups were coupled that allowed binding peptides to thiopropyl-Sepharose beads. In contrast to an earlier affinity-based isolation protocol (6), this improved enrichment procedure was more effective and led to the characterization of 150 tyrosine nitration sites in 102 proteins using a total of 3.1 mg of a mouse brain homogenate sample that was in vitro nitrated (10). However, this procedure requires many modification steps, which, when incomplete, will introduce artifacts (see “Results”). Among others, these explain the rather low number of identified nitrated tyrosines peptides using the high amount of starting material that was in vitro nitrated.

Our laboratory adapted diagonal chromatography (17) for contemporary mass spectrometry-driven proteomics. Central in our combined fractional diagonal chromatography (CO-
FRAFRADIC\(^1\) (18) approach is a chemical or enzymatic step that changes the reverse-phase column retention properties of a set of peptides such that these can be isolated. Among others, we developed COFRADIC protocols for isolating peptides carrying amino acid modifications such as phosphorylation (19), N-glycosylation (20), and sialylation (21) or peptides that are the result of protein processing (22–24). Here we describe a COFRADIC procedure for sorting peptides carrying nitrated tyrosines. Peptide sorting is based on a hydrophilic shift after reducing the nitro group to its amino counterpart. The applicability of COFRADIC for analyzing this modification is illustrated by characterization of four 3-nitrotyrosines in BSA treated with tetrinitromethane, the mapping of 335 different nitration sites in 267 different proteins starting from 300 \(\mu\)g of 

**EXPERIMENTAL PROCEDURES**

Reduction of 3-Nitrotyrosine Peptides by Sodium Dithionite and Analysis by RP-HPLC—The peptide EY(NO\(_2\))OQLYYDPSSR (Y(NO\(_2\)) = 3-nitrotyrosine) was synthesized using Fmoc (\(N\)-(9-fluorenyl)methoxy-carbonyl) chemistry on a 433A peptide synthesizer (Applied Biosystems, Framingham, MA). 2 nmol of this peptide was dissolved in 75 \(\mu\)l of water/acetonitrile (3:97, v/v) and lyophilized. To this was added 20 nmol of sodium dithionite and the sample was allowed for 10 min on ice followed by centrifugation at 16,000 \(\times\) g for 30 min at 4 °C to remove cellular debris. 500 \(\mu\)l of this lyase was loaded on a NAP-5 column, and the proteins were collected in 1 ml of 100 mM ammonium bicarbonate buffer at pH 7.8. Protein concentration was determined using the Bradford assay.

Peroxynitrite was freshly prepared as described (27). Briefly, 10 ml of 0.6 M HCl (Sigma-Aldrich) and 0.7 M H\(_2\)O\(_2\) (Sigma-Aldrich) was mixed with 10 ml of 0.6 M NaNO\(_2\) and immediately neutralized with 20 ml of 1.5 M NaOH. Excess H\(_2\)O\(_2\) was removed by adding 10 mg of MnO\(_2\) for 10 min at 25 °C. To determine the concentration of the peroxynitrite stock solution, the absorbance at 302 nm was measured, and the following formula was used to determine the molar concentration of peroxynitrite: [ONOO\(^-\)] = \(A_{302}/1.670\). For nitration of proteins, 25 \(\mu\)l of 40 mM peroxynitrite solution was added for 10 min at 25 °C. Because of the instability of peroxynitrite, this step was repeated once.

Then, 500 \(\mu\)l of the nitrated protein mixture was taken out, and cysteines were reduced with DTT (Sigma-Aldrich; 20 mM final concentration) for 15 min at 37 °C followed by alkylation of cysteines by iodoacetamide (Sigma-Aldrich; 60 mM final concentration) for 30 min at 37 °C. Proteins were then precipitated by adding 9 volumes of ice-cold ethanol and incubation for 4 h at –80 °C followed by centrifugation for 1 h at 100,000 \(\times\) g (4 °C). The resulting protein pellet was redissolved in 50 \(\mu\)l of 50 mM triethylammonium bicarbonate (pH 7.8) (Sigma-Aldrich). Trypsin was added in a 1:100 ratio (w/w, enzyme/substrate) for overnight digestion at 37 °C.

100 \(\mu\)l of the protein digest was then acidified with 5 \(\mu\)l of 10% acetic acid and treated with 0.5% (w/v) H\(_2\)O\(_2\) (final concentration) for 30 min at 30 °C. This oxidation step uniformly converts methionines to their sulfoxide derivatives (18), hence preventing that methionyl peptide shift during the secondary separations upon spontaneous oxidation. The treated peptide mixture was then separated by RP-HPLC as described above and fractionated into 48 1-min fractions (80 \(\mu\)l each) between 30 and 78 min. To reduce the number of secondary separations, primary fractions that were separated by 16 min were pooled, dried, redissolved, treated with dithionite as indicated above, and separated a second time. Per primary fraction, peptides shifting upon reduction by dithionite were collected in 10-min-wide time intervals starting 13 min before the starting point of collection of each primary fraction. All primary and secondary fractions were sampled by MALDI-MS using an Ultraflex II TOF/TOF mass spectrometer (Bruker Daltonics Inc., Bremen, Germany) operated as described before (26).

In Vitro Nitration of Lysate from Jurkat Cells by Peroxynitrite—200 ml of a Jurkat cell suspension (ATCC, Manassas, VA), at 150 \(\times\) 10\(^6\) cells/ml, was centrifuged for 5 min at 1,500 \(\times\) g. The pellet was washed twice with 50 ml of ice-cold PBS and lysed with 600 \(\mu\)l of lysis buffer (50 mM Hepes (pH 7.4), 0.9% CHAPS (Sigma-Aldrich), 150 mM NaCl, 1 mM EDTA (Sigma-Aldrich)) and the appropriate amount of a protease inhibitor mixture (Roche Applied Science). Cell lysis was allowed for 10 min on ice followed by centrifugation at 16,000 \(\times\) g for 30 min at 4 °C to remove cellular debris. 500 \(\mu\)l of this lysate was loaded on a NAP-5 column, and the proteins were collected in 1 ml of 100 mM ammonium bicarbonate buffer at pH 7.8. Protein concentration was determined using the Bradford assay.

Peroxynitrite was freshly prepared as described (27). Briefly, 10 ml of 0.6 M HCl (Sigma-Aldrich) and 0.7 M H\(_2\)O\(_2\) (Sigma-Aldrich) was mixed with 10 ml of 0.6 M NaNO\(_2\) and immediately neutralized with 20 ml of 1.5 M NaOH. Excess H\(_2\)O\(_2\) was removed by adding 10 mg of MnO\(_2\) for 10 min at 25 °C. To determine the concentration of the peroxynitrite stock solution, the absorbance at 302 nm was measured, and the following formula was used to determine the molar concentration of peroxynitrite: [ONOO\(^-\)] = \(A_{302}/1.670\). For nitration of proteins, 25 \(\mu\)l of 40 mM peroxynitrite solution was added for 10 min at 25 °C. Because of the instability of peroxynitrite, this step was repeated once.

Then, 500 \(\mu\)l of the nitrated protein mixture was taken out, and cysteines were reduced with DTT (Sigma-Aldrich; 20 mM final concentration) for 15 min at 37 °C followed by alkylation of cysteines by iodoacetamide (Sigma-Aldrich; 60 mM final concentration) for 30 min at 37 °C. Proteins were then precipitated by adding 9 volumes of ice-cold ethanol and incubation for 4 h at –80 °C followed by centrifugation for 1 h at 100,000 \(\times\) g (4 °C). The resulting protein pellet was redissolved in 50 \(\mu\)l of 50 mM triethylammonium bicarbonate (pH 7.8) (Sigma-Aldrich). Trypsin was added in a 1:100 ratio (w/w, enzyme/substrate) for overnight digestion at 37 °C.

One-tenth of the generated peptide mixture (~4 nmol of BSA) was treated with 0.5% (w/v) H\(_2\)O\(_2\) (final concentration) for 30 min at 30 °C. This oxidation step uniformly converts methionines to their sulfoxide derivatives (18), hence preventing that methionyl peptide shift during the secondary separations upon spontaneous oxidation. The treated peptide mixture was then separated by RP-HPLC as described above and fractionated into 48 1-min fractions (80 \(\mu\)l each) between 30 and 78 min. To reduce the number of secondary separations, primary fractions that were separated by 16 min were pooled, dried, redissolved, treated with dithionite as indicated above, and separated a second time. Per primary fraction, peptides shifting upon reduction by dithionite were collected in 10-min-wide time intervals starting 13 min before the starting point of collection of each primary fraction. All primary and secondary fractions were sampled by MALDI-MS using an Ultraflex II TOF/TOF mass spectrometer (Bruker Daltonics Inc., Bremen, Germany) operated as described before (26).

\(^1\) The abbreviations used are: COFRADIC, combined fractional diagonal chromatography; RP, reverse-phase; LTQ, linear trap quadrupole; FDR, false discovery rate.
fraction. A total of 360 secondary fractions were in this way collected, and to reduce mass spectrometer analysis time, secondary fractions that were separated by 15 min were repoled, resulting in 90 fractions for further analysis.

Identification of Tyrosine Nitration Sites in Serum Proteins of Sepsis Mouse Model—Female C57BL6/J mice were from Janvier (Le Genest-St. Isle, France) and used at the age of 8–10 weeks. The mice were maintained in a temperature-controlled, air-conditioned specific-pathogen-free animal facility with 14/10 h light/dark cycles and received food and water ad libitum. All experiments were approved by the animal ethics committee of the Faculty of Sciences of Ghent University (Belgium) and performed according to its guidelines.

Pathogenic Salmonella enteritidis (serovar typhimurium) were from ATCC. Bacteria were diluted in lipopolysaccharide-free PBS to a concentration of 4 × 10^7 colony-forming units/ml, and 0.25 ml was intravenously injected per mouse in the right tail vein. Mice were bled daily at 9 a.m. at the retro-orbital plexus. Blood was allowed to clot for 60 min at 37 °C and further overnight at 4 °C. The clot was removed, and cells were pelleted by centrifugation at 14,000 rpm for 15 min. Serum was separated and stored at −20 °C until further use. Five serum samples taken at days 4 and 5 were pooled to obtain a total volume of 150 μl of crude serum followed by depletion of the top three most abundant proteins: albumin, immunoglobulin G, and transferrin. A commercially available affinity system was used for this purpose (Multiple Affinity Removal System, Agilent Technologies). 2 ml of the depleted serum was reduced to 900 μl by vacuum drying. The pH of the solution was adjusted to 8.7 by addition of 100 μl of 1 M Tris-HCl (pH 8.7). Alkylation of cysteines was carried out using final concentrations of 60 μl iodoacetamide and 30 μl of 2-carboxyethylphosphine for 30 min at 37 °C. Excess reagents were removed by desalting over a NAP-10 (Amersham Biosciences) desalting column in 1.5 ml of 50 μm triethylammonium bicarbonate buffer (pH 7.8). The protein concentration was determined using the Bradford assay for proteins.

The pH of the solution was adjusted to 8.7 by addition of 100 μl of 1 M Tris-HCl (pH 8.7). Alkylation of cysteines was carried out using final concentrations of 60 μl iodoacetamide and 30 μl of 2-carboxyethylphosphine for 30 min at 37 °C. Excess reagents were removed by desalting over a NAP-10 (Amersham Biosciences) desalting column in 1.5 ml of 50 μm triethylammonium bicarbonate buffer (pH 7.8). The protein concentration was determined using the Bradford assay for proteins.

In this LTQ, MS/MS scans were recorded in profile mode at a target value of 75 μm-inner diameter × 150 mm reverse-phase column (PepMap C18, Dionex). Peptides were eluted with a linear gradient of a 1.8% solvent B (0.05% formic acid in water/acetonitrile (2:8, v/v)) increase per minute at a constant flow rate of 300 nl/min.

The mass spectrometer was operated in data-dependent mode, automatically switching between MS and MS/MS acquisition for the six most abundant ions per MS spectrum. Full-scan MS spectra were acquired at a target value of 1e6 with a resolution of 30,000. The six most intense ions were then isolated for fragmentation in the linear ion trap. In the LTQ, MS/MS scans were recorded in profile mode at a target value of 5,000. Peptides were fragmented after filling the ion trap with a maximum ion time of 10 ms and a maximum of 1e4 ion counts. From the MS/MS data in each LC-run, Mascot generic files (mgf) were created using the Mascot Distiller software (version 2.2.1.0, Matrix Science Ltd.). When generating these peak lists, grouping of spectra was performed with a maximum intermediate retention time of 30 s and maximum intermediate scan count of 5. Grouping was further done with 0.1-Da precursor ion tolerance. A peak list was only generated when the spectrum contained more than 10 peaks. There was no deisotoping, and the relative signal to noise limit for both precursor ions and fragment ions was set to 2. These peak lists were then searched with Mascot using the Mascot Daemon interface (version 2.2.0, Matrix Science Ltd.). The Mascot search parameters were as follows. Searches were performed in the Swiss-Prot database with taxonomy set to human (database version 56.4; 20,328 human protein sequences). Methionine oxidation to its sulfoxide was set as a fixed modification. Variable modifications were 3-aminotyrosine, sulfation of 3-aminotyrosine, acetylation of protein N termini, carbamidomethylation of cysteine, and oxidation of the carbamidomethylated cysteine. Trypsin was set as the enzyme used (one missed cleavage was allowed), the mass tolerance on the precursor ion was set to 10 ppm, and the mass tolerance on the fragment ions was set to 0.5 Da. In addition, the C13 setting of Mascot was set to 1. For the estimation of the false discovery rate of peptides identified by Mascot, searches were performed in a concatenated decoy database consisting of a reversed Swiss-Prot database (28) at the 95% confidence level.

LC-MS/MS Analysis of Mouse Serum Peptides—Secondary fractions were redissolved in 15 μl of 2% acetonitrile, and 8 μl was used for LC-MS/MS analysis using an Ultimate 3000 HPLC system (Dionex, Amsterdam, The Netherlands) in line connected to an LTQ Orbitrap XL mass spectrometer (Thermo Electron). Peptides were first trapped on a trapping column (PepMap , 0.3-mm inner diameter × 5 mm (Dionex)), and following back-flushing from the trapping column, the sample was loaded on a 75-μm-inner diameter × 150-mm reverse-phase column (PepMap C18, Dionex). Peptides were eluted with a linear gradient of a 1.8% solvent B (0.05% formic acid in water/acetonitrile (2:8, v/v)) increase per minute at a constant flow rate of 300 nl/min.

The mass spectrometer was operated in data-dependent mode, automatically switching between MS and MS/MS acquisition for the six most abundant ions per MS spectrum. Full-scan MS spectra were acquired at a target value of 1e6 with a resolution of 30,000. The six most intense ions were then isolated for fragmentation in the linear ion trap. In the LTQ, MS/MS scans were recorded in profile mode at a target value of 5,000. Peptides were fragmented after filling the ion trap with a maximum ion time of 10 ms and a maximum of 1e4 ion counts. From the MS/MS data in each LC-run, Mascot generic files (mgf) were created using the Mascot Distiller software (version 2.2.1.0, Matrix Science Ltd.). When generating these peak lists, grouping of spectra was performed with a maximum intermediate retention time of 30 s and maximum intermediate scan count of 5. Grouping was further done with 0.1-Da precursor ion tolerance. A peak list was only generated when the spectrum contained more than 10 peaks, no deisotoping was performed, and the relative signal to noise limit was set to 2. Such generated peak lists were then searched with Mascot using the Mascot Daemon interface (version 2.2.0, Matrix Science Ltd.). Spectra were searched against the Swiss-Prot database, and taxonomy was set to Mus musculus (database version 56.4; 15,988 mouse protein sequences). Variable modifications were set to methionine oxidation, pyroglutamate formation of N-terminal glutamine, carbamidomethylation of cysteines, oxidation of the carbamidomethylated cysteine, acetylation of the N terminus, and deamidation of glutamine.
or asparagines. Mass tolerance of the precursor ions was set to 10 ppm, and mass tolerance of the fragment ions was set to 0.5 Da. The peptide charge was set to 1+, 2+, or 3+, and one missed tryptic cleavage site was allowed. Also, the C13 setting of Mascot was to 1. Only peptides that were ranked 1 and scored above the threshold score set at 95% confidence were withheld. The FDR was estimated as indicated above for the LTQ data.

Quality Assessment of Mascot-identified Tyrosine-nitrated Peptides Using Peptizer Algorithm—Following Mascot database searching of the Jurkat MS/MS data, 683 MS/MS spectra were identified to belong to peptides containing 3-aminotyrosine or sulfated 3-aminotyrosine. To assess the quality of these peptide identifications and thus reduce the number of potential false positive identifications, the following quality assumptions were further inspected by Peptizer (29). The peptide identification scored more than 10 units above the 95% identity threshold, the peptide was longer than 8 amino acids, only a single peptide was allowed to score above the 95% identity threshold, at least 30% of b ions and y ions were observed, and an (optional) MS/MS-unstable proline peptide bond produced dominant b ions or y ions. If two or more of the above assumptions were not fulfilled by a suggested identified peptide, this peptide was not included in the high quality list. The Peptizer profile used is available upon request. Supplemental Table 1 lists the identifications that passed the Peptizer criteria (note that whenever a peptide was linked to several protein sequences stored in the database this is indicated in the table). The supplemental information lists the identified MS/MS spectra from both the Jurkat and the mouse serum study.

RESULTS
Diagonal Reverse-phase Chromatographic Isolation of 3-Nitrotyrosine Peptides after Reduction to 3-Aminotyrosine Peptides by Na₂S₂O₄—A previous report suggests that 3-aminotyrosine peptides are more hydrophilic than their nitrated counterparts (30). Here this is illustrated by the UV absorbance chromatograms shown in Fig. 1: the 3-nitrotyrosine peptide eluted at about 48 min (solid line), and the 3-aminotyrosine-containing peptide undergoes a hydrophilic shift of about 7 min (dotted line).

![Fig. 1. Reduction of 3-nitrotyrosine to 3-aminotyrosine causes a hydrophilic shift.](image_url)

A synthetic peptide holding one nitrated tyrosine residue (NH₂-EY(NO₂)QLQYDPSR) was separated on a reverse-phase column, and the UV absorbance trace at 214 nm is shown (solid line). Upon reduction with sodium dithionite, the 3-aminotyrosine-containing peptide undergoes a hydrophilic shift of about 7 min (dotted line). mAU, milliabsorbance units.
Tyrosine Nitration Revealed by COFRADIC
phic shift may depend on the sequence of the peptide, using the RP-HPLC setup described above, the evoked shift was typically more than 4 min, implying sufficient separation between sorted (3-aminotyrosyl) and unsorted peptides. In general, the average yield of the reduction of the nitrotyrosine was over 95% as judged by the drop of the UV absorbance intensity of the nitrated peptide (Fig. 1).

**Identification of Tyrosine Nitration Sites in Tetrinitromethane-treated BSA**—In a second study, BSA was in *vitro* nitrated and digested with trypsin, and the resulting peptide mixture was separated by RP-HPLC (Fig. 2A). Primary fraction 32 contained a peptide ion with a mass of 1,612.774 Da (Fig. 2C), corresponding to the expected mass of singly nitrated DAFLSFLYEYSR, a tryptic peptide spanning residues 347–359 of BSA. Characteristic satellite peaks 16, 30, and 32 Da lower than the mass of the intact peptide ion were previously observed in MALDI-MS spectra of 3-nitrotyrosyl peptides (25, 32) and confirm tyrosine nitration of this peptide. Upon treatment with dithionite and reseparation by RP-HPLC (Fig. 2B), a peptide ion with a mass of 1,582.653 Da appears in the MALDI-MS spectrum of the secondary fraction containing the sorted peptide (Fig. 2D). This mass can be linked to DAFLSFLYEYSR, now with one 3-aminotyrosine residue. MALDI-PSD analysis further confirmed the nature of this peptide and indicated that tyrosine 357 was indeed present in its amino form and was thus in *vitro* nitrated by tetrinitromethane (Fig. 2E). Interestingly, tyrosine 355 was not nitrated, although it is only two residues distant from the nitrated tyrosine on position 357.

Secreted BSA contains 20 tyrosines, and following MALDI-MS analysis of all secondary fractions and MALDI-PSD analysis on sorted peptides, we identified four tyrosines that were nitrated by tetrinitromethane: tyrosines 161, 357, 364, and 424. Interestingly, these tyrosines meet nitration criteria that were previously set: all are surface-accessible and have negatively charged amino acids but no cysteines nearby (33). We could not show nitration of other tyrosines, which may be due to several reasons such as the fact that they fail to meet *in vitro* protein nitration criteria or they reside in peptides that are not analyzable by LC-MS/MS.

**Protein-bound in Vitro Tyrosine Nitration in Jurkat Cell Lysate**—We then isolated and identified 3-nitrotyrosine-containing peptides in a much more complex mixture, a Jurkat cell lysate treated with peroxynitrite. Because earlier reports (13, 34) showed that reduction of 3-nitrotyrosine by sodium dithionite may lead to two end products, 3-aminotyrosine and sulfated 3-aminotyrosine (probably caused by the addition of an SO$_3$H group onto the ortho position of the aromatic benzene ring), we further accounted for sulfation of 3-aminotyrosine side chains during database searching. Using 300 µg of starting material for the COFRADIC procedure, we initially identified 683 LTQ-FT MS/MS spectra belonging to 443 unique sequences from 342 different proteins. Recently Stevens et al. (14) reported several factors that lead to misidentification of 3-nitrotyrosine-containing peptides. Therefore, we introduced two approaches to create a high quality list of the identified spectra and thus filter out possible false positive identifications. First, we estimated the FDR of Mascot identifications using a concatenated decoy database search, resulting in an FDR value of 2.6%. Next, we used the in-house developed Peptizer program (29) to filter out all peptides that did not meet strict criteria such as the presence of flanking b and y fragment ions at the 3-aminotyrosine residue (or its sulfated counterpart). The result is a high quality list of 491 LTQ-FT MS/MS spectra that were assigned to 335 different 3-nitrotyrosine peptides from 267 unique proteins (see supplemental Table 1). Importantly, Peptizer not only removed all spectra linked to 3-aminotyrosine-containing peptides identified in the decoy database but also removed peptides identified in the “forward” database that were highly likely to be false. An example of the latter is the N-terminal peptide of fructose-bisphosphate aldolase 1. Interestingly, the murine N-terminal aldolase peptide (nitrated on Tyr$^3$) was identified in another study (10), and here we initially identified its human counterpart (nitrated on Tyr$^3$ and Tyr$^5$), but Peptizer removed it from the list of identifications as it did not meet the criteria of Peptizer and was therefore considered as a possible false positive identification.

Our list represents a map of Jurkat tyrosines that are targeted by the peroxynitrite radical, and among the 335 different nitratated sequences, we found previously identified nitration sites (both from *in vitro* as *in vivo* experiments) such as Tyr$^{148}$ in the sequence ITLDNAY*MEK* (where Y* is 3-aminotyrosine) from pyruvate kinase (see Fig. 3A), which was previously identified in skeletal muscle tissue from 34-month-old Fisher 344/Brown Norway F1 rats (35). Interestingly, we also picked up its sulfated form, and Fig. 3B clearly shows an 80-Da increase on the peptide fragment ions carrying the

Fig. 2. **Nitrotyrosyl COFRADIC applied on tryptic digest of in *vitro* nitrated BSA**. A, primary RP-HPLC separation of a tryptic digest of nitrated BSA (UV absorbance trace at 214 nm is shown). Peptides eluting between 20 and 68 min were collected in 1-min fractions. The highlighted fractions 16, 32, and 48 were pooled, reduced by dithionite, and separated a second time under identical conditions. Peptides undergoing a hydrophilic shift by reduction (indicated by an asterisk) were collected in intervals of 10 min starting 13 min prior to the collection of each primary fraction (B). C, MALDI-MS spectrum of primary fraction 32 indicating a peptide with a mass of 1,612.774 Da, which theoretically corresponds to DAFLSFLYEYSR with one nitrated tyrosine. D, MALDI-MS spectrum of the secondary fraction eluting between 45 and 46 min. This fraction contains a peptide with a mass of 1,582.653 Da corresponding to the 3-aminotyrosyl counterpart of the nitrated peptide. E, MALDI-PSD spectrum of the 3-aminotyrosyl peptide in D. As can be judged from the y fragment ions, tyrosine 357 and not tyrosine 355 is a 3-aminotyrosine (mass difference of 178.03 Da between $y_3$ (440.10 Da) and $y_9$ (262.07 Da) ions), and hence this residue was in *vitro* nitrated by tetrinitromethane (for the sake of clarity, only y-type fragment ions are indicated). mAU, milliabsorbance units.
sulfated 3-aminotyrosine. 75.6% of the reduced nitration sequences (371 of 491 spectra) were actually identified to carry sulfated 3-aminotyrosine. On the peptide level, this means that 51 peptides (~15%) were identified in their non-sulfated form, 45 peptides (~13%) were identified in both forms, and the majority (239 peptides or ~71%) were identified as sulfated 3-aminotyrosine peptides, which illustrates the importance of accounting for this modification in database searches. Using the precursor ion intensities of peptides that were found both sulfated and non-sulfated, we estimated that the degree of sulfation ranged from 5 to 90%, again showing that sulfation of aminotyrosine residues is an important artifact when dithionite is used. On the other hand, however, we currently do not hold conclusive proof on how aminotyrosine residues become sulfated in our system but noticed that by increasing the excess of dithionite in the reduction reaction the degree of sulfation was also increased, hinting to a role of dithionite (or its oxidized products) in this sulfation reaction.

Looking more into detail in the list of identified peptides, Tyr^{161} and Tyr^{357} of the tubulin α-1 β chain were here identified as nitrated. Both tyrosines were found by Tedeschi et al. (36) to play an important role during nerve growth factor-induced neuronal differentiation. In addition, nitration of Tyr^{224} found here was previously described by Fiore et al. (37), specifically during grade IV human glioma. Haqqani et al. (38) reported histone nitration, and we confirmed Tyr^{72} of histone H4 and Tyr^{43} of histone H2B as nitration spots upon in vitro peroxynitrite treatment.

Thus, because tyrosine nitration sites previously found in vivo were also identified in our study, it is tempting to state that in vitro nitration by peroxynitrite does not target tyrosines randomly but might well resemble physiological nitration found in living cells. Hence, we asked whether sequence or structural features could help explain preferred sites of peroxynitrite nitration of tyrosines. It is believed that nitration targets reside in specific protein segments containing negatively charged or turn-inducing amino acids (33, 39). At first sight, many of the peptides we identified appeared to contain such amino acids; however, using the Two Sample Logo tool (40), we found no absolute preferences of specific amino acids surrounding the tyrosine nitration site (supplemental Fig. 1A), an observation that was also made by Souza et al. (5). This possibly points to the local three-dimensional protein structure as an important determinant for tyrosine nitration rather than the primary protein sequence. Considering secondary structure, a slight increase of nitration sites in coiled regions is observed (supplemental Fig. 1B).

Several reports also point to a dynamic interplay between protein tyrosine nitration and phosphorylation (41, 42). 22 identified peptides (about 6.5%) are modified on tyrosines that are known to be phosphorylated by a wide diversity of tyrosine kinases. This, combined with the chance that a random tyrosine-containing peptide is phosphorylated (0.46%), indicates that our list contains a tendency toward tyrosines that are phosphorylated or indicates that nitration, similar to phosphorylation, occurs on tyrosine residues that are surface-accessible. Note that the value of 0.46% was calculated by dividing the number of experimentally found tyrosine phosphorylation sites in human proteins (1,387 sites stored in Phospho.ELM) by the number of tyrosines found in human proteins (299,816 found in SwissProt).

Tyrosine Nitration of Serum Proteins during Salmonella-induced Septic Shock—Intravenous administration of Salmonella into mice causes severe septic shock and leads to oxidative burst (43). As a result, oxygen- and nitrogen-derived radicals are produced to create a toxic environment for the invading pathogen. The excess radicals produced are scavenged by serum proteins and cause modifications such as nitration of tyrosines.

Using our COFRADIC approach, we investigated whether we could identify nitrated tyrosine residues in serum proteins of mice infected with Salmonella. Here we linked 28 MS/MS spectra to peptides potentially containing in vivo nitrated tyrosines. The spectra were then filtered by Peptizer to eliminate possible false positive spectra using the same agents as described above. Finally, eight MS/MS spectra were withheld pointing to six different peptides from four unique serum proteins (Table I).

Three sites in α2-macroglobulin were identified: tyrosines 1019, 1024, and 1330. Earlier reports indeed indicated that α2-macroglobulin was susceptible for nitration (44) and thus probably serves as a scavenger for excess radical formation. Apolipoprotein A-I is also known to be nitrated during conditions of oxidative stress because of its close association with myeloperoxidase (45–48), and Tyr^{43} of this protein was found nitrated. The study of Parastatidis et al. (47) clearly showed that apolipoprotein A-I serves as a protector protein that scavenges radicals and controls the oxidative burden. Tyr^{66} from haptoglobin was also nitrated in the sepsis model, and nitration of haptoglobin is known to influence its function. Finally, we identified Tyr^{110} from the vitamin D-binding protein as a nitration site during sepsis.

DISCUSSION

We developed a novel technology for studying tyrosine nitration in a diagonal chromatography setup. Central in our
Tyrosine Nitration Revealed by COFRADIC

| Protein                      | Swiss-Prot accession no. | Nitrated tyrosine peptides | Score (threshold) |
|------------------------------|--------------------------|---------------------------|-------------------|
| α2-Macroglobulin             | Q61838                   | 1016AINYLISGYQR1026       | 43 (30)           |
|                              |                          | 1016AINYLISGYQR1026       | 42 (30)           |
|                              |                          | 132LPDLPGNYVTK1333        | 45 (30)           |
| Apolipoprotein A-I           | Q00623                   | 30DFANVYTVDAVK46          | 42 (30)           |
| Haptoglobin                  | Q61646                   | 50LRAEGDGVYTLNDEK72       | 39 (32)           |
|                              |                          | 60AEGDGVYTLNDEK72         | 47 (31)           |
| Vitamin D-binding protein    | P21614                   | 403GQEMCADY*SENTFTEYK419 | 42 (27)           |
|                              |                          | 403GQEMCADY*SENTFTEYK419 | 33 (26)           |

Our technology led to the identification of four nitration sites on albumin treated with tetranitromethane. Next, a whole proteome, a lysate from Jurkat cells treated in vitro with peroxynitrite, was analyzed. We initially identified 683 MS/MS spectra containing nitrated tyrosine; however, to improve the quality of reported peptides, we used the PepTizer algorithm to remove suspicious identifications among others based on the fact that in MS/MS spectra the aminotyrosine residue should be covered on both sides by either b or y fragment ions. This resulted in a trimmed, although high quality list of 491 MS/MS spectra pointing to 336 peptides from 277 proteins. We used our list of tyrosine nitration targets to check whether we could find sequence preferences for nitration, but no conclusive preferences were found, likely indicating that the three-dimensional environment determines whether or not a tyrosine is susceptible for nitration. For our study, only 300 μg of protein material was used; this is much less material compared with the amount of material used by another MS-driven proteomics technology (49). However, in the latter study, using an LTQ ion trap mass spectrometer, 150 different nitrotyrosine peptides were identified in 102 unique proteins starting from 3 mg of proteins that needed to undergo a series of chemical steps before nitrated peptides were isolated. Sulfation of aminotyrosines was not considered in that study, which might explain its apparent overall lower sensitivity.

Finally, we identified tyrosine nitration in mouse serum proteins depleted of the three most abundant serum proteins (albumin, IgGs, and serotransferrin) from mice that were injected intravenously with Salmonella. We picked up targets from as little as 10 μl of undepleted mouse serum. α2-Macroglobulin and apolipoprotein A-I are important proteins that control the oxidative burden; especially apolipoprotein A-I, which is associated with myeloperoxidase, an enzyme generating oxygen-derived radicals, appears as an “expected” target for nitration. Such proteins are abundant serum proteins, thus pointing to an important role for serum proteins in easing oxidative stress by scavenging radicals. Furthermore, our results also suggest that nitrated serum proteins could be interesting candidates for further biomarker analysis studies.

As our approach is gel-free, it centers on peptides instead of traditional, gel-based approaches that are protein-centric. As such, the major advantage of our approach is that peptides carrying a nitrated tyrosine are directly isolated, and peptide fragmentation spectra readily point to the residue that is modified. Such information is highly important as it can help our understanding of how and why tyrosine nitration alters the structure or function of affected proteins. Furthermore, our sorting procedure is straightforward, involving a simple reduction step, and was shown here to not only identify in vitro nitration sites that are probably “easily” picked up (Jurkat study) but more importantly also identified in vivo nitration of proteins in what is probably the most complex proteome, serum.

* This work was supported by research grants from the Fund for Scientific Research-Flanders (Belgium) (Projects G.0156.05, G.0077.06, and G.0042.07), the Concerted Research Actions (Project BOF07/GOA/012) from Ghent University, the Inter University Attraction Poles (IUAP06), and the European Union Interaction Proteome (6th Framework Program).§ The on-line version of this article (available at http://www.mcponline.org) contains supplemental Fig. 1 and Table 1.
¶ A postdoctoral research fellow of the Fund for Scientific Research-Flanders (Belgium).
§§ To whom correspondence should be addressed: Dept. of Medical Protein Research and Biochemistry, VIB and Faculty of Medicine and Health Sciences, Ghent University, A. Baertsoenkaai 3, B-9000 Ghent, Belgium. Tel.: 32-92649274; Fax: 32-92649496; E-mail: kris.gevaert@UGent.be.
REFERENCES

1. Ischiropoulos, H. (1998) Biological tyrosine nitration: a pathophysiological function of nitric oxide and reactive oxygen species. Arch. Biochem. Biophys. 356, 1–11.

2. Greenacre, S. A., and Ischiropoulos, H. (2001) Tyrosine nitration: localisation, quantification, consequences for protein function and signal transduction. Free Radic. Res. 34, 541–581.

3. van der Vliet, A., Esierich, J. P., Halliwell, B., and Cross, C. E. (1997) Formation of reactive nitrogen species during peroxidase-catalyzed oxidation of nitrite. A potential additional mechanism of nitric oxide-dependent toxicity. J. Biol. Chem. 272, 7617–7625.

4. Ischiropoulos, H. (2003) Biological selectivity and functional aspects of protein tyrosine nitration. Biochem. Biophys. Res. Commun. 305, 776–783.

5. Souza, J. M., Peluffo, G., and Radl, R. (2008) Protein tyrosine nitration—functional alteration or just a biomarker? Free Radic. Biol. Med. 45, 357–366.

6. Yeo, W. S., Lee, S. J., Lee, J. R., and Kim, K. P. (2008) Nitrosative protein tyrosine modifications: biochemistry and functional significance. BMB Rep. 41, 194–203.

7. Perry, G., Raina, A. K., Nunomura, A., Wataya, T., Sayre, L. M., and Smith, M. A. (2000) How important is oxidative damage? Lessons from Alzheimer’s disease. Free Radic. Biol. Med. 28, 831–834.

8. Lee, P. P., Liberman, M., Sandoli de Brito, F., and Laurindo, F. R. (2004) Redox processes underlining the vascular repair reaction. World J. Surg. 28, 331–336.

9. Castegna, A., Thongboonkerd, V., Klein, J. B., Lynn, B., Markesbery, W. R., and Butterfield, D. A. (2003) Proteomic identification of nitrated proteins in Alzheimer’s disease brain. J. Neurochem. 85, 1394–1401.

10. Zhang, G., Qian, W. J., Knyushko, T. V., Clauss, T. R., Purvine, S. O., Moore, R. J., Sacksteder, C. A., Chin, M. H., Smith, D. J., Camp, D. G., 2nd, Bigelow, D. J., and Smith, R. D. (2007) A method for selecting enrichment and analysis of nitrosyc-containing peptides in complex proteome samples. J. Proteome Res. 6, 2257–2268.

11. Desiderio, D. M., and Zhan, X. (2003) The human pituitary proteome: the characterization of differentially expressed proteins in an adenoma compared to a control. Cell. Mol. Biol. 49, 689–712.

12. Sacksteder, C. A., Qian, W. J., Knyushko, T. V., Wang, H., Chin, M. H., Lacan, G., Melega, W. P., Camp, D. G., 2nd, Smith, R. D., Smith, D. J., Squier, T. C., and Bigelow, D. J. (2006) Endogenously nitrated proteins in mouse brain: links to neurodegenerative disease. Biochemistry 45, 8009–8022.

13. Ghersiqhre, B., Gothela, M., Van Damme, J., Staas, A., Timmerman, E., Vandekerckhove, J., and Gevaert, K. (2006) Improved tandem mass spectrometric characterization of 3-nitrotyrosine sites in peptides. Rapid Commun. Mass Spectrom. 20, 2885–2893.

14. Stevens, S. M., Jr., Prokai-Tatrai, K., and Prokai, L. (2007) Factors that contribute to the misidentification of tyrosine nitration by shotgun proteomics. Mol. Cell. Proteomics 7, 2442–2451.

15. Deckers-Hebestreit, G., Schmid, R., Kiltz, H. H., and Altendorf, K. (1987) F0 portion of Escherichia coli ATP synthase: orientation of subunit c in the membrane. Biochemistry 26, 5486–5492.

16. Nikov, G., Bhat, V., Wishnok, J. S., and Tannenbaum, S. R. (2003) Caspase-specific and non-specific in vivo protein processing during Fas-induced apoptosis. Nat. Methods 2, 771–777.

17. Lamkanfi, M., Kanneganti, T. D., Van Damme, P., Vanden Berghe, T., Vanoverberge, I., Vandekerckhove, J., Vandenebelle, P., Gevaert, K., and Núñez, G. (2008) Targeted peptidomic proteomics reveals caspase-7 as a substrate of the caspase-1 inflammasomes. Mol. Cell. Proteomics 7, 2350–2363.

18. Van Damme, P., Mauer-Stroh, S., Plasman, K., Van Durme, J., Colaert, N., Timmerman, E., De Bock, P. J., Goethals, M., Rousseau, F., Schymkowitz, J., Vandekerckhove, J., and Gevaert, K. (2009) Analysis of protein processing by N-terminal proteomics reveals novel species-specific substrate determinants of granzyme B orthologs. Mol. Cell. Proteomics 8, 258–272.

19. Arrels, A., Scheffler, N. K., Sheltar, M. D., and Gibson, B. W. (2001) Analysis of peptides and proteins containing nitrosotyrosine by matrix-assisted laser desorption/ionization mass spectrometry. J. Am. Soc. Mass Spectrom. 12, 554–564.

20. Gevaert, K., Pinxteren, J., Demol, H., Hugelier, K., Staas, A., Van Damme, J., Martens, L., and Vandekerckhove, J. (2006) Four stage liquid chromatographic separation of methionyl peptides for peptide-centric proteome analysis: the proteome of human multipotent adult progenitor cells. J. Proteome Res. 5, 1415–1428.

21. Beckman, J. S., Chen, J., Ischiropoulos, H., and Crow, J. P. (1994) Oxidative chemistry of peroxynitrite. Methods Enzymol. 233, 229–240.

22. Elias, J. E., and Gygi, S. P. (2007) Target-decoy search strategy for increased confidence in large-scale protein identifications by mass spectrometry. Nat. Methods 4, 207–214.

23. Helsens, K., Timmerman, E., Vandekerckhove, J., Gevaert, K., and Martens, L. (2008) Pepitizer, a tool for assessing false positive peptide identifications and manually validating selected results. Mol. Cell. Proteomics 7, 2364–2372.

24. Hoogenbain, B., Kamisaki, Y., Martin, E., and Murad, F. (1999) Requirements for heme and thios for the nonenzymatic modification of nitrosotyrosine. Proc. Natl. Acad. Sci. U.S.A. 96, 13136–13141.

25. Liu, P., Feasley, C. L., and Regnier, F. E. (2004) Optimization of diagonal chromatography for recognizing post-translational modifications. J. Chromatogr. A 1047, 221–227.

26. Petersson, A. S., Steen, H., Kalume, D. E., Caidahl, K., and Roepstorff, P. (2001) Investigation of tyrosine nitration in proteins by mass spectrometry. J. Mass Spectrom. 36, 616–625.

27. Souza, J. M., Daikin, E., Yudkoff, M., Raman, C. S., and Ischiropoulos, H. (1999) Factors determining the selectivity of protein tyrosine nitration. Arch. Biochem. Biophys. 371, 169–178.

28. Riordan, J. F., and Sokolovsky, M. (1971) Reduction of nitrosotyrosyl residues in proteins. Biochim. Biophys. Acta 236, 161–163.

29. Kanski, J., and Schöneich, C. (2005) Protein nitration in biological aging: proteomic and tandem mass spectrometric characterization of nitrated sites. Methods Enzymol. 396, 160–171.

30. Tedeschi, G., Cappelletti, G., Negri, A., Pagliato, L., Maggioni, M. G., Maci, R., and Ronchi, S. (2005) Characterization of nitroproteome in neuron-like PC12 cells differentiated with nerve growth factor: identification of two nitration sites in alpha-tubulin. Proteins 59, 2422–2432.

31. Fiore, G., Di Cristo, C., Monti, G., Amoresano, A., Columbano, L., Pucci, P., Cugno, S., Di Cosimo, S., and Palumbo, A., and d’Iachia, M. (2006) Tubulin nitration in human gliomas. Neurosci. Lett. 394, 57–62.

32. Haqqani, A. S., Kelly, J. F., and Birnboim, H. C. (2002) Selective nitration of histone tyrosine residues in vivo in mutantats tumors. J. Biol. Chem. 277, 3614–3621.

33. Kanski, J., Behring, A., Pelling, J., and Schöneich, C. (2005) Proteomic identification of 3-nitrotyrosine-containing rat cardiac proteins: effects of biological aging. Am. J. Phys. Heart Circ. Physiol. 286, H371–H381.

34. Vacic, V., Iakoucheva, L. M., and Radivojac, P. (2006) Two Sample Logo: a graphical representation of the differences between two sets of sequence alignments. Bioinformatics 22, 1536–1537.

35. Klotz, L. O., Schroeder, P., and Sies, H. (2002) Peroxynitrite signaling: receptor tyrosine kinases and activation of stress-responsive pathways. Free Radic. Biol. Med. 33, 737–743.
42. Brito, C., Naviliat, M., Tiscornia, A. C., Vuillier, F., Gualco, G., Dighiero, G., Radi, R., and Cayota, A. M. (1999) Peroxynitrite inhibits T lymphocyte activation and proliferation by promoting impairment of tyrosine phosphorylation and peroxynitrite-driven apoptotic death. *J. Immunol.* **162**, 3356–3366

43. Van Amersfoort, E. S., Van Berkel, T. J., and Kuiper, J. (2003) Receptors, mediators, and mechanisms involved in bacterial sepsis and septic shock. *Clin. Microbiol. Rev.* **16**, 379–414

44. Kuo, W. N., Kreahling, J. M., Shanbhag, V. P., Shanbhag, P. P., and Mewar, M. (2000) Protein nitration. *Mol. Cell. Biochem.* **214**, 121–129

45. Shao, B., Bergt, C., Fu, X., Green, P., Voss, J. C., Oda, M. N., Oram, J. F., and Heinecke, J. W. (2005) Tyrosine 192 in apolipoprotein A-I is the major site of nitration and chlorination by myeloperoxidase, but only chlorination markedly impairs ABCA1-dependent cholesterol transport. *J. Biol. Chem.* **280**, 5983–5993

46. Hermo, R., Mier, C., Mazzotta, M., Tsuji, M., Kimura, S., and Gugliucci, A. (2005) Circulating levels of nitrated apolipoprotein A-I are increased in type 2 diabetic patients. *Clin. Chem. Lab. Med.* **43**, 601–606

47. Parastatidis, I., Thomson, L., Fries, D. M., Moore, R. E., Tohyama, J., Fu, X., Hazen, S. L., Heijnen, H. F., Dennehy, M. K., Liebler, D. C., Rader, D. J., and Ischiropoulos, H. (2007) Increased protein nitration burden in the atherosclerotic lesions and plasma of apolipoprotein A-I deficient mice. *Circ. Res.* **101**, 368–376

48. Zheng, L., Nukuna, B., Brennan, M. L., Sun, M., Goormastic, M., Settle, M., Schmitt, D., Fu, X., Thomson, L., Fox, P. L., Ischiropoulos, H., Smith, J. D., Kinter, M., and Hazen, S. L. (2004) Apolipoprotein A-I is a selective target for myeloperoxidase-catalyzed oxidation and functional impairment in subjects with cardiovascular disease. *J. Clin. Investig.* **114**, 529–541

49. Dobryszycka, W., and Bec-Katnik, I. (1975) Effect of modification on physico-chemical and biological properties of haptoglobin. Acetylation, iodination and nitration. *Acta Biochim. Pol.* **22**, 143–153