Chemical labeling for the analysis of proteins, peptides and metabolites by mass spectrometry
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Protein tyrosine nitration (PTN) is a post-translational modification occurring under the action of a nitrating agent. Tyrosine is modified in the 3-position of the phenolic ring through the addition of a nitro group (NO$_2$). In the present article, we review the main nitration reactions and elucidate why nitration is not a random chemical process. The particular physical and chemical properties of 3-nitrotyrosine (e.g. pK$_a$, spectrophotometric properties, reduction to aminotyrosine) will be discussed as well as the biological consequences of PTN (e.g. modification of enzymatic activity, sensitivity to proteolytic degradation, impact on protein phosphorylation, immunogenicity and implication in disease). Recent data indicate the possibility of an in vivo denitration process, which will be discussed with respect to the different reaction mechanisms that have been proposed. The second part of this article reviews the major challenges in developing analytical methods to determine PTN in complex proteomes.


1 INTRODUCTION

3-Nitrotyrosine is a protein post-translational modification occurring under the action of a nitrating agent resulting in the addition of a nitro group (NO₂) in ortho position to the phenolic hydroxyl group (Fig. 1). Tyrosine may be nitrated through several chemical reactions and the process has been shown to be selective with respect to the proteins that are tyrosine-nitrated and the affected tyrosine residues in a given protein. The addition of a nitro group confers particular physicochemical properties to the modified amino acid and the corresponding proteins, which may have important functional consequences. Even though protein tyrosine nitration (PTN) has been considered to be a stable post-translational modification, there is increasing evidence of an in vivo denitration process. Finally, the determination of in vivo protein targets of tyrosine nitration remains an analytical challenge, and several approaches have been developed to investigate this.

Figure 1: Formation of 3-nitrotyrosine under the action of a nitrating agent

2 NITRATION REACTIONS

2.1 Tetranitromethane

Although tetranitromethane-mediated nitration cannot be considered biologically relevant, this chemical has been used in the late sixties to experimentally nitrate tyrosine residues in proteins.1-5 It has still been used later on, but peroxynitrite has been generally preferred, likely because of its supposedly greater biological relevance, the possibility of its in-house production and the fact that there are risks associated with the use of tetranitromethane (e.g. toxic, carcinogenic, explosive). Next to the modification of other amino acid residues, tetranitromethane may result in tyrosine nitrosylation (i.e. the addition of –NO) by a radical-mediated reaction.6

2.2 Peroxynitrite and its derivatives

In vivo, the peroxynitrite anion (ONOO•) is formed rapidly through reaction of the superoxide anion (O₂•⁻) with nitric oxide (NO•).7 The superoxide anion is generated in a number of biochemical reactions including cellular respiration, in activated...
polymorphonuclear leucocytes, macrophages, epithelial and endothelial cells and as a result of the mitochondrial electron flux.\(^8\) Alternatively, it can be formed by the reaction of the nitroxyanion (NO\(^-\)) with molecular oxygen.\(^9\) Since peroxynitrite (pKa of 6.5-6.8) decomposes rapidly to nitrate with a k\(_d\) of 1.25 +/- 0.05 s\(^{-1}\) at 25 °C when protonated,\(^10\) it must be stored under basic conditions in its anionic form.

Peroxynitrite is highly reactive and modifies biomolecules such as lipids, DNA or proteins. An excess of peroxynitrite is consequently involved in a number of pathological mechanisms including inflammation, cell damage (apoptosis and cytotoxicity), and may interfere with NO\(^-\)-mediated signaling.\(^8,11\) The complex chemistry of peroxynitrite has therefore been investigated ever since the discovery of NO\(^-\) as a signaling molecule.\(^12-14\)

Peroxynitrite reacts with several amino acids. Cysteine, methionine and tryptophan react directly, whereas tyrosine, phenylalanine and histidine are modified through intermediary secondary species.\(^15-18\) Protein sulphhydryls\(^19\) and tyrosyl residues are the principal targets of peroxynitrite in proteins.\(^20,21\) Oxidation of thiols is faster than tyrosine nitration,\(^15,16\) but several factors, most of them relevant under physiological conditions, can modify peroxynitrite reactivity in favor of an acceleration of tyrosine nitration. Peroxynitrite chemistry is highly pH dependent. Nitration is maximal at physiological pH (about pH 7.4) and its yield decreases quickly under more acidic or more basic conditions, whereas sulphhydryl oxidation increases from pH 5 to pH 9.\(^9,21\) Carbon dioxide and bicarbonate (respectively found at 1.3 mM and 25 mM in plasma)\(^22\) strongly influence peroxynitrite-mediated reactions.\(^22-26\) They enhance nitration of aromatic ring systems as in tyrosine (they can also promote nitration in the presence of antioxidants such as uric acid, ascorbate and thiols, which normally prevent nitration), while partially inhibiting the oxidation of thiols. Carbon dioxide reacts with peroxynitrite to form the nitrosperoxycarbonate anion (ONOOCO\(_2^-\)), which subsequently rearranges to form the nitrocarbonate anion (O\(_2\)NOCO\(_2^-\)). The latter is considered to be the direct oxidant of peroxynitrite-mediated reagents in biological environments.\(^26\) Peroxynitrite-mediated tyrosine nitration is also accelerated in the presence of transition metal ions, either in their free form (Cu\(^{2+}\), Fe\(^{3+}\), Fe\(^{2+}\)), or as complexes involving protoporphyrin IX (hemin), or certain chelators (cyanide (CN\(^-\)), ethylene diamine tetraacetic acid (EDTA)).\(^27-29\) However, Fe\(^{3+}\) complexed with diethylene triamine pentaacetic acid (Fe\(^{3+}\)-DTPA) does not react with peroxynitrite (DTPA is therefore used to obtain peroxynitrite solutions free of trace levels of metal ions).\(^12\) Metal catalysis in general plays an important role in the nitration of protein-bound tyrosine.\(^30\) Interestingly, peroxynitrite, peroxynitrite-derivatives or peroxynitrite-generating species have all been detected in cigarette smoke. These oxidants may pass the alveolar wall to raise the level of systemic oxidative stress.\(^31-33\)

Next to the fact that peroxynitrite is probably the main nitrating agent in vivo, it has the further advantage to be easily synthesized by mixing nitrite with an acidified solution of hydrogen peroxide (formation of peroxynitrous acid) and subsequent stabilization at alkaline pH as peroxynitrite anion.\(^12,34-39\) Synthetic alternatives, often more hazardous, associated with lower reaction yields or with higher levels of byproducts, exist (e.g. by bubbling ozone gas into alkaline solutions of azide, or by grinding potassium superoxide at -77 °C into liquid ammonia in a potentially explosive reaction to obtain solid, contaminant-free tetramethylammonium peroxynitrite) but are usually outside the
experimental scope of the average biochemistry laboratory. A constant flux of peroxynitrite at low-concentrations can be obtained by cogeneration of superoxide and nitric oxide through xanthine-oxidase-mediated degradation of hypoxanthine or by decomposition of spermine NONOate. Alternatively, the decomposition of the NO-generating vasodilator 3-morpholinosydnimine (SIN-1) can be used to produce low steady-state levels of peroxynitrite. SIN-1 undergoes base-catalyzed ring opening prior to reaction with an electron acceptor (generally O2) to yield superoxide and nitric oxide, that further combines to form peroxynitrite.

2.3 Other tyrosine nitration pathways

Although nitrotyrosine has been considered for some time as a direct footprint of peroxynitrite activity, it appears that other nitrating pathways, not related to peroxynitrite, can also lead to the formation of nitrotyrosine. It is actually likely that several nitrating pathways operate simultaneously, the preferred mechanism being determined by the reactive species present, kinetics, secondary reactions with antioxidants and scavengers, the presence of inflammatory cells and compartmentalization of the various components of the reaction. The details of these chemical reactions are outside the scope of this review but have been reviewed elsewhere.

Nitrotyrosine is therefore likely not a footprint for peroxynitrite alone but more generally a marker of nitrative stress. An important aspect of the multiplicity of sources for reactive species that can result in tyrosine nitration is the effect on selectivity and the eventual differences in protein nitration patterns between in vivo models and in vitro experiments, which are generally performed with peroxynitrite or tetranitromethane.

3 Selectivity of nitration

3.1 Abundance of proteins and of tyrosine residues in proteins

An important aspect of PTN is the fact that it does not occur at random. Despite the fact that most proteins contain tyrosine residues (natural abundance: 3.2 %), neither the abundance of a protein nor the abundance of tyrosine residues in a given protein can predict whether it is a target for PTN. Not all tyrosine residues are available for nitration, depending on their accessibility to solvent. With a hydrophathy index of ~1.3, tyrosine is mildly hydrophilic, a characteristic that is explained by the aromatic ring carrying a hydrophilic hydroxyl group. As a consequence tyrosine is often

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surface-exposed in proteins (only 15% of tyrosine residues are at least 95% buried) and should thus be available for nitration.\textsuperscript{51,52}

That nitration is not a random process can be exemplified with human serum albumin. This protein is a target for PTN, but despite being the most abundant plasma protein on a molar basis, it is less extensively nitrated than other plasma proteins.\textsuperscript{46} While human serum albumin contains 18 tyrosine residues, an \textit{in vitro} study of peroxynitrite-mediated PTN showed that only two tyrosines are particularly susceptible to nitration.\textsuperscript{54}

### 3.2 The effect of primary sequence and local environment

PTN does not seem to be promoted by a specific consensus sequence. Protein secondary structure and the local environment of the tyrosine residue seem to be important in determining the site of PTN.\textsuperscript{51,52} Most nitrated tyrosines are found in loop structures with turn-inducing residues (e.g. proline or glycine) in their vicinity. The proximity of a negative charge is also beneficial.\textsuperscript{51,52} Peptides with a glutamate residue in the immediate vicinity of tyrosine have been shown to be most easily nitrated.\textsuperscript{51,52} The presence of sulfur-containing residues (i.e. cysteine or methionine) in the vicinity of tyrosine residues decreases the probability of PTN, since these amino acids are alternate targets for reaction with nitrating agents and can be considered as intramolecular scavengers.\textsuperscript{51,52} Although Souza et al.\textsuperscript{51} stated that there is no apparent sequence homology between residues located at positions \(-5\) to \(+5\) around nitrated tyrosine residues, some investigators proposed possible preferred sequence motives, albeit based on a rather limited number of proteins. Elfering et al. observed in 10 identified nitrated peptides a consensus sequence \([\text{LMVI}]-X-[\text{DE}]-[\text{LMVI}]-X(2,3)-[\text{FVLI}]-X(3,5)-Y\), where \(X\) is any amino acid and \(Y\) is the target tyrosine.\textsuperscript{55} Hydrophobicity of the amino acids surrounding the target tyrosine residue seems to play an important role in determining susceptibility towards PTN.\textsuperscript{51} Based on the observation of the recurrent presence of hydrophobic residues, Elfering et al. simplified their putative consensus sequence to \(H-X-[\text{DE}]-H-(2)-X(2,4)-Y\), in which \(H\) represents a hydrophobic residue (such as L, M, V, I, P, A, F, or W).\textsuperscript{55} Other reports support selective PTN in protein transmembrane domains.\textsuperscript{56-60} Since metal catalysis plays an important role in the peroxynitrite-mediated PTN of protein-bound tyrosine,\textsuperscript{30} metalloproteins that have a metal center or a heme group do not follow the general rules presented above.\textsuperscript{51}

### 3.3 Cellular and subcellular localization of PTN

Due to the rather short-lived nature of most nitrating species, it is conceivable that the site of generation of a nitrating agent with respect to the target protein plays a role in determining which proteins become nitrated and possibly also in defining the primary nitration sites. In several disease models, nitrated proteins have been detected at the site of injury or within specific cell types.\textsuperscript{52} It is not surprising that nitrated proteins have been mainly observed in subcellular compartments that are known to generate nitrating species. For instance, nitrotyrosine-containing proteins were identified by immuno-electron microscopy in (i) peroxidase-containing secretory granules, in quiescent eosinophils and neutrophils in the bone marrow, (ii) in the cytosol of circulating erythrocytes, (iii) in mitochondria and the endoplasmic reticulum of
endothelial cells, fibroblasts and smooth muscle cells, in the vasculature, (iv) in association with the cytoplasmic interface of the endoplasmic reticulum membrane in cartilage chondrocytes and (v) in the peroxisomes of liver hepatocytes and of secretory cells in the lachrymal gland.\textsuperscript{62} Based on proteomics techniques aiming at identifying nitrated proteins, Kanski et al. identified mainly cytosolic nitrated proteins in skeletal muscle, whereas nitrated proteins in the heart were principally mitochondrial.\textsuperscript{63}

Mitochondria appear to be a primary locus of PTN. Peroxynitrite can diffuse from extra-mitochondrial compartments into the mitochondria or be formed inside mitochondria.\textsuperscript{64} This is not surprising, since one of the main sources of reactive oxygen species are complexes I-III of the mitochondrial respiratory chain.\textsuperscript{65} Within the mitochondrion, PTN occurs largely in the matrix\textsuperscript{62, 66, 67} whereas it is typically found in association with the cytosolic side of the membrane in the endoplasmic reticulum.\textsuperscript{62}

### 3.4 Nitrating agents and selectivity

As summarized above, peroxynitrite is not the sole nitrating agent \textit{in vivo}. The reactivity of a tyrosine residue may thus also depend on the nature of the reactive species.\textsuperscript{51} While peroxynitrite and tetranitromethane nitrate certain proteins in a comparable manner,\textsuperscript{68} there are differences in PTN patterns in other proteins.\textsuperscript{59, 70} For instance, a strong inhibition of MnSOD catalytic activity by peroxynitrite-mediated PTN has been reported and explained by nitration of the essential tyrosine residue 34,\textsuperscript{71, 72} while PTN via a cytochrome C/H$_2$O$_2$/NO$_2^-$-mediated reaction resulted also in PTN of MnSOD, but not in a significant loss of activity indicating that Tyr34 was not nitrated in this case.\textsuperscript{73} There is clearly much more work ahead to understand the effect of the chemical nature of the nitrating agent on selectivity but these examples show that it can play an important role next to the compartmentalization of the reaction.

### 3.5 Contribution of preferential protein denitration or degradation to the overall abundance of PTN-modified proteins

The accumulation of specific tyrosine-nitrated proteins is the result of the PTN reaction, a putative denitration process (for details on the denitration reaction see section 6) and the degradation rate of tyrosine-nitrated proteins. Elfering et al. observed in fractions of rat liver mitochondria that PTN in mitochondrial fractions does not correlate with the mean half-live of proteins, indicating that the longer-lived proteins do not necessarily have a higher probability of being nitrated.\textsuperscript{55}

### 3.6 Correlation of \textit{in vivo} and \textit{in vitro} PTN

Frequently, proteins have been exposed to nitrating agents \textit{in vitro} to predict their potential sensitivity toward PTN \textit{in vivo} and to obtain standard nitrated peptides for the screening of biological samples. However, in some instances these two approaches have yielded quite different results.\textsuperscript{63, 74} The distinctly different patterns may be caused by one or more of the following factors. (i) Specific isoforms of nitrated proteins may be eliminated \textit{in vivo} through proteasome-mediated degradation or denitration.\textsuperscript{63, 74} (ii) As discussed previously, peroxynitrite is likely not the only nitrating agent \textit{in vivo}, and the
nature of the nitrating agent may influence selectivity. Moreover, the concentration of the nitrating agent in vitro may be quite different from the local concentration in vivo and thus affect selectivity. It has, for instance, been shown that the preference for PTN of two tyrosine residues in human serum albumin was more pronounced when the relative concentration of peroxynitrite with respect to albumin was decreased.54 (iii) Target proteins may exist in vitro in complexes with other proteins or small molecular weight compounds (phosphocreatine, ATP), which can affect the chemical selectivity of PTN.63, 74 For instance, a protective effect of phosphocreatine and ATP was observed on the modification of creatine kinase by peroxynitrite.75 (iv) Finally, although in vitro PTN conditions can be chosen to mimic the physiological conditions, they can never reproduce the local conditions within a cell or a tissue. Differing conditions might cause conformational changes in target proteins when compared with the in vivo situation, which is potentially associated with an altered susceptibility of specific tyrosine residues to PTN.63 Moreover, since peroxynitrite is highly susceptible to certain factors (e.g. bicarbonate, metal ions), it is no easy task to reproduce the exact in vivo conditions in vitro.

4 PHYSICOCHEMICAL PROPERTIES OF NITROTYROSINE

Tyrosine nitration has a slight effect on the bulkiness of the residue, which becomes 30 Å³ larger than the 205 Å³ of non-modified tyrosine.77 Other physicochemical properties that are affected by tyrosine nitration are: a decrease in pKa of the phenolic OH-group, a change in light absorption and the possibility to be reduced to aminotyrosine, which has again another set of physicochemical properties. From an analytical point of view it is also of interest to note that the nitro group influences the ionization process of nitrotyrosine prior to mass spectrometry (see section 7.2).

4.1 Effect of nitration on the pKa of the phenolic hydroxyl group

The major effect of nitration of tyrosine on its physicochemical properties is possibly the shift of the pKa value of the hydroxyl group (10.1 in tyrosine and 7.2 in nitrotyrosine)78 (a report by the same authors published in the same year reported a value of 6.8 for nitrotyrosine).3 The hydroxyl group of nitrotyrosine is about 50% charged at physiological pH, whereas tyrosine is neutral. At acidic pH, where the hydroxyl group is uncharged, nitrotyrosine is more hydrophobic than tyrosine, resulting, for instance, in increased retention times of tyrosine-nitrated peptides as compared to their non-modified counterparts upon reverse-phase liquid chromatography (RPLC). On the contrary, at basic pH, the higher tendency of nitrotyrosine to carry a negative charge renders it much more hydrophilic than tyrosine.76 Both of these effects, steric hindrance and pKa change, affect the reactivity of tyrosine residues and prevent, for example, its phosphorylation.

4.2 Spectroscopic properties

Nitrotyrosine can form an internal hydrogen bond between the nitro and the phenolic OH-group causing its absorption properties to be strongly pH-dependent.76 At
acidic pH (pH < 6), nitrotyrosine has an absorption maximum at 357-360 nm. Consequently 360 ± 5 nm has been used as the wavelength for detection of nitrotyrosine-containing peptides by HPLC-UV under acidic conditions. It should be noted that protein modifications other than tyrosine nitration (e.g. tryptophan oxidation, other nitration or nitrosation products) may contribute to absorbance at this wavelength. Under basic conditions (pH > 8), nitrotyrosine has an absorption maximum at 427-430 nm giving it a yellow color. Moreover, absorbance at 276 nm, the maximum of absorbance common to all aromatic amino acids, decreases.

Nitrotyrosine is essentially nonfluorescent and absorbs at a wavelength where tyrosine and tryptophan emit. It may therefore function as a potential energy acceptor in Förster resonance energy transfer (FRET) studies and quench the emitted fluorescence from tryptophan or tyrosine residues depending on their proximity. The reduction of nitrotyrosine to aminotyrosine renders it fluorescent with emission maxima at 308 nm (such as tyrosine) as well as at 350 nm between pH 3.0 and 3.5. This opens the possibility to detect nitrotyrosine indirectly after reduction to aminotyrosine.

4.3 Reduction of nitrotyrosine to aminotyrosine

Nitrotyrosine can be reduced to aminotyrosine by different means. Based on the knowledge that sodium dithionite (also known as sodium hydrosulfite) catalyzes the reduction of nitrophenol to aminophenol, Sokolovsky et al. reported the use of this reagent for the reduction of nitrotyrosine to aminotyrosine under conditions that are mild enough for protein modification. Riordan and Sokolovsky reported a reaction yield of about 90% and observed another, more acidic by-product. Using elemental analysis (showing the presence of sulfur), absorption spectra under basic and acidic conditions (showing that the phenolic hydroxyl group can be ionized and is therefore not substituted), and the analysis of aminosulfonic acid and its N-substituted derivatives, they concluded that the by-product is an aminotyrosine derivative that is N-substituted with -SO₂H, -SO₃H or -S₂O₃H. Observing an 80-Da mass increase after dithionite-mediated reduction of nitrotyrosine Ghesquière et al. suggested the formation of aminotyrosine O-sulfate as a by-product. Based on both of these reports, it is likely that this by-product is actually an N-substituted aminotyrosine derivative with -SO₃H.

To avoid false positive results, the specificity of anti-nitrotyrosine antibodies must be checked. Reduction with dithionite has been proposed to differentiate between genuine detection of nitrotyrosine and artifacts in immunohistochemistry (IHC), immunoprecipitation and immunoblotting experiments. Dithionite reduction has been extensively used in immunoblotting experiments despite its reactivity and thus instability under atmospheric conditions. This can result in incomplete reduction of proteins on surfaces or membranes. Moreover, aminotyrosine can reoxidize to nitrotyrosine over time resulting in weak signals even on dithionite-treated membranes. Since the nitro group itself is not very reactive, reduction to aminotyrosine is often used prior to derivatization with a tag to study free and protein-bound nitrotyrosine. Some of these techniques are depicted in greater detail in section 7.1.7.

Dithionite-mediated reduction of nitrotyrosine has also been used for analysis of nitrotyrosine by HPLC followed by electrochemical detection. Reducibility by dithionite
is, however, not sufficient proof for the presence of nitrotyrosine, since the presence of a closely eluting dithionite-reducible peak, which is not nitrotyrosine, was described in the HPLC analysis of human brain tissue.\textsuperscript{97, 98} Since aminotyrosine is poorly retained on C\textsubscript{18} reversed phase columns, its modification to an N-acetyl derivative (followed by the reversal of O-acetylation) was required prior to analysis.\textsuperscript{99, 100} To avoid these extra steps, on-line electrochemical reduction of nitrotyrosine to aminotyrosine prior to electrochemical detection has been developed using a platinum column as electrode,\textsuperscript{101} a gold amalgam electrode,\textsuperscript{102} or glassy carbon electrodes.\textsuperscript{103, 104}

The dithionite-based reduction of nitrotyrosine is not free of side reactions, as described above, which asks for alternatives especially when working with complex biological mixtures. Balabanli et al. showed more recently that dithiothreitol (DTT) in combination with heme can reduce nitrotyrosine to aminotyrosine quantitatively at 100°C.\textsuperscript{105} This phenomenon may cause underestimation of the PTN level in immunoblotting experiments after polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate (SDS-PAGE), since samples are often boiled in a so-called “sample buffer” containing DTT and complex biological samples often contain heme-containing proteins.\textsuperscript{106} Although much slower, reduction occurs also at 37°C and may explain the observed partial loss of nitrotyrosine \textit{in vivo} referred to as “denitration” (see section 6 for further details).\textsuperscript{105, 107} Ghesquière et al. reduced nitrotyrosine based on the report of Balabanli and coworkers with Fe\textsuperscript{2+}-charged cytochrome C as the heme-containing protein, showing this reaction to be generally preferable to reduction with dithionite due to a better control of the reaction and the absence of by-products.\textsuperscript{84}

5 \hspace{0.915cm} \textbf{BIOLOGICAL CONSEQUENCES OF PTN}

5.1 \hspace{0.915cm} \textbf{Effect on protein function}

PTN can result in a loss, an increase, or no effect on protein function. Pacher et al.\textsuperscript{108} and Souza et al.\textsuperscript{61} recently reviewed the literature with respect to the effect of PTN on protein activity. It should be noted, however, that not all studies showed unambiguously that PTN was the cause for altered protein function, which is critical before drawing conclusions, since nitrating reagents can also alter other amino acids (e.g. cysteine and methionine).\textsuperscript{61}

5.2 \hspace{0.915cm} \textbf{Sensitivity to proteolytic degradation}

Oxidative modifications have been shown to increase the susceptibility of modified proteins to proteolysis. The removal of oxidized proteins can be seen as a defense mechanism against the consequences of oxidative stress.\textsuperscript{109} An increase in proteolytic degradation as a result of exposure of isolated proteins or cells in culture to peroxynitrite was indeed observed.\textsuperscript{109-112} This effect was shown to be due to a faster degradation of tyrosine-nitrated proteins by the proteasome. Steady-state protein levels were restored by incubation with an antisense oligodeoxynucleotide to the initiation codon region of the C2 subunit of the proteasome or by lactacystin (a proteasome inhibitor),\textsuperscript{106, 111} but not by leupeptin (inhibitor of trypsin and trypsin-like proteases,}
PMSF (serine protease inhibitor) or calpeptin (calpain inhibitor). Of the four reports cited above, two attributed this effect to PTN, although only one of them proved this unambiguously in the case of bovine Cu,Zn superoxide dismutase, where exposure to peroxynitrite resulted in nitration of a single tyrosine residue without any significant other amino acid modifications. Since other amino acids can be modified by peroxynitrite, further work is needed to substantiate the relationship between PTN and protein degradation. Elfering et al. observed in rat liver mitochondria that nitrating conditions increased protein turnover significantly from days to hours. Based on immunoblotting, the authors suggested that PTN might trigger mitochondrial proteolytic enzymes. However, another plausible explanation is that nitrated proteins are “denitrated”, since an active denitration process has been shown in mitochondria (see section 6).

5.3 Influence on tyrosine phosphorylation

Next to alterations of the structure and function of proteins, PTN might affect cellular function by interfering with tyrosine phosphorylation. The latter is an important regulator of signal transduction in cells and has been implicated in cellular responses to growth factors, cytokines, and calcium ionophores. The shift in pH of the phenolic hydroxyl group due to nitration may be the major cause for this interference (tyrosine kinases phosphorylate the neutral phenolic hydroxyl group rather than the negatively charged phenolate) but steric hindrance and a distortion of the local protein structure may also contribute. The reverse situation has also been observed, in that phosphorylation of tyrosine prevents subsequent PTN. Therefore, nitration and phosphorylation are two competing modifications at a given tyrosine residue. However, other reports suggest that peroxynitrite, the main reactive species to promote PTN, could also promote phosphorylation at the cellular level. Based on these observations and increasing evidence for a putative enzymatic denitration process, a new concept arises. The influence of PTN on tyrosine phosphorylation and vice versa may play a role in modulating signal transduction in addition to the well-known phosphorylation-dephosphorylation reaction that is mediated through kinases and phosphatases. The relationship between PTN and phosphorylation has been reviewed by Monteiro et al.

5.4 Immunogenicity

Endogenous proteins are normally not immunogenic due to immunological tolerance. However, post-translational modifications of self-proteins may lead to the generation or unmasking of epitopes, resulting in the triggering of an immune response, which may induce autoimmune disease. Studies of the recognition of tyrosine nitrated proteins by T-cells showed that PTN of a tyrosine residue in a T-cell receptor (TCR) contact position may result in the formation of an immunogenic neoepitope. Moreover, it was shown that nitration of tyrosines located in non-TCR-contact positions can have an indirect yet major impact on stimulation of the immune system by affecting interactions of the TCR with the peptide-loaded major histocompatibility complex. Nitrotyrosine is furthermore structurally similar to a widely used synthetic hapten, 4-hydroxy-3-nitrophénylacetyl, which some anti-DNA antibodies cross-react with.
A variety of post-translationally modified (including nitrated) proteins have been shown to accumulate in apoptotic or inflamed tissue. The accumulation in inflamed tissue of nitrotyrosine-containing autologous proteins, that appear as foreign to the immune system, might induce an autoimmune response and sustain a chronic inflammatory response. Elevated levels of anti-nitrotyrosine antibodies have indeed been measured in synovial fluid of patients with rheumatoid arthritis and osteoarthritis, as well as in the serum of patients with systemic lupus erythematosus (a multisystem autoimmune disease) or after acute lung injury.

PTN of endogenous proteins may not only trigger deleterious, abnormal immune reactions but also impair beneficial, normal responses due to impaired TCR recognition, or due to PTN of the TCR itself. Oxidative stress as a result of the accumulation of myeloid-derived suppressor cells has been shown to provoke TCR nitration, leading to CD8+ T-cell tolerance, which is a major cause for the escape of tumor cells from immune recognition.

Finally, PTN may play an important role in inducing and sustaining the chronic inflammatory condition that leads to airway obstruction in several inflammatory lung disorders including chronic obstructive pulmonary disease (COPD). Macrophage stimulation by inflammatory signals or oxidative stress can lead to the targeted increase of chromatin modifications, such as histone acetylation, resulting in the transcription of genes that drive the inflammatory response. Histone deacetylase-2 (HDAC2) can normally be recruited by corticosteroids to reverse histone acetylation and switch the transcription of inflammatory genes off. One hypothesis to explain the resilience of COPD patients to treatment with corticosteroid-based anti-inflammatory drugs, as compared to asthma patients, is that PTN-induced inactivation of HDAC2 curbs the possibility of this enzyme to turn inflammatory gene transcription off in response to corticosteroids.

5.5 PTN and mitochondria

Mitochondria (more exactly the mitochondrial matrix) appear to be the primary locus for PTN. Peroxynitrite can diffuse from extramitochondrial compartments into mitochondria or be formed intramitochondrially through the generation of reactive oxygen species due to the activity of complexes I-III of the mitochondrial respiratory chain. Several reports have investigated the PTN of mitochondrial proteins and its possible consequences. PTN and inactivation of manganese superoxide dismutase (MnSOD) was shown to occur long before the onset of renal dysfunction in a rat model of chronic allograft nephropathy. MnSOD normally counteracts peroxynitrite formation by dismutating superoxide. However, the fact that it is in itself sensitive and inactivated by PTN can lead to a vicious circle, where increased peroxynitrite formation leads to further oxidative damage. Such a cycle was observed in an animal model of renal ischemia/reperfusion, where MnSOD was rapidly nitrated leading to further renal injury and PTN of other mitochondrial proteins. PTN of mitochondrial proteins may then lead to further structural damage and to alteration in energy production, antioxidant defense, and apoptosis.

Initially the generation of nitrotyrosine was considered to be an irreversible process. However, recent reports indicate that there is a protease-independent but oxygen-
tension-dependent denitration system in isolated mitochondria suggesting an organelle-specific mechanism against NO-mediated oxidative stress at decreasing oxygen tension. This important phenomenon will be discussed in section 6. An extension of this hypothesis proposes that in mitochondria there is a signaling nitration–denitration pathway that fulfills four basic criteria: (i) specific modification of target proteins, (ii) altered activity/functionality of the modified protein, (iii) reversibility of the modification, and (iv) nitration/denitration occurring on a physiological timescale.

5.6 Implication of PTN in disease

Nitrotyrosine in its free or protein-bound form has been detected in association with at least 50 diseases and more than 80 animal models of disease or cell culture systems. Details may be found in an extensive review by Greenacre and Ischiropoulos. It has been observed, for instance, in association with cardiovascular disease, lung disease, diabetes, and neurodegenerative disease. PTN is in most cases a result of oxidative stress and may serve as biomarker but it may also aggravate the disease process as outlined above in the case of renal ischemia/reperfusion injury.

6 Denitration

Because of its chemical stability over a wide pH range (from 3.5 to 9.0), against prolonged incubation at 100°C, and the absence of a known enzymatic process removing or converting the nitro group, PTN was initially believed to be a stable post-translational protein modification. However, there is increasing evidence of a biological denitrating system. Some reports showed that plasma, isolated platelets, LDL- and HDL-containing plasma lipoprotein fractions, activated macrophages, and tissue homogenates or crude extracts from diverse organs (liver, brain, lung, heart, spleen and prostate) reduce the level of nitrotyrosine in a time-, concentration- and temperature-dependent manner in the absence of apparent proteolytic activity (e.g. no effect of the proteasome-inhibitor lactacystin). Denitration is LPS-inducible and sensitive to heat, trypsin and peroxynitrite treatment. The activity was retained after filtration through a 10-kDa cutoff membrane. It is presently unclear whether an enzymatic “denitrase” complex exists and what its constituents might be.

Denitration shows some selectivity both at the tissue and the protein level. With homogenates of rat kidney, rat liver or human erythrocytes a decrease of nitrated BSA was observed in conjunction with proteolysis, while denitration was impaired in the presence of protease inhibitors. On the contrary, a loss of nitrated BSA was observed by immunoblotting with an anti-nitrotyrosine antibody with homogenates of spleen or lung tissue in the presence of protease inhibitors. The denitration reaction exhibits different kinetic profiles towards different substrates. Histone H1.2 and calmodulin were shown to be preferential targets for denitration. Several reports showed denitration of certain nitrated proteins (e.g. bovine serum albumin) as substrate but not of free nitrotyrosine. Endogenous nitrated proteins of spleen homogenates, used to denitrate nitrotyrosine-containing albumin, were shown not to be substrates for this
reaction, because the intensity of staining of these nitrotyrosine-containing proteins with anti-nitrotyrosine antibodies upon Western blotting did not decrease during incubation.\textsuperscript{150} It may thus be hypothesized that there is a balance between nitration and denitration, which, in combination with protein turnover, results in the fact that only subsets of nitrated proteins accumulate.\textsuperscript{153}

In isolated mitochondria, denitration was not affected by protease inhibitors but was oxygen tension-dependent.\textsuperscript{65, 88} Freshly isolated mitochondria showed a background of PTN that disappeared rapidly during hypoxia/anoxia treatment. After reoxygenation (a phase known to be associated with a burst of reactive oxygen species), PTN was again increased finally exceeding the level in the starting sample. This process was NO•-dependent, since it occurred in presence of L-arginine but not D-arginine, which is not a substrate of mitochondrial nitric oxide synthase.\textsuperscript{65, 88} In contrast to what is observed in mitochondria, total PTN was reported to increase in whole organs exposed to ischemic hypoxia. This leads to the hypothesis that mitochondrial denitration would be an organelle-specific mechanism against nitrative stress under decreasing oxygen tension, because mitochondria are one of the main sources of reactive oxygen species resulting from the activity of complexes I-III of the mitochondrial respiratory chain.\textsuperscript{65}

Loss of nitrotyrosine recognition through antibodies likely results from the reduction of nitrotyrosine to aminotyrosine, or the complete removal of the nitro moiety although other modifications cannot be excluded.\textsuperscript{65, 148, 150} As stated before, nitrotyrosine can be reduced to aminotyrosine in a purely chemical reaction between Fe\textsuperscript{3+}-containing heme and a reducing agent such as DTT\textsuperscript{107} (a reaction that has also been described in the case of the reduction of nitrated deoxynucleobases to their amino analogues)\textsuperscript{154} under physiological conditions (pH 7.2, 37 °C). This has also been observed with heme-containing proteins such as hemoglobin and myoglobin as well as with the cobalt-containing cyanocobalamin. Aminotyrosine appeared as the end-product while no unmodified tyrosine was detected.\textsuperscript{107} This is in agreement with the observation of aminotyrosine-containing peptides in α-synuclein of rotenone-exposed neurons.\textsuperscript{155}

Interestingly, a Ca\textsuperscript{2+}-dependent denitration process was observed in homogenates of brain and heart,\textsuperscript{149} and in LDL- and HDL-containing plasma samples\textsuperscript{147} with a concomitant stoichiometric increase of nitrate ion concentration. Notably, addition of calcium to freshly isolated platelets accelerated the denitration process.\textsuperscript{146} The authors proposed the following putative denitration reaction mechanism: Tyr-NO\textsubscript{2} + H\textsubscript{2}O \rightarrow Tyr-H + H\textsuperscript{+} + NO\textsubscript{3}-, which involves the direct removal of the nitro group without prior reduction to aminotyrosine.\textsuperscript{147, 149}

Based on all these data, there may exist two mechanisms of denitration. Observing that denitration activity decreased by 70% in predialyzed brain homogenates, Kuo et al. suggested the presence of two types of enzyme systems: one that is dependent on additional reducing agents and another type that does not require such agents.\textsuperscript{149} Based on the recent report of in vivo heme-mediated reduction of nitrated proteins,\textsuperscript{107} a reductant-dependent, hemoprotein-dependent non-enzymatic conversion of nitrotyrosine to aminotyrosine as well as a reductant-independent, Ca\textsuperscript{2+}-dependent enzymatic conversion of nitrotyrosine to tyrosine may be postulated. Free nitrotyrosine may be further metabolized by O-sulfation mediated by the cytosolic sulfotransferase
SULT1A3.\(^{145}\) It was not reported by the authors whether this reaction can also occur with protein-bound nitrotyrosine.

7 **ANALYTICAL METHODS FOR THE DETECTION OF PTN IN COMPLEX BIOLOGICAL SAMPLES**

Many methods developed so far have focused on the detection and quantification of 3-nitrotyrosine at the amino acid level either in its circulating free form or after release by protein hydrolysis (see reviews by Herce-Pagliai et al.,\(^{156}\) Greenacre and Ischiropoulos,\(^{132}\) Duncan,\(^{157}\) Tsikas and Caidahl\(^{158}\) and Ryberg and Caidahl\(^{159}\)). Depending on the disease and the tissue of interest, a 2-10 fold increase in the level of tyrosine-nitrated proteins and a 1.5-2 fold increase in free nitrotyrosine have been reported.\(^{132}\)

While these analyses are of relevance to assess NO•-dependent oxidative stress, a major drawback of the analysis of free nitrotyrosine is that it does not provide information concerning the identity and concentration of individual nitrated proteins.\(^{160}\) Although a much more challenging analytical task, obtaining site-specific information is key to relating nitrotyrosine formation to biological mechanisms.\(^{161}\) Such information would also open the possibility to establish a relationship between defined nitrated proteins and a pathological phenotype.\(^{52}\) In the following, we review methods used for the analysis and identification of tyrosine-nitrated proteins and the localization of PTN sites, extending previous reviews by Kanski and Schöneich\(^{89}\) and Nuriel et al.\(^{162}\)

7.1 **Separation and enrichment methods**

7.1.1 **Two-dimensional polyacrylamide gel electrophoresis (2DE)**

Study of the “nitroproteome” by 2DE has resulted in most of the identifications of endogenously nitrated proteins to date (see Table 1). 2DE followed by immunoblotting, in-gel digestion and MS identification has been widely used for nitroproteome studies.\(^{86, 87}\) The clone 1A6 monoclonal antibody is commonly used for immunoblotting.\(^{89}\) It has been well characterized and is less prone to problems of non-specific recognition and batch-to-batch variability than a polyclonal antibody.\(^{86}\) Other antibodies are available and have been compared with respect to specificity and the recognition of nitrotyrosine.\(^{163, 164}\) To control for nonspecific binding, sodium dithionite-mediated reduction of nitrotyrosine is recommended.\(^{86}\) Although this method has been extensively used, it is technically difficult to achieve adequate reduction of proteins on the membrane due to the sensitivity of sodium dithionite to molecular oxygen.\(^{85}\) \(^{88}\) and the fact that aminotyrosine can reoxidize to nitrotyrosine over time, which may result in weak signals even on dithionite-treated membranes.\(^{89}\)

2DE has, however, several shortcomings (e.g. limited loadability of the gels, poor recovery of membrane proteins) that explain why the identified, nitrated proteins are generally limited to abundant and soluble proteins. The failure of many 2DE approaches to actually detect the peptides carrying the PTN site is likely due to a number of reasons:
(i) the limited protein load combined with the low steady-state levels of nitrotyrosine on a given protein and the low abundance of some of the nitrotyrosine-containing proteins, (ii) the solubility, size, and/or extreme pI values of proteins, which may compromise isoelectric focusing, and (iii) the recovery of nitrotyrosine-containing peptides from the gels and/or HPLC columns during subsequent LC-MS/MS analysis. The difficulty to analyze hydrophobic membrane proteins with the 2DE approach is an important limitation when it comes to studying PTN, since the selective nitration of tyrosine in protein transmembrane domains has been reported. Finally, it is sometimes not possible to assign immunopositive spots on the blotting membrane to stained spots in the gel or to the identified proteins, since a spot may contain multiple proteins. It is therefore indispensable to confirm PTN by mass spectrometry as a final proof.

### 7.1.2 Solution isoelectric focusing / SDS-PAGE (IEF/1DE)

In-solution isoelectric focusing followed by SDS-PAGE or direct protein digestion in solution has allowed MS/MS identification of nitrated proteins when this was not possible using immunoprecipitation or 2DE. The substitution of the first dimension of 2DE by in-solution isoelectric focusing followed by SDS-PAGE allows higher sample loads, up to several mg in the first dimension (i.e. an increase of sample load by a factor of about 10), when compared to in-gel isoelectric focusing, where protein loads were generally on the order of about 0.5 mg. Moreover, in-solution isoelectric focusing is more suitable for hydrophobic membrane proteins. However, a drawback of this technique is a lower resolution compared to 2DE, manifested by the presence of proteins of interest across several collected fractions.

### 7.1.3 HPLC / SDS-PAGE

Another approach developed to overcome the limitation of 2DE in terms of protein loading capacity and resolution of hydrophobic proteins combines pre-fractionation of proteins by reverse phase HPLC on C4-columns with SDS-PAGE. Collected HPLC fractions are further separated by SDS-PAGE and nitrated proteins detected by Western blot analysis.

### 7.1.4 Two-dimensional liquid chromatography

Multidimensional chromatographic separations based on a combination of ion-exchange and reverse phase HPLC of digested protein mixtures followed by tandem MS-based protein identification have been successfully used for nitroproteome studies. This technique overcomes some of the limitations of gel-based analyses and allows the quantification of the extent of PTN. However, this technique produces large amounts of data that require extensive data processing and analysis.
7.1.5 Immunoprecipitation / SDS-PAGE (IP/1DE)

MacMillan-Crow and Thompson were the first to describe a method for the immunoprecipitation of nitrotyrosine-containing proteins based on the use of a monoclonal anti-nitrotyrosine antibody (clone 1A6) followed by SDS-PAGE and Western blot analysis. According to Guo et al., immunoprecipitation with an anti-nitrotyrosine antibody is the preferred technique for detecting unknown tyrosine-nitrated proteins in tissue. Importantly, unlike 2DE, IP/1DE does not discriminate against proteins with very basic isoelectric points or membrane proteins. Immunoprecipitation has, however, the inconvenience to be lengthy (16 h for the incubation alone) and to use more antibody than typical Western blot analyses. Furthermore, the recovery and identification of proteins having similar molecular weights as the heavy and the light chains of the anti-nitrotyrosine antibody (55 and 25 kDa) may be difficult, although this problem can be circumvented by the use of covalently immobilized anti-nitrotyrosine antibodies that are now commercially available. Finally, IP is no guarantee that only tyrosine-nitrated proteins will be precipitated, since most antibodies, as well as the supports that they are bound to, exhibit considerable non-specific binding. It is thus indispensable to identify peptides containing a nitrotyrosine residue to be certain. Some studies reported also that immunoprecipitation of nitrated proteins resulted in insufficient amounts for gel electrophoretic and/or MS/MS analysis, a common problem with identification of low-abundance, post-translationally modified proteins.

7.1.6 Immunoaffinity chromatography

In the early 1970s Helman and Givol developed a column of anti-nitrotyrosine antibodies immobilized on Sepharose for the affinity capture of nitrated peptides from in vitro tetranitromethane-treated lysozyme following trypsin digestion. Based on this report, other investigators conducted similar experiments for the study of nitrotyrosine-containing proteins generated either by tetranitromethane-mediated PTN or after chemical coupling of nitrotyrosine to carboxylic acid groups through reaction of nitrotyrosyl ethyl ester after activation with 1-cyclohexyl-3-(2-morpholinoethyl) carbodiimide metho-p-toluenesulfonate. The recovery of nitrated peptides was, however, variable reaching 55% in the case of Helman and Givol’s report, based on UV absorbance at 381 nm. Later Zhan et al. reported the identification of endogenous, nitrated proteins from pituitary adenoma tissue using an immobilized anti-nitrotyrosine antibody column. They sought to increase sensitivity by allowing the sample to incubate for 19 h with the immunoaffinity resin and enhance selectivity by using a stringent washing procedure.

7.1.7 Chemical modification of nitrotyrosine followed by affinity capture

Based on the pKₐ difference between the aromatic amine of aminotyrosine (pKₐ = 4.75) and other aliphatic amines present in proteins (pKₐ of 8.0 for α-amines and
10.5 for ε-amines), some investigators sought to selectively modify aminotyrosine at pH 5, (generally with an affinity tag) after nitrotyrosine reduction. In the late sixties, Sokolovsky et al. reported an experiment where they derivatized aminotyrosine-containing angiotensin II with succinic anhydride (20% of the α-amino group was also modified, however). Bustin and Givol reported the modification of aminotyrosine-containing lysozyme with fluorodinitrobenzene to form dinitrophenylaminotyrosyl-derivatives (yield: 63%) to make use of anti-dinitrophenyl antibodies for detection, since anti-nitrotyrosine antibodies were not readily available at that time. More than three decades later, Nikov et al. investigated this possibility with N-hydroxysuccinimide biotin ester (NHS-biotin) by first converting nitrotyrosine to aminotyrosine using sodium dithionite followed by labeling of the aromatic amino group with NHS-biotin at pH 5.

The selectivity of a labeling method is of primary importance when it comes to the study of low abundance post-translational modifications. Although pH-controlled selectivity of NHS-based acylations has been attempted previously to label α- but not ε- amines, it was also reported that selectivity of NHS esters for the preferential labeling of N-terminal amines is rather poor. In agreement with reports by other investigators, we observed that aliphatic amines reacted with NHS esters to a certain extent at pH 5 indicating that the method of Nikov et al. is not selective enough to enrich nitrotyrosine-containing proteins from complex biological matrices.

Recently Zhang et al. published an approach for the chemical labeling and enrichment of nitrotyrosine-containing peptides based on acetylation of primary amines with acetic acid anhydride followed by reduction of nitrotyrosine with sodium dithionite and the acylation of aminotyrosine with NHS S-acetylthioacetate (SATA). Independently of this work, we developed a chemical approach for the biotinylation of nitrotyrosine residues in peptides with each step reaching a yield close to 100% as determined by LC-MS (see Chapter 5 of this thesis). Having a quantitative and selective method is critical to avoid labeling of other functional groups, such as primary amines, thiols or hydroxyl groups, which are much more abundant than nitrotyrosine and would thus lead to the enrichment of many non-nitrotyrosine-containing peptides. Very recently, Nuriel et al. proposed another approach based on reductive dimethylation of primary amines followed by reduction of nitrotyrosine and biotinylation of aminotyrosine with NHS-SS-biotin at pH 5. However, no applications based on the use of this method have been reported at present.

7.2 Mass spectrometric analysis of nitrotyrosine-containing proteins and peptides

7.2.1 Matrix-assisted laser desorption ionization (MALDI)

Matrix-assisted laser desorption ionization (MALDI) with laser light at 337 nm combined with mass spectrometry (MS) is a widely used technique for the analysis of proteins and peptides. Nitrotyrosine-containing peptides are, however, sensitive to light of this wavelength and undergo a decomposition process yielding, besides the expected mass increase of 45 amu for the addition of a nitro group (Tyr-NO₂ [M+H⁺+45]), other major peaks corresponding to nitrosotyrosine ((Tyr-NO) [M+H⁺+29]), aminotyrosine ((Tyr-NH₂) [M+H⁺+15]), and nitrenetyrosine ((Tyr-N) [M+H⁺+13]). This can be
explained by the fact that the absorbance maximum of nitrotyrosine under acidic conditions is ~360 nm (see section 4.2), which is in the vicinity of the emission wavelength of the nitrogen ion laser (337 nm). The absorbance maximum shifts to ~430 nm under basic conditions.183

While on the one hand this phenomenon constitutes a unique fingerprint for the detection of nitrated peptides, it also impairs the sensitivity of detection, since it spreads the molecular ion signal over at least three major species, hence reducing the signal by as much as 60-70%, which may result in the failure to observe nitrated peptides in complex peptide maps.164, 178-186 This is aggravated by the fact that PTN is a low-abundance protein post-translational modification with an occurrence of approximately 1 in 10000 tyrosines in vivo.48

The pH-dependence of nitrotyrosine photodegradation is related to the MALDI matrix and its respective pKₐ. Petersson et al. observed less fragmentation with the neutral matrix 2,4,6-trihydroxyacetophenone (THAP) than with acidic matrices such as α-cyano-4-hydroxycinnamic acid (CHCA) or dihydroxybenzoic acid (DHB).183 Sheeley et al. investigated this further with the tyrosine-nitrated α-bag cell peptide.187 They did not observe a peak corresponding to the intact nitrated peptide (+45 amu) with DHB (pKₐ = 2.9) independent of laser intensity. MALDI with CHCA (pKₐ = 3.0) gave only a small peak for the intact nitrated peptide whereas this peak was clearly visible in matrix preparations containing sinapinic acid (SA; pKₐ = 4.4). However, matrix-dependance is not solely due to the pKₐ, since the nitrated peptide was also not observed in 2-amino-3-hydroxypyridine (AHP, pKₐ = 7.3).

Reduction to aminotyrosine can circumvent this problem, since it yields defined molecular ions upon MALDI,161 although it raises an additional issue in tandem MS analysis (see section 7.2.3).84 An alternative is to analyze nitrotyrosine-containing peptides with IR-MALDI (emission at 2.94 µm (infrared area) exciting water as the matrix), which generally leads to less photodecomposition than UV-MALDI, since the emitted wavelength of the laser is far from the absorbance maximum of nitrotyrosine.184, 188

7.2.2 Electrospray ionization (ESI)

ESI has the advantage that it can generate ions from the effluent of a reverse phase HPLC column (LC-MS). An LC-MS configuration including an absorbance detector allows furthermore the detection of nitrated peptides at 360 ± 5 nm under acidic conditions prior to MS.51, 54, 79-81 It should be noted, however, that protein modifications other than tyrosine nitration (e.g. tryptophan oxidation, other nitration or nitrosylation products) may contribute to an absorbance change at this wavelength,81 and that sensitivity is limited. Due to the low amount of nitrated proteins, that is usually obtained after gel electrophoresis or after affinity enrichment, nanoESI is recommended to enhance MS sensitivity.89
7.2.3 Tandem MS analysis

Assignment of PTN has often been based on MALDI-TOF-MS-based peptide mass fingerprinting, but even when a putative nitrated peptides is observed with a 45-Da mass increase, it is not certain that the corresponding protein is tyrosine-nitrated, since, for example, tryptophan can also be nitrated. Mass fingerprinting alone is therefore not sufficient proof of PTN. The gold standard to ascertain that a protein is tyrosine nitrated is to localize the modified amino acid in the protein sequence.

Precursor ion scanning in a triple quadrupole mass spectrometer based on the selective detection of the nitrotyrosine-derived immonium ion (181.06 amu) can be a reliable and fast method to identify nitrated peptides in complex peptide maps. Petersson et al. found precursor ion scanning to be a sensitive and specific method to identify nitrated peptides in a tryptic digest of BSA, however, there was interference from other fragment ions when analyzing native BSA, which was attributed to the high collision energy required to generate the immonium ion. Amoresano et al. recently proposed an elegant approach to combine chemical labeling of aminotyrosine with precursor ion scanning MS after the pH-controlled labeling of nitrated milk proteins or nitrated E. coli proteins with dansyl chloride. Nitrotyrosine-containing peptides were detected after reduction to aminotyrosine followed by dansylation and a combination of precursor ion and MS3 scan modes on a linear ion trap mass spectrometer to detect dansyl-specific fragment ions.

Recently, Ghesquière et al. reported that tandem MS analysis of aminotyrosine-containing peptides tends to result in mass differences of more than 1 Da between the measured and calculated mass. Moreover, this “fragment mass shuffling” is inconsistent, and MS/MS data could not be matched to databases using common search algorithms. The authors showed that after acetylation of all amines, these aminotyrosine-containing peptides presented an enhanced and more uniform peptide fragmentation by tandem MS analysis, yielding MS/MS spectra of better quality than when analyzing nitrotyrosine-containing peptides directly.

7.2.4 MS-based quantification of nitrated peptides

The native reference peptide method, an LC-MS technique that quantifies modified peptides in a protein digest by selecting another reference peptide from the protein of interest as the internal standard, has been applied to follow the kinetics of PTN of human serum albumin at tyrosine 162. The reference peptide must (i) be a proper tryptic peptide, (ii) contain no modifiable amino acids such as methionine or cysteine, (iii) have a retention time similar to the analyte peptides, and (iv) give response characteristics that are similar to the analyte peptides. This method showed a good precision (relative standard deviation < 10%) and a limit of detection of 5 fmol.

Van Haandel et al. recently reported the stable isotope labeling of dithionite-reduced nitrotyrosine with 2H-containing phenylisothiocyanate. This coupling reaction was quantitative when performed at pH 3, conditions resulting in a better selectivity for the aromatic amine than at pH 5 as previously used with other methods. Photochemically-mediated cyclization of the obtained tyrosyl-3-thiourea resulted in a
2-anilino-benzoxazole derivative (yield: 80%), a process requiring the presence of the neighboring phenolic hydroxyl group, thus increasing overall labeling selectivity. Relative quantitation was based on the mass increase due to the labeling with light or heavy phenylisothiocyanate (+116 or +121 amu with respect to nitrotyrosine). While the reagent as such does not allow affinity chromatography, it is possible, according to the authors, to include an affinity tag in the reagent structure.96

7.3 Application of analytical methods to the identification of nitrated proteins

Table 1 summarizes various proteomics approaches that have been used for the identification of nitrated proteins. Articles reporting the PTN of a single protein or that do not show protein identification by peptide mass fingerprinting, tandem MS or N-terminal sequencing by Edman degradation were not included.

Analysis of these reports reveals that gel-based methods are by far the most widely used with the following distribution: two-dimensional gel electrophoresis (2DE; 21 reports), SDS-PAGE (1DE; 2 reports), immunoprecipitation/SDS-PAGE (IP/1DE; 2 reports) and in-solution isoelectric focusing/SDS-PAGE (IEF/1DE; 2 reports). The other approaches were based on IEF without subsequent 1DE, anti-nitrotyrosine affinity columns, enrichment after affinity tag-based chemical labeling, dansyl chloride labeling, or two-dimensional chromatography. Most proteins were only identified by a single analytical approach but some authors used different techniques and combined the results (see Table 1).166 Gokurangan et al., who studied rat brain tissue by 2DE, IP/1DE and IEF/1DE, identified 9 nitrated proteins by 2DE, 14 by IP/1DE, 3 by IEF/1DE, 7 by both 2DE and IP/1DE, and 1 by both IP/1DE and IEF/1DE. Interestingly no protein was identified by all three approaches indicating that coverage of the nitroproteome by any of the presently used methods is at best partial (see Fig. 2). Kanski et al., who applied 2DE and IEF/1DE to heart homogenate and samples from heart mitochondria190 identified 13 proteins by 2DE, 9 by IP/1DE, and 1 by both methods in heart homogenate. Eleven proteins were identified by 2DE, 20 by IP/1DE, and 3 by both methods in heart mitochondria (see Fig. 3). It is noteworthy that a later study by the same group using IEF/1DE to identify nitrated proteins in heart homogenate found only one protein (tropomyosin 1, a chain) that was in common between both studies.167 The phenomenon, that different sample preparation, separation and identification techniques lead to the identification of different proteins, is a common observation in proteomics, notably with respect to PTMs, pointing towards the incomplete coverage of the sample in question using a single analytical method.191

Interestingly, Hong et al. reported that the predicted molecular weights of the proteins identified by MS/MS analysis and those of the immunopositive bands or spots upon Western blot analysis did not correlate.167 Proposed explanations are that in vivo proteolysis of nitrated proteins may have occurred or that not all immunoresponsive proteins on Western blot contain sufficient amounts of nitrotyrosine for a positive MS/MS identification of nitrated peptides. It is thus pivotal to localize the nitrotyrosine residue in the peptide to have proof that the protein in question is really tyrosine-nitrated.
Figure 2: Nitrotyrosine-containing proteins from aging rat brain tissue identified by 2DE, IP/SDS-PAGE and IEF-SDS/PAGE. Data derived from a study by Gokulrangan et al., 2007.166

Figure 3: Identification of nitrotyrosine-containing proteins from aging rat heart tissue (homogenate or mitochondrial fraction) by 2DE and IEF-SDS/PAGE. Data derived from a study by Kansi et al., 2005.190
Several nitrated proteins shown in Table 1 were reported as being nitrated based on peptide mass fingerprint analysis of immunopositive spots in 2DE gels. Although reduction with sodium dithionite was used to reduce the risk of a lack of antibody specificity, the claim that these proteins contain nitrotyrosine must be taken with caution, since nitrotyrosine was not localized in the protein sequence. An additional risk factor is that a gel spot may contain several proteins and that the identified protein is actually not tyrosine-nitrated but just co-migrating with a less abundant protein that is. Photodegradation-based fragment mass fingerprints upon MALDI-TOF-MS analysis indicated the presence of peptides with a mass increase corresponding to one or several nitro group(s) adding confidence to the original identifications. The presence of nitrotyrosine was further confirmed by IP/SDS-PAGE, which provided further support. Although indirect evidence is helpful in deciding whether a protein is nitrated, the gold standard is to sequence and identify the modified amino acid in the protein sequence. In 15 out of the 29 references cited (particularly the most recent ones), PTN in at least part of the identified proteins was unambiguously confirmed by sequencing the nitrotyrosine-containing peptides.

Table 1: Overview of proteomics methods for the identification of tyrosine-nitrated proteins.

The following results were included in this survey: (i) reporting more than one nitrated protein, (ii) identifying proteins by peptide mass fingerprinting, tandem MS, or N-terminal sequencing. As nitrotyrosine-containing peptides were considered all peptides that were sequenced by MS/MS localizing the nitrotyrosine moiety. Peptides that were considered “nitrated” based on a 45-Da mass increase with respect to the theoretical mass were not considered.

**Abbreviations:** 1DE: one-dimensional (SDS) polyacrylamide gel electrophoresis; 2DE: two-dimensional polyacrylamide gel electrophoresis; AC: affinity chromatography; CL: chemical labeling; ESI: electrospray ionization; IEF: isoelectric focusing; IP: immunoprecipitation; LC: (reverse-phase) liquid chromatography; MALDI: matrix-assisted laser desorption ionization; MPO: myeloperoxidase; MS: mass spectrometry; MS/MS: tandem MS; nESI: nano-ESI; N-term. sequencing: N-terminal sequencing; ONOO−: peroxynitrite; pub. date: publication date; Q-IT: quadrupole-IT; TOF: time-of-flight; Q-TOF: quadrupole-TOF; TNM: tetranitromethane; SCX: strong cation exchange chromatography.
## Protein Tyrosine Nitration: a Review

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1 All 10 proteins identified by MALDI-TOF-MS are also part of the 21 proteins identified by LC-ESI-MS/MS.
2 Some nitrated proteins were confirmed by IP.
3 3 proteins were identified by MALDI-TOF-MS, 1 by LC-ESI-MS/MS, and 5 by both methods.
4 Some peptides were assigned based on a 45-Da mass increase, but there was insufficient material for MS/MS sequencing. IP was also done, but there was also not enough material for MS/MS analysis.
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<th>Methodology</th>
<th>Proteins Identified</th>
<th>Partly/Not Identified</th>
<th>MS/MS Methodology</th>
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<tr>
<td>Elfering et al.</td>
<td>01/2004</td>
<td>rat</td>
<td>liver mitochondria</td>
<td>endogenous NO•-mediated stimulation</td>
<td>2DE, MALDI-TOF-MS</td>
<td>37</td>
<td>partly (8/37) (8)</td>
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<td>Koeck et al.</td>
<td>06/2004</td>
<td>rat</td>
<td>liver mitochondria</td>
<td>in vitro hypoxia/anoxia/reoxygenation</td>
<td>2DE, MALDI-TOF-MS</td>
<td>8</td>
<td>no</td>
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<tr>
<td>Koeck et al.</td>
<td>06/2004</td>
<td>human</td>
<td>skin fibroblast</td>
<td>in vitro nitration with ONOO</td>
<td>2DE, MALDI-TOF-MS</td>
<td>7</td>
<td>no</td>
<td>-</td>
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<tr>
<td>Zhan and Desiderio</td>
<td>12/2004</td>
<td>human</td>
<td>brain (pituitary)</td>
<td>normal post-mortem sample</td>
<td>2DE, LC-ESI-MS/MS (Q-IT)</td>
<td>4</td>
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<td>Kanski et al.</td>
<td>01/2005</td>
<td>rat</td>
<td>heart homogenate</td>
<td>aging</td>
<td>2DE, LC-nESI-MS/MS (Q-IT)</td>
<td>13(4)</td>
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<td>Kanski et al.</td>
<td>06/2005</td>
<td>rat</td>
<td>skeletal muscle</td>
<td>aging</td>
<td>IEF/1DE, LC-nESI-MS/MS (Q-IT)</td>
<td>14(6)</td>
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<td>Suzuki et al.</td>
<td>09/2005</td>
<td>rat</td>
<td>Brain (cerebral cortex)</td>
<td>normal brain</td>
<td>2DE/LC, N-term sequencing</td>
<td>9(6)</td>
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<td>Sultana et al.</td>
<td>04/2006</td>
<td>human</td>
<td>brain (hippocampus)</td>
<td>Alzheimer's disease model</td>
<td>2DE, MALDI-TOF-MS</td>
<td>21(6)</td>
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<td>Ghosh et al.</td>
<td>05/2006</td>
<td>mouse</td>
<td>lung</td>
<td>asthma</td>
<td>2DE, LC-ESI-MS/MS Q-IT (Q-IT)</td>
<td>30</td>
<td>no</td>
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<td>Zhan and Desiderio</td>
<td>06/2006</td>
<td>human</td>
<td>brain (pituitary)</td>
<td>cancer</td>
<td>AC, vMALDI-LTQ (MS/MS)</td>
<td>9</td>
<td>yes</td>
<td>10</td>
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<td>Sacksteder et al.</td>
<td>07/2006</td>
<td>mouse</td>
<td>brain</td>
<td>Parkinson's disease model</td>
<td>SCX/LC, ESI-MS/MS (LTQ IT)</td>
<td>29</td>
<td>yes</td>
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5 12 proteins of 37 showed an increase in nitration upon NO•-mediated stimulation and were selected for MS/MS analysis.
6 In heart homogenate, 13 proteins were identified by 2DE, 9 by IP/1DE, and 1 by both methods. In heart mitochondria, 11 proteins were identified by 2DE, 20 by IP/1DE, and 3 by both methods.
<table>
<thead>
<tr>
<th>Study</th>
<th>Year</th>
<th>Species</th>
<th>Tissue/Cell Type</th>
<th>Condition</th>
<th>Technique</th>
<th>Protein Identification</th>
<th>Number of Proteins</th>
<th>Validation Method</th>
<th>Additional Notes</th>
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<td>Zhan and Desiderio</td>
<td>2007</td>
<td>Human</td>
<td>Brain (pituitary)</td>
<td>Normal post-mortem sample</td>
<td>2DE</td>
<td>vMALDI-LTQ (MS/MS)</td>
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<td>Gokulrangan et al.</td>
<td>2007</td>
<td>Rat</td>
<td>Brain (cerebellum)</td>
<td>Aging</td>
<td>2DE</td>
<td>LC-nESI-MS/MS (Q-IT)</td>
<td>16(7)</td>
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<tr>
<td>Hong et al.</td>
<td>2007</td>
<td>Rat</td>
<td>Heart</td>
<td>Aging</td>
<td>IEF</td>
<td>LC-nESI-MS/MS</td>
<td>10</td>
<td>Yes</td>
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<tr>
<td>Tedeschi et al.</td>
<td>2007</td>
<td>Rat</td>
<td>PC12 (neurone-like) cells</td>
<td>Neuronal differentiation</td>
<td>2DE</td>
<td>MALDI-TOF-MS</td>
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<td>Zhang et al.</td>
<td>2007</td>
<td>Mouse</td>
<td>Brain</td>
<td>In vitro nitration with ONOO-</td>
<td>CL/AC</td>
<td>LC-ESI-MS/MS (LTQ IT)</td>
<td>102</td>
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<td>150</td>
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<td>Amoresano et al.</td>
<td>2007</td>
<td>Cow</td>
<td>Milk</td>
<td>In vitro nitration with TNM</td>
<td>CL</td>
<td>MALDI-TOF-MS (Q-IT)</td>
<td>5</td>
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<td>Distler et al.</td>
<td>2007</td>
<td>Rat</td>
<td>Liver mitochondria</td>
<td>Physiological conditions</td>
<td>1DE</td>
<td>MALDI-TOF-MS (LTQ IT)</td>
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<td>Tyther et al.</td>
<td>2007</td>
<td>Rat</td>
<td>Kidney</td>
<td>Hypertension model</td>
<td>2DE</td>
<td>LC-ESI-MS/MS</td>
<td>19</td>
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<td>Reed et al.</td>
<td>2008</td>
<td>Rat</td>
<td>Brain</td>
<td>Traumatic brain injury treated post-injury with gamma-glutamylcysteine ethyl ester</td>
<td>2DE</td>
<td>MALDI-TOF-MS</td>
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<td>No</td>
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<tr>
<td>Reed et al.</td>
<td>2008</td>
<td>Human</td>
<td>Brain (inferior parietal lobule)</td>
<td>Early Alzheimer disease</td>
<td>2DE</td>
<td>MALDI-TOF-MS</td>
<td>8</td>
<td>No</td>
<td></td>
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</tbody>
</table>

7 9 proteins were identified by 2DE, 14 by IP/1DE, 4 by IEF (of which 1 was followed by 1DE/in-gel digestion instead of in-solution digestion), 7 by 2DE and IP/1DE, 1 by IP/1DE and IEF/1DE.
The labeling and enrichment technique employed by Zhang et al. is particularly promising. It resulted in the identification of 102 nitrated proteins (whereas 2DE-based studies reported 12 on average), with 150 confirmed nitrotyrosine-containing peptides. It should be noted, however, that the level of PTN in the analyzed proteome was boosted through addition of 2 mM peroxynitrite, whereas most of the other reports studied nitrated proteins at their endogenous levels. Moreover, as noted by Nuriel et al., the identified 150 peptides represent only 35% of all MS/MS-sequenced peptides. In other words, 65% of the identified peptides were not nitrated. Still, this result shows that non-gel-based approaches have the potential to provide a more comprehensive overview over the extent of PTN in a proteome at a higher throughput than gel-based methods. To do so, it is essential to develop more effective and selective labeling and/or enrichment strategies, as the abundance of false positives will otherwise make the discovery of truly nitrated proteins cumbersome.

8 CONCLUSIONS

The study of PTN has intensified over the last 20 years leading to a better understanding of this post-translational modification (see Fig. 4). Although initially considered to be a stable post-translational modification, there is a growing number of indications that it is in fact a reversible process. While the biological significance of PTN was not clear in the beginning, there is now evidence that it can significantly alter cellular functions next to being an indicator of NO•-mediated oxidative stress. Therefore the identification of in vivo targets of PTN may help in understanding the development and progression of disease.

Whereas many analytical methods have been developed for the analysis of nitrotyrosine, either in its free form or after its release by protein hydrolysis, the identification of the actual protein targets of PTN is a much more challenging analytical task. This has led to a number of innovative approaches for the analysis of nitrotyrosine-containing proteins or peptides in recent years to detect this low-abundance post-translational modification (estimated frequency of 1 in 10000 tyrosines under inflammatory conditions). Next to the classical gel- and antibody-based approaches, (generally based on two-dimensional gel electrophoresis and immunoblotting), which have reached their limits in terms of sensitivity and throughput, there is now a range of techniques based on the development of highly controlled labeling chemistry for enrichment, detection, and quantification using gel-free workflows. Finally, although much less studied than tyrosine nitration, it is noteworthy that nitration of tryptophan also occurs in vivo and has attracted increasing recent interest.
Figure 4: Number of hits for the keyword “nitrotyrosine” in the Pubmed database (http://pubmed.org) between 1988 and 2007.
Chapter 2

9 REFERENCES


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