Chapter 2

Protein Tyrosine Nitration: Consequences of its formation in S. cerevisiae.
2.1. Introduction

The formation of 3-nitrotyrosine (3-NT) in proteins occurs as an in vivo post translational modification. Tyrosine is modified in the 3-position of the phenolic ring through the addition of a nitro group. It is believed that tyrosine nitration involves a two-step process where the initial step is the oxidation of the phenolic ring of Tyr to yield the one electron oxidation product, Tyr radical (Tyr•). Several one-electron oxidants known to occur in vivo such as CO\textsubscript{3}•, •OH, •NO\textsubscript{2} or compound I of peroxidases can accomplish this task. The second step involves the addition of •NO\textsubscript{2} to the Tyr• in a radical-radical termination reaction [Radi, 2004; Ischiropoulos \textit{et al.}, 1992; Ischiropoulos \textit{et al.}, 1992; Beckman \textit{et al.}, 1992; Smith \textit{et al.}, 1992; Ischiropoulos, 2009]. There are two proximal nitrating agents that account for nitration in vivo. One nitrating agent is peroxynitrite which is formed by the fast reaction between nitric oxide (NO) and superoxide (O\textsubscript{2}\textsuperscript{•−}). The other proximal nitrating agents involve hemeperoxidases such as myeloperoxidase or eosinophil peroxidase in the presence of hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}) and nitrite (NO\textsubscript{2}\textsuperscript{−}) [Souza \textit{et al.}, 2008; Radi, 2013]. Collectively, the published work revealed the existence of multiple biochemical pathways (peroxidases, hemeproteins) leading to nitration. Nitrated proteins have been detected in a number of physiological and pathological settings. Both in vivo and in vitro data support a selective process in which the local structural environment of specific tyrosine residues governs the selectivity. Advancements in analytical biochemistry have also enabled the discovery of specific proteins modified by nitration in vivo using monoclonal and polyclonal antibodies. There are three major effects on protein function that can be observed due to protein nitration: (1) No changes in protein function, (2) loss of function and (3) gain of function. A series of recent publications have extensively analyzed biologically relevant nitration under different conditions and environments and its pathological relevance [Quijano \textit{et al.}, 2005; Bartesaghi \textit{et al.}, 2007; Peluffo and Radi, 2007; Sacksteder \textit{et al.}, 2006; Reynolds \textit{et al.}, 2007]. However, the significance of protein nitration in protein turnover, protein localization and signal transduction processes is under investigation.

Yeast has become a very important model organism to study biochemistry and molecular biology of mammalian cells and to answer fundamental questions concerning the mechanisms of many processes [Sahoo \textit{et al.}, 2003; Sahoo \textit{et al.}, 2006; Sahoo \textit{et al.}, 2009, Bhattacharjee \textit{et al.}, 2009; Bhattacharjee \textit{et al.}, 2010; Majumdar \textit{et al.}, 2012].
The origin of NO in yeast cells is still a matter of debate essentially because of the lack of mammalian nitric oxide synthase (NOS) orthologues in the yeast genome. NO mediated apoptosis and NOS like activity in yeast have been demonstrated [Almeida et al., 2007]. Bhattacharjee et al. first time showed that protein tyrosine nitration occurs in vivo in *Saccharomyces cerevisiae* cells grown under respiratory proficient conditions [Bhattacharjee et al., 2009]. The protein tyrosine nitration profile of both cytosol and mitochondria of wild type and flavohemoglobin deleted strain of *S. cerevisiae* were compared in this study. Flavohemoglobin is known to play important role both in oxidative stress and nitrosative stress [Liu et al., 2000; Zhao et al., 1996]. Many immunopositive spots were observed both in cytosol and in mitochondria from wild type as well as in flavohemoglobin deleted strain of *S. cerevisiae* when they were run in two dimensional gel electrophoresis followed by western blotting using monoclonal anti-3-nitrotyrosine antibody [Bhattacharjee et al., 2009]. The results indicated that a basal level of NO or nitrite or peroxynitrite is produced in yeast system. Furthermore, Sahoo et al. also showed a novel role of catalase in peroxynitrite detoxification in *S. cerevisiae* [Sahoo et al., 2009]. Generation of either peroxynitrite or nitrite is essential for protein tyrosine nitration in *S. cerevisiae*. Concomitant release of NO and O$_2^{-}$ is prerequisite for peroxynitrite formation. Several studies indicated that NO can be produced in *S. cerevisiae*. Superoxide anion O$_2^{-}$ is mainly generated from the leakage of electrons from the mitochondrial respiratory chain as a normal consequence of aerobic respiration [Bhattacharjee et al., 2009, Almeida et al., 2007]. Hydrogen peroxide (H$_2$O$_2$) is produced during the detoxification of superoxide anion catalyzed by superoxide dismutases and also in yeast during the oxidation of fatty acids in the peroxisome [Perrone et al., 2008]. There is still no information available till date regarding the mechanism of protein tyrosine nitration formation in *S. cerevisiae* and its functional significance.

This study for the first time show that protein tyrosine nitration (PTN) occurs in petite mutants of *S. cerevisiae*. This indicated that protein tyrosine nitration can occur even in the absence of functional mitochondria of *S. cerevisiae*. An investigation on protein tyrosine nitration in *S. cerevisiae* might provide insights and knowledge to elevate protein tyrosine nitration from a biomarker to a biologically important post translation modification.
2.2. Results

2.2.1. Protein tyrosine nitration (PTN) is associated with growth phases of *S. cerevisiae*

To check if protein tyrosine nitration (PTN) is associated with the growth phases of *S. cerevisiae* or not, wild type strain of *S. cerevisiae* (Y190) was grown in YPGlycerol media under respiratory proficient conditions up to stationery phase of growth. FACS analysis was also done to observe the cell cycle profile of *S. cerevisiae* (Figure 2.1). There was no significant difference in band patterns between them. Multiple numbers of immunopositive protein bands were visible in Y190 cell lysate. Few nitrated proteins were found to be conserved among different growth phases of *S. cerevisiae*; however, their individual nitrated band intensity varied. Thus it was concluded that PTN was a normal physiological event of Y190 cells grown in respiratory proficient media.

2.2.2. PTN in *S. cerevisiae* is not dependent on the cell growth media

Wild type Y190 cells were grown in synthetic medium containing 0.067% (wt/vol) yeast nitrogen base without amino acids and ammonium sulfate (Difco), 2% (wt/vol) glucose, and with the necessary supplements. Figure 2.2 represents protein tyrosine nitration profile of different growth phases of *S. cerevisiae* cells grown in synthetic media. Interestingly, PTN profile of cells grown in YPG medium was significantly different than the cells grown in synthetic media under fermentative conditions. Next the Y190 cells were grown in yeast carbon base (YCB) followed by Western blot analysis with monoclonal anti-3-nitrotyrosine antibody. Surprisingly, immunopositive protein bands also appeared in cell lysate of *S. cerevisiae* grown in YCB media.

2.2.3. Specificity of Protein tyrosine nitration

To investigate the specificity of protein tyrosine nitration, wild type Y190 cells were grown in fermentative media (YPD) up to the log phase of growth and cell lysate was subjected to incubate with graded increase in concentrations of peroxynitrite (0.125 mM, 0.25 mM and 0.5 mM) for 20 min under in vitro conditions. The PTN status of these
Figure 2.1 Cell lysates from early log, mid log, late log and stationary phase cultures of wild type Y190 strain were subjected to western blot analysis using monoclonal anti 3-Nitrotyrosine antibody. The arrows indicate the proteins whose nitration pattern altered during different growth phases (A). FACS profile of Y190 cells in YPGlycerol medium at various phases in its growth (B).
FIGURE 2.3 Wild type Y190 cell lysates were treated *in vitro* with increasing concentrations of peroxynitrite for 15 mins. Western blot analysis using monoclonal anti 3-Nitrotyrosine antibody showed basal level nitration increases with increasing concentration of peroxynitrite (A). (B) is the corresponding coomassie stained gel.
FIGURE 2.4 Growth curve of Y190, flavohemoglobin deleted ΔYhb1 and their corresponding petite (pet) mutants in YPDextrose medium.

FIGURE 2.5 Cell lysates of wild type Y190, its isogenic flavohemoglobin deleted ΔYhb1 strains and their corresponding petite mutant strains were subjected to western blot analysis using monoclonal anti 3-Nitrotyrosine antibody.
peroxynitrite treated crude cell lysates were followed by western blot with monoclonal anti-3-nitrotyrosine antibody (Figure 2.3). From the analysis, it was revealed that overall protein tyrosine nitration profile was gradually increased with increasing concentrations of peroxynitrite.

2.2.4. **Protein tyrosine nitration occurs in petite mutant of *S. cerevisiae***

To investigate the role of mitochondria in protein tyrosine nitration, petite mutants of wild type and flavohemoglobin deleted strain of *S. cerevisiae* were generated and the mutants were unable to grow in media containing glycerol as sole carbon source. Immunoblot analysis was done using cell lysates of Y190, ΔYhb1, Y190 Petite and ΔYhb1 Petite grown in YPD media using monoclonal anti-3-nitrotyrosine antibody. Surprisingly, petite mutants also showed protein tyrosine nitration like wild type and ΔYhb1 indicating even in absence of functional mitochondria, this post translational modification occurred in *S. cerevisiae* (Figure 2.4 and 2.5).

2.2.5. **Detection of nitrated proteins in immune histochemical study***

Immunohistochemistry was carried out in wild type strain of *S. cerevisiae* (Y190) using monoclonal anti-3-nitrotyrosine antibody to visualize the distribution of protein tyrosine nitration in yeast cell. Bright fluorescent spots were very much prominent in spheroplasts of *S. cerevisiae* cells treated with primary monoclonal anti-3-nitrotyrosine antibody followed by Alexa fluor conjugated secondary antibody. All cells showed similar bright green fluorescence irrespective of the media used for growth of *S. cerevisiae* cells which corroborated well with the previous western blot experimental results. Fluorescence micrograph of spheroplasts generated from Y190, ΔYhb1, Y190 Petite, ΔYhb1 Petite clearly showed the distribution of nitrated proteins in yeast cells (Figure 2.6).

To check the specificity of the observed fluorescence spheroplasts of *S. cerevisiae* cells were treated with anti-3-nitrotyrosine antibody preincubated with 5 mM 3-nitrotyrosine for 15 min which showed decrease in fluorescence. The nucleus was counterstained with DAPI (Figure 2.7).
FIGURE 2.6 *In vivo* protein nitration was analyzed in wild type Y190 strains by immunofluorescence method using monoclonal anti 3-Nitrotyrosine antibody and Alexa flour conjugated secondary antibody (excitation at 495 nm and emission at 515 nm). Bright green spots of nitrated proteins were observed in Y190 and its isogenic flavohemoglobin deleted Δ Yhb1 strain as well as their corresponding petite mutants. Nitration were also present in different growth conditions.
FIGURE 2.7 Immunofluorescence microscopy of wild type Y190 strain with anti-3-Nitrotyrosine antibody preincubated with 5 mM 3-Nitrotyrosine and visualized using Alexaflour 488 conjugated secondary antibody. The nucleus was counterstained with DAPI.
FIGURE 2.8 Wild type BY4742, its isogenic proteasomal defective mutants ΔPre9 and ΔRpn10 were grown in presence of 0.1 mM canavanine (Cav). Cell lysates of the control and treated were subjected to western blot analysis using monoclonal anti 3-Nitrotyrosine antibody (A). (B) represents the corresponding coomassie stained gel image.
2.2.6. Fate of nitrated proteins is independent of proteosomal clearing

To investigate the fate of protein tyrosine nitration in S. cerevisiae, ΔRpn10 and ΔPre9 and its corresponding wild type BY4742 of S. cerevisiae to follow the protein tyrosine nitration accumulation in defective proteasome containing system were used. Growth rate of Rpn10Δ and Pre9Δ strain of S. cerevisiae was much slower than its corresponding wild type in synthetic media. Interestingly, there is no significant difference in protein tyrosine nitration pattern among the ΔRpn10, ΔPre9 and BY4742 of S. cerevisiae. However, the protein nitrated band intensities of ΔPre9 were high compared to BY4742 of S. cerevisiae. On the other hand, protein nitrated band intensities of Pre9Δ were high compared to BY4742 of S. cerevisiae which indicated that the fate of protein tyrosine nitration in S. cerevisiae is not dependent on proteosomal clearing. Canavanine is an arginine analog which induces misfolding. Canavanine (0.1 mM) treated BY4742, Rpn10Δ, Pre9Δ strain of S. cerevisiae showed an altered misfolded protein profile. Protein nitration profile indicated that 0.1 mM canavanine treated BY4742, ΔRpn10, ΔPre9 strain of S. cerevisiae produced misfolded proteins which can also be nitratated at the 3 position of the Tyr residue of a protein (Figure 2.8).

2.2.7. Identifying nitrated peptides by fluorinated carbon tags

Bovine catalase has 21 tyrosine residues in its sequence. The tryptic peptides were enriched using fluorinated carbon column, and the peptides analyzed in LC/MS. The M/Z values obtained were then compared with a theoretically digested catalase sequence with fixed and variable modifications as present in the original LC/MS run. It was found that only 8 out of the 21 tyrosine residue are probable candidates for nitration. These were at position 84, 137, 215, 236, 308, 386, 447 and 512 respectively. The result was also compared with GPS-YNO2 in silico protein tyrosine nitration prediction tool. GPS-YNO2 predicts tyrosine nitration sites in proteins. GPS-YNO2 demonstrated a promising accuracy of 76.51%, a sensitivity of 50.09% and a specificity of 80.18% [Liu et al., 2011]. It also predicted position 447 and 512 are most likely to be nitratated (Figure 2.9 and 2.10).
Figure 2.2  Cell lysates of wild type Y190 grown in synthetic complete medium at different phases of growth (A) and in yeast carbon base (C) were analyzed by western blot using monoclonal anti 3-Nitrotyrosine antibody. Catalase is used as a positive control. (B) and (D) represents the ponceau stained membrane and coomassie stained gel as loading control.

FIGURE 2.10 Bos taurus liver catalase protein sequence showing tyrosine sites in the protein residue and crystal structure of the bovine catalase consisting four equal subunit, each with a 60 kDa molecular weight.
2.3. Discussion

Yeast is an excellent system to study protein nitration because it is possible to grow the microorganisms both in respiratory proficient conditions as well as in fermentative conditions. Although the endogenous source of NO in the yeast cell remains a mystery, but it is certain that protein tyrosine nitration is mediated by secondary products of nitric oxide metabolism. In this study protein tyrosine nitration was found to occur in *S. cerevisiae* both in respiratory proficient conditions as well as fermentative conditions. It is important to note that protein tyrosine nitration in *S. cerevisiae* was solely dependent on intrinsic reactive nitrogen species as it occurred in synthetic media without any extrinsic nitrite source. It is postulated that nitric oxide may also act as a signaling molecule in unicellular eukaryotes like the yeast [Tuteja *et al.*, 2004; Almeida *et al.*, 2007; Kig *et al.*, 2009]. Additionally nitric oxide signaling was claimed to be implicated in the programmed cell death during chronological aging of the yeast [Almeida *et al.*, 2007]. However, in another study it was shown that elevation of apoptosis during chronological aging in the yeast was nitric oxide independent. Additionally it was claimed that yeast cells did not suffer nitrosative stress during chronological aging since the pattern of protein tyrosine nitration was slightly decreased [Lewinska *et al.*, 2011]. From this study it is evident that protein tyrosine nitration was a normal physiological event irrespective of the growth phases of *S. cerevisiae*. An important aspect of protein tyrosine nitration is the fact that it does not occur at random. It is very much selective. Even though protein tyrosine nitration has been considered to be a stable post-translational modification, there is increasing evidence of an in vivo denitration process. The accumulation of specific tyrosine nitrated proteins is the result of the protein tyrosine reaction as well as a putative denitration process and the degradation rate of tyrosine nitrated proteins [Koeck *et al.*, 2004].

Mitochondrion is the main endogenous source of intracellular free radicals and various reactive oxygen and reactive nitrogen species in eukaryotic cells. Mitochondrial catalase is an important component of *S. cerevisiae* antioxidant defense [Petrova *et al.*, 2002]. Under respiratory growth conditions in the presence of a non-fermentable carbon source, mitochondrial respiration is maximally induced, reactive oxygen species (ROS) and reactive nitrogen species (RNS) are rapidly accumulating and their detoxification by catalase in vivo would be advantageous for cells.
Yeast flavohemoglobin (YHb), a homologue of mammalian cytohemoglobin is a NO oxidoreductase, which plays an essential role in both oxidative and nitrosative stress response. It is conceivable that the extent of free radical generation would be less in fermentative media than the cells grown in the presence of non-fermentable sugar. It has been reported that when *S. cerevisiae* cells were grown in non-fermentable glycerol media, catalase (~4 fold) activity was increased in flavohemoglobin deleted strain of the same [Bhattacharjee et al., 2010]. However, previous reports showed that in yeast cells that had been cultivated on the fermentable carbon source like glucose, catalase activity in peroxisome and mitochondria was significantly lower, but Cta1p protein level was equally distributed in both organelles indicating less ROS formation [Petrova et al., 2004]. Thus the possibility of peroxynitrite generation would be much less under the nonfunctional state of mitochondria.

From the experimental results it can be conceived that in vivo mechanism of protein nitration in yeast may not involve peroxynitrite because petite mutants also showed nitrated proteins. In petite mutants, protein nitration mechanism may involve nitrite radical and tyrosine radical. So it is possible that nitrite is converted to nitrite radical to mediate 3 nitrotyrosine formations if the tyrosine radical is available in a suitable protein microenvironment. Although there is no motif available for the protein nitration still it has been indicated that local environment influences protein nitration and thus maintain the specificity. Tyrosine nitration reaction is highly pH dependent. Acid catalyzed nitration by NO$_2^-$ may be a plausible mechanism of tyrosine nitration in gastric lumen [Quijano et al., 2005]. However, the process is likely to be dependent on the formation of NO$_2^-$ . Similar pathway may also become operative in yeast system.

Recently published results showed that sulfite efflux permease Ssu1 and Ssu2 are also able to excrete nitrite and nitrate. The role of nitrite excretion out of the *S. cerevisiae* cells could be the response of yeast cells to protect it from the nitrite toxicity. It has also been shown that when ssu2 deleted strain of *H. polymorpha* and *S. cerevisiae* cells were grown in presence of nitrate, nitrite efflux rate was increased not the nitrate indicating nitrite was found to be more toxic to cell than the nitrate [Cabrera et al., 2014].

The degradation of ubiquitinated proteins is mediated by 26S proteasome which contains the 20S proteasome plus a 19S regulatory complex. Peroxynitrite treatment of aconitase augmented its degradation by the 20S proteasome. However, supraphysiological
concentrations of peroxynitrite induced a decrease of the enzyme degradation by the proteasome. The nitration of Tyr108 of bovine CuZnSOD has been shown to double the speed of degradation by the 20S proteasome when compared with the unmodified SOD [Ischiropoulos 2009; Souza et al., 2013]. In a number of neurodegenerative diseases accumulation of 3-nitrotyrosine in the form of insoluble aggregates is observed, like the Lewy bodies in Parkinson’s disease which indicates that aggregated protein became a poor substance of the proteasome. There is also evidence that it could be a decrease in the activity of the ubiquitin–proteasome system in Parkinson’s disease [Gow et al., 1994]. This study indicated that the fate of protein tyrosine nitration in *S. cerevisiae* is not dependent on proteosomal clearing.

In conclusion, protein tyrosine nitration is a protein modification characteristic of peroxynitrite and other NO-related oxidants, but the functional consequences of this modification are still unclear. The immunoblotting and immunolocalization data first time provide a sound foundation to further investigate the mechanisms of *in vivo* protein tyrosine nitration in *S. cerevisiae*. 