Nitration of tyrosine residues 368 and 345 in the $\beta$-subunit elicits $F_0F_1$-ATPase activity loss

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Tyrosine nitration is a covalent post-translational protein modification associated with various diseases related to oxidative/nitrative stress. A role for nitration of tyrosine in protein inactivation has been proposed; however, few studies have established a direct link between this modification and loss of protein function. In the present study, we determined the effect of nitration of Tyr$^{345}$ and Tyr$^{368}$ in the $\beta$-subunit of the $F_1$-ATPase using site-directed mutagenesis. Nitration of the $\beta$-subunit, achieved by using TNM (tetratrimethane), resulted in 66% ATPase activity loss. This treatment resulted in the modification of several asparagine, methionine and tyrosine residues. However, nitrated tyrosine and ATPase inactivation were decreased in reconstituted $F_1$ with Y368F (54%), Y345F (28%) and Y345,368F (1%) $\beta$-subunits, indicating a clear link between nitration at these positions and activity loss, regardless of the presence of other modifications. Kinetic studies indicated that an $F_1$ with one nitrated tyrosine residue (Tyr$^{345}$ or Tyr$^{368}$) or two Tyr$^{368}$ residues was sufficient to grant inactivation. Tyr$^{368}$ was four times more reactive to nitration due to its lower $pK_a$. Inactivation was attributed mainly to steric hindrance caused by adding a bulky residue more than the presence of a charged group or change in the phenolic $pK_a$ due to the introduction of a nitro group. Nitration at this residue would be more relevant under conditions of low nitrative stress. Conversely, at high nitrative stress conditions, both tyrosine residues would contribute equally to ATPase inactivation.

Key words: aging, ATPase, mitochondria, nitrative stress, structure–activity relationship, tyrosine nitration.

INTRODUCTION

Protein nitration constitutes a post-translational modification that occurs mainly at Tyr (tyrosine) residues in vivo as a result of nitrative/oxidative stress. Proteomic studies support protein nitration as a tissue-specific footprint of nitrative/oxidative stress, and increases in protein nitration have been associated with over 50 diseases and aging [1–5]. The formation of 3-nitro-L-Tyr in vivo is an established marker for nitrative stress.

Mitochondria have been recognized as a target of protein Tyr nitration via the endogenous production of reactive oxygen and nitrogen species [6–8]. We have previously shown that certain mitochondrial Tyr-containing proteins acquired a nitro group under conditions of a sustained NO production by mitochondrial NOS (nitric oxide synthase) [9]. This agreed with other reports identifying a considerable number of nitrated proteins in mitochondria under different conditions, including in vivo situations [4,8,10].

Under physiological conditions, the production of NO has important implications for the maintenance of cellular metabolism [7]. However, overproduction of NO may lead to pathological processes. The radical recombination of NO and superoxide anion leads to the formation of the highly reactive species peroxynitrite [8,11,12]. Peroxynitrite-mediated inactivation of mitochondrial proteins has been investigated intensively. Several studies indicated that oxygen uptake by isolated mitochondria and cells was inhibited when exposed to peroxynitrite. This inhibition was attributed to inactivation of Complex I, II and V, possibly through nitration or oxidation of critical residues [11,13–15]. Complex V (ATPase) has also been found to be nitrated under high NO production [10,13,14].

Despite several attempts in vitro to link mitochondrial activity loss and nitration {e.g. Complex I [14–17], Complex II [14], cytochrome c [18–20], MnSOD (manganese-superoxide dismutase) [21,22] and Complex V [14]}, the exact role of nitration remains to be investigated. The concomitant occurrence of nitrotyrosine(s) and protein activity loss is not enough to prove a cause–effect link between Tyr nitration and inactivation. Peroxynitrite-treated MnSOD and indoleamine 2,3-dioxygenase resulted in Tyr nitration and activity loss; however, by using site-directed mutagenesis, it was demonstrated that this modification was not required for protein inactivation [21–23]. Several studies indicated that, when using peroxynitrite as the ‘nitrating’ agent, most of the changes in activity seemed to be related to cysteine oxidation rather than Tyr nitration [24,25]. In addition, chemical nitration has been mainly performed under relatively extreme conditions [13,14,26], giving limited attention to optimization in the use and selection of the nitrating agent (i.e. minimizing protein modifications other than nitration). For example, nitration of cytochrome c with a 40-fold molar excess of TNM (tetratrimethane) resulted in the formation of one or two nitrotyrosines without impairment of protein function [27], whereas nitration of one Tyr residue, obtained with a 2.5 M [20] or 15 M [19] excess of peroxynitrite, was found to be enough for inactivation or development of a pseudo-peroxidase activity; however, the amount of nitrated Tyr required for inactivation, and/or changes in methionine (or tryptophan) relative to the iron ion of the haem moiety that may change protein function independently of Tyr remained unanswered. It is clear that protein mutagenesis is one of the ideal approaches to show the role of Tyr nitration in protein inactivation.

One of the indications of the biological impact of nitrative stress in vivo is that, during aging, Tyr nitration of the $\beta$-subunit of $F_1$-ATPase increases with a concomitant decrease in activity [9]. Furthermore, several studies support Complex V (ATPase) as one of the important targets of protein nitration [8,10,28].

Abbreviations used: IPTG, isopropyl $\beta$-D-thiogalactoside; LB broth, Luria–Bertani broth; LC-MS/MS, liquid chromatography-tandem MS; MnSOD, manganese-superoxide dismutase; Ni-NTA resin, Ni$^{2+}$-nitrilotriacetate resin; TFA, trifluoroacetic acid; Tyr, tyrosine; TNM, tetratrimethane; WT, wild-type.

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Site-directed mutagenesis for single and double mutations

For the single base change for Y345F and Y368F, site-directed mutagenesis was performed using the following primer pairs of which T was replaced by A at 1206 (GenBank accession number NM_134364): mut 1 (5′-GGGCATCTTTCCACGCTG-TGG-3′) and mut 2 (5′-GATCCACAGCTGAAAGATGC-3′) for Y345F, and at 1275: mut 3 (5′-GGCAGTGGACATTGTA-GTTGCTGAGG-3′) and mut 4 (5′-CCAGACGCACA-TCAAATGCTCAGTCC-3′) for Y368F (mismatches are underlined). The expression vector for WT (wild-type) β-subunit was amplified for 30 cycles (denaturation at 94 °C for 15 s, annealing at 60 °C for 15 s, extension at 68 °C for 5 min) with high-fidelity KOD Hot Start DNA Polymerase (Novagen), following an initial heating step at 94 °C for 2 min. The PCR product was treated with DpnI at 37 °C for 1 h to degrade the template expression vector for WT β-subunit before being transformed into XL10-Gold ultracompetent cells (Stratagene). Direct sequencing was performed on the expression vector for the β-subunit with either amino acid mutation, Y345F or Y368F. The sequencing verified that the final β-subunit cDNA expression vector was identical to the WT one, except for the substitution 1206T > A (Y345F) or 1275T > A (Y368F) respectively. The expression vector construct for the double mutations, Y345F and Y368F, was created with the same method using the vector for β-subunit with Y368F and the primer set of mut 1 and mut 2. The sequencing for the final construct for double mutations confirmed 1206T > A (Y345F) and 1275T > A (Y368F).

Protein expression and purification

The expression and purification of His6-tagged recombinant proteins were performed according to the manufacturer’s protocol (The QiAexpressionist; Qiagen). M15 [pREP4] Escherichia coli cells were transformed with 200 ng of expression vector and selected on LB (Luria–Bertani)-agar plates containing 25 μg/ml of kanamycin and 100 μg/ml of ampicillin. A single colony inoculated into 50 ml of LB broth with the same concentration of antibiotics was grown at 37 °C with continuous shaking at 250 rev./min overnight. Subsequently, 20 ml from the overnight bacterial culture was inoculated into 1 litre of LB broth (containing the same concentration of antibiotics). The cells were grown at 37 °C with continuous shaking at 250 rev./min until the ΔAbs reached 0.5–0.6 (2–2.5 h). Protein expression was induced by addition of 0, 0.005, 0.05, 0.1, 0.5 or 1 mM IPTG (isopropyl β-D-thiogalactoside) (Sigma–Aldrich) into the exponentially growing E. coli at 20, 25 or 37 °C for 1, 2, 4 or 12 h to establish optimum conditions for protein expression. The induced E. coli pellet was harvested by centrifugation (4 °C, 4000 g, 20 min), resuspended into lysis buffer [20 mM imidazole, 300 mM NaCl, 50 mM NaH2PO4, 1% CHAPS, 10% glycerol plus protease inhibitor cocktail set III (EMD Biosciences) containing 1 μM ABEFS [4-(2-aminoethyl)benzenesulfonyl fluoride] 40 nM aprotinin, 2.5 μM bestatin, 0.8 μM E-64 protease inhibitor, 1 μM hemisulfate leupeptin and 0.5 μM pepstatin A], The resuspended bacteria were treated with 1 mg/ml lysozyme for 30 min at 4 °C, and subsequently sonicated (six times 10 s sonication with a 10 s interval between each sonication). The lysed bacterial solution was incubated with 10 μg/ml of RNase and 5 μg/ml of DNase at 4 °C for 15 min. Soluble fractions were separated by centrifugation (20 min at 4 °C, 10000 g) and were incubated with Ni-NTA (Ni2+-nitrilotriacetae) resin (Qiagen) for 2 h at 4 °C with constant shaking. The Ni-NTA incubated with soluble fractions was packed on Poly-Prep Chromatography Columns (Bio-Rad). After washing the Ni-NTA resin extensively

and considering that this Complex requires critical Tyr residues to sustain activity [29–31], no study had shown a clear link between residue contribution, stoichiometry and mechanism of nitration of a specific Tyr residue and ATPase inactivation. To address this issue, we used a combination of two approaches: one, refine the choice and use of the nitrating agent; and two, site-directed mutagenesis of the β-subunits in which critical Tyr residues were substituted with phenylalanine (Y345F, Y368F and Y345,368F). Note that the residue numbers used in the present manuscript correspond to the bovine mature ATPase β-subunit protein (P00829). Tyr345 and Tyr368 correspond to residues 395 and 418 in the rat mature β-subunit (P10719) and to residues 358 and 381 in the His-tagged recombinant β-subunit. Previously, we indicated that two Tyr residues, namely Tyr345 and Tyr368, in the β-subunit of F1-ATPase, were significantly nitrated under conditions of a sustained production of NO [9] and in aging [32]. Studies performed using nucleotide analogues to modify conditions of a sustained production of NO [9] and in aging [9] found in relevant medical conditions, has not been studied in detail in Complex V. We optimized the procedure of chemical nitration using TNM to maximize the nitration of these Tyr residues. We characterized the biochemical reactions of TNM with the β-subunit and, finally, we determined the stoichiometry of inactivation and the effect of nitration of these residues on F1-ATPase activity. In the present study, we determine unequivocally the role of nitration at each of these Tyr residues in F1-ATPase β-subunits.

EXPERIMENTAL

Chemicals and reagents

Pyruvate kinase/lactic dehydrogenase enzymes, phosphoenolpyruvic acid trisodium salt hydrate, NADH, ATP, Tris/succinate, deoxyferoxamine mesylate, Hepes and NH4HCO3 were purchased from Sigma–Aldrich. CHAPS was purchased from G-Biosciences. Imidazole was purchased from EMD Biosciences. Goat serum was purchased from Biocell Laboratories.  

Cloning of α-, β- and γ-F1-ATPase subunits

Full-length α-, β- and γ-F1-ATPase subunit cDNAs were amplified by KOD Hot Start DNA Polymerase (Novagen) from rat liver QUICK-Clone cDNA (BD Biosciences), which originated from Sprague-Dawley rats, using the following primer pairs: A-U1: 5′-CACAGAATTCATTAAAGAGGAAGAATTAATGTTAGATGATTACCACCAATCCATGATATGattaagctggaggagagggagtgacctgtttgatgtacagag-3′ and A-L1: 5′-GATCAAGCTTGTGACATGGGAGGACACAAAAGAGGAGGTAACATCTGAGGCACACATCCATGACACAC-3′; and BML1: 5′-CGCTTGTGACATGGGAGGACACAAAAGAGGAGGTAACATCTGAGGCACACATCCATGACACAC-3′ and B-L1: 5′-GATCAAGCTTGTGACATGGGAGGACACAAAAGAGGAGGTAACATCTGAGGCACACATCCATGACACAC-3′ for the β-subunit; G-U1: 5′-CACAGAATTCATTAAAGAGGAAGAATTAATGTTAGATGATTACCACCAATCCATGATATGattaagctggaggagagggagtgacctgtttgatgtacagag-3′ and G-L1: 5′-GATCAAGCTTGTGACATGGGAGGACACAAAAGAGGAGGTAACATCTGAGGCACACATCCATGACACAC-3′ for the γ-subunit. The cDNA fragments were individually subcloned into the prokaryote expression vector TAGZyme pQE-1 (Qiagen). Sequencing verified that the α-, β-, and γ-subunit cDNA constructs encoded an initiation codon, a His6 tag and the (expected) mature sequences.

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with the washing buffer (same components as lysis buffer), His$_6$-tagged recombinant proteins were eluted by adding elution buffer consisting of lysis buffer containing higher concentrations of imidazole. Lastly, the recombinant proteins were dialysed against dialysis buffer (150 mM NaCl, 0.2 mM 2-mercaptoethanol, 10% glycerol, 1% CHAPS and 50 mM potassium phosphate buffer, pH 7.4) in Spectra/Por Dialysis Membranes (molecular weight cut-off of 25 kDa or 12–14 kDa; Spectrum Laboratories) to dialyse out imidazole and NaCl. The conditions for the induction of protein expression were optimized to obtain the highest protein yield in soluble fractions. The yield was evaluated by changing the temperature (20, 25 or 37°C), the duration (1, 2, 4 or 12 h), or the IPTG concentrations (0, 0.005, 0.05, 0.1, 0.5 or 1 mM) of induction. It was concluded that relatively low temperature (20°C), short induction time (2–2.5 h) and low IPTG concentration (0.05 mM) produced the most efficient recovery of the recombinant proteins for all three subunits in soluble fractions (results not shown). For the β-subunit recombinant protein, imidazole and NaCl were removed by dialysis against phosphate buffer; however, the recombinant protein solutions of α and γ precipitated in the same dialysis procedure, despite several efforts at changing the components of the dialysis buffer. Therefore, the recombinant α- and γ-subunits were immediately stored at −80°C without the step of dialysis until used for reconstitution.

**Coomassie Blue staining and immunoblotting of purified recombinant proteins**

The proteins were separated by SDS/PAGE using 12.5% polyacrylamide gels. The bands were visualized with Coomassie Blue stain and processed using Kodak Image Station 2000MM (Eastman Kodak). The molecular weight of the bands was calculated by comparison with standard protein molecular weight markers (SeeBlue Plus2; Invitrogen). Western blot analysis was performed by transferring proteins to a PVDF membrane, Immobilon-P Transfer Membranes (Millipore), using a semi-dry blotting method at 20 V for 20 min. Membranes to be probed for His$_6$-tag, α- and β-subunit antibodies and F$_1$-serum were blocked for 1 h in a solution containing 5% (w/v) non-fat dried skimmed milk powder in TBS-T [Tris-buffered saline with Tween 20; 150 mM NaCl, 20 mM Tris/HCl, pH 7.5, and 0.05% Tween 20]. These membranes were incubated for 1–2 h with each primary antibody [anti-His$_6$ mouse monoclonal antibody (1:10000); clone BMG-His-1, Roche Diagnostics), anti-F$_1$, ATPase α-subunit (1:5000; anti-OxPhos Complex V subunit, monoclonal 7H10, Invitrogen), anti-F$_1$, ATPase β-subunit (1:5000; anti-OxPhos Complex V subunit, monoclonal 3D5, Invitrogen) and antiserum against purified mitochondrial F$_1$-ATPase was provided by Dr Imanaka (Department of Microbiology and Molecular Pathology, School of Pharmaceutical Sciences, Teikyo University, Kanagawa, Japan) (1:1000) [33]] for 1–2 h at room temperature (21°C). The membranes were washed in TBS-T, and then incubated with secondary antibodies (1:5000) of either HRP (horseradish peroxidase)-goat anti-mouse IgG or HRP-goat anti-rabbit IgG (Zymed Laboratories) for 1 h at room temperature. Membranes were extensively washed with TBS-T, and were developed using an enhanced chemiluminescence detection system (GE Healthcare).

**Nitration of β-subunits**

Nitration of β-subunits was performed by reaction with TNM (Sigma–Aldrich). To 6 μM each recombinant β-subunit (WT, Y345F, Y368F and Y345,368F), suspended in 200 μl of reaction buffer (0.1 M Tris/HCl, 0.1 M KCl; pH 8.0), TNM was added at room temperature and incubated for 30 min. To optimize this treatment, this process was carried at several concentrations of TNM [molar ratios of 250, 100, 50, 25, 10, 5, 2.5 and 1 TNM/β-subunit or NO$_2$/12 Tyr respectively]. The reaction was stopped by replacing the reaction buffer with reconstitution buffer (10% glycerol, 0.2 mM 2-mercaptoethanol, 10 mM Tris/succinate, pH 6.0) using Microcon YM-30 (Millipore). The recombinant proteins modified by TNM yielded the characteristic absorbance properties of 3-nitrotrosine using N-acetyl-3-nitrotyrosine ethyl ester as model, namely an isosbestic point of 381 nm, a maximum at 360 nm in acidic buffer and a peak at 420 nm in base [34], allowing other putative chromophores to be excluded. The concentration of nitrotyrosine in β-subunits was determined at 428 nm (pH 8.5) using a Cary 1E spectrophotometer (Varian) using ε$_{428}$ = 4100 M$^{-1}$·cm$^{-1}$ [35].

**LC-MS/MS (liquid chromatography-tandem MS) analysis for purified recombinant proteins and nitrated β-subunits**

Each recombinant protein (α, β and γ) was identified by LC-MS/MS from in-gel digestion with trypsin as previously described [36,37]. Protein spots were excised from the one-dimensional SDS/PAGE gel as precisely as possible and divided into smaller pieces. Gel pieces were washed with 200 μl of 50 mM NH$_4$HCO$_3$ and dehydrated with 100 μl of 50% acetonitrile three times. After washing with 100 μl of 50 mM NH$_4$HCO$_3$ and 100 μl of 50% acetonitrile, gel pieces were dried in a vacuum centrifuge for 15 min. A volume of 10 mM dithiothreitol (Sigma–Aldrich) in 100 mM NH$_4$HCO$_3$ sufficient to cover the gel pieces was added, and the proteins were reduced for 30 min at 37°C. Then the gel pieces were dehydrated again with 100 μl of 100% acetonitrile (Mallinckrodt Baker) and subsequently dried in a vacuum centrifuge for 15 min. After a 20 min incubation in 55 mM lodoacetamide (EMD Chemicals) in 100 mM NH$_4$HCO$_3$ at room temperature in the dark, the gel pieces were washed with 100 μl of 50 mM NH$_4$HCO$_3$ and 50% acetonitrile for 30 min at 37°C in order to destain these completely. Thereafter, the gel pieces were digested overnight at 37°C by incubation with 20 ng/μl trypsin (Gold, Mass Spectrometry Grade; Promega). Proteins were extracted on the following day by adding 60 μl of 60% acetonitrile/1% TFA (trifluoroacetic acid; EMD Chemicals). The gel pieces were sonicated with a Branson 3510 ultrasonic water bath for 10 min to improve the protein recovery, and the supernatant was collected by centrifugation for 30 s, and dried in a vacuum centrifuge. The protein samples were dissolved in 8 μl of 3% TFA for LC-MS/MS analysis. The nitrated β-subunits treated with various molar ratios of TNM followed by in-gel digestion with trypsin excised from the one-dimensional SDS/PAGE gel were subjected to LC-MS/MS analysis. Scaffold Software (Proteome Software) was used to validate MS/MS-based peptide and protein identifications by using the Peptide Prophet algorithm [38,39]. The 1500+ spectra were analysed manually using Scaffold Software for background, signal-to-noise ratio, contiguous ladders, b and y prominent peaks, and other characteristics as indicated by Lonscher and Turner ([www.proteomsoftware.com, Validating Sequences]). Relative changes in nitration abundance in MS-based analysis were performed by spectral counting [40].

Reconstitution of recombinant rat F$_1$

F$_1$ was reconstituted by using 3:3:1 molar ratios of α-, β- and γ-subunits with a total protein concentration of 0.1 mg/ml according to published procedures [41,42]. Briefly, a mixture
of α-, γ- and one of the β-subunits (WT, Y345F, Y368F and Y345,368F, with or without TNM treatment) in the reconstitution dissolving buffer (10 mM Tris/succinate, pH 6.0, 0.2 mM 2-mercaptoethanol, 10% glycerol and 1% CHAPS) were dialysed against the reconstitution dialysis buffer (50 mM Tris/succinate, pH 6.0, 50 μM deferoxamine mesylate, 5 mM ATP, 2 mM MgCl₂, 0.2 mM 2-mercaptoethanol, 10% glycerol and 1% CHAPS) under constant stirring at room temperature overnight using a Spectra/Por dialysis membrane (molecular weight cut-off of 15 kDa; Spectrum Lab). The reconstituted ATPase prepared from isolated subunits was separated from weight cut-off of 15 kDa; Spectrum Lab). The reconstituted WT F₁ was 40–42 μmol/min per mg of protein.

The specific activity of the decomposition of control samples containing BSA, but no protein concentration tested. The specific activity of the ATPase activity was calculated from the following equation:

\[ \text{ATPase activity} \times \text{[mmol of NADH/min · mg]} = -\frac{\text{dA}_{340}}{\text{dt}} [\text{μM · mg}] \times \varepsilon_{340}^{-1} \times 0.15 [\text{ml}] \times \alpha^{-1} [\text{cm}] \times 1000 \mu g/\text{mg} \]

where \( \varepsilon_{340} \) is the extinction coefficient for NADH, 6.22 mM⁻¹ cm⁻¹ and \( \alpha \) is the path length.

For 150 μl of well fill volume, experimental \( \alpha \) was equal to 0.5 cm. The rates were corrected for background NADH decomposition of control samples containing BSA, but no ATPase. The initial rates of ATPase activity were linear within the protein concentration tested. The specific activity of the reconstituted WT F₁ was 40–42 μmol/min per mg of protein.

Statistical analyses

The results presented were expressed as means ± S.D. Each experiment was carried out a minimum of three times, and the results represented all results obtained. Student’s \( t \) test was used for statistical analysis. A \( P < 0.05 \) was considered to represent a statistical significant difference.

RESULTS AND DISCUSSION

Analysis of purified recombinant F₁ proteins

The three major subunits of F₁ (α, β and γ) were expressed as His₃-tag recombinant proteins and purified by Ni-NTA affinity chromatography. The purified proteins were identified by using three different methods. Coomassie Blue staining of SDS/PAGE gels showed that the molecular weights for the major bands were consistent with the expected size of the mature, His-tag α, β and γ recombinant proteins: 59, 57 and 31 kDa respectively (Figure 1A). Expression of the α, β and γ recombinant proteins was confirmed by Western blot analysis with anti-His tag monoclonal antibody (Figure 1B). The α- and β- recombinant proteins crossreacted with their respective monoclonal antibodies (Figures 1C and 1D respectively), whereas the recombinant γ-subunit was probed with antiserum against purified mitochondrial F₁-ATPase (results not shown). LC-MS/MS analysis of the excised bands confirmed the identification of the proteins with the reported rat amino acid sequence NP_075581 (α), NP_599191 (β) and NP_446277 (γ). The coverage of the recombinant proteins obtained by LC-MS/MS was 53%, 69% and 27% for α, β and γ respectively.

Effect of variation of the molar excess of TNM on the nitration of the recombinant WT β-subunit

TNM was used for chemical nitration because it is specific for nitrating Tyr residues under mild conditions [26,34]. The β-subunit was treated with various molar excess concentrations of TNM. Increasing TNM concentrations increased the nitration of the recombinant WT β-subunit. A dose–response curve consistent with the presence of at least two Tyr residues with distinct reactivity to TNM (Figure 2A, black closed circles) was obtained. A highly reactive Tyr residue that was nitrated at low TNM concentrations was seen at molar ratios <25 TNM/β-subunit (Figure 2A, grey circles), whereas a less reactive Tyr residue was significantly nitrated at molar ratios higher than 50 (Figure 2A, open circles).

The nitrated WT β-subunit, treated with a molar ratio of 25 TNM/β-subunit, contained 1.34 nitrotyrosine/β-subunit. The presence of a nitrated β-subunit significantly decreased the ATPase activity (62% and 66% decrease compared with controls; Table 1). The increase of the molar ratio from 25 to 100 (TNM/β-subunit) resulted in an increase of 2.1 nitrotyrosines/β-subunit with a minor effect on activity. These results indicated that the molar ratio of 25 was sufficient to induce most of the changes to grant ATPase inactivation. To achieve a 50% inhibition of ATPase inactivation, a molar ratio of 17 TNM/β-subunit was required (linear part of Figure 2B). Interpolating this ratio in Figure 2(A), 1.2 nitrotyrosine/β-subunit would be required for 50% inactivation. Since the product of the reaction between TNM and a protein is a nitroformate ion, we evaluated the amount of nitroformate ion formed by spectrophotometry at 350 nm (ε = 14400 M⁻¹ cm⁻¹ [34]). Under our experimental conditions, an average of 8% of the TNM was recovered as nitroformate ion. By using these numbers (1.2 nitrotyrosine/β-subunit divided by 8% of 17 TNM/β-subunit or 1.36 nitroformate ion/β-subunit), the stoichiometry of the reaction can be calculated, resulting in 0.88 nitrotyrosine/nitroformate ion, indicating that the nitration reaction occurs with one Tyr residue and only one of the four potentially reactive nitro groups of TNM, as has been also observed in a previous study [46].
Tyrosine nitration and ATPase activity loss

Figure 1 Separation of F1 recombinant proteins by gel electrophoresis

Each recombinant protein (3 µg) was run on a Tris/glycine SDS/12.5% PAGE under reducing conditions and stained with Coomassie Blue (A). Western blot analyses were performed using His6-antibody to α-, β- and γ-subunits (B), or protein-specific antibodies to α-(C) and β-subunits (D). Molecular mass markers shown next to each gel are in kDa.

Figure 2 Dose-response curve (TNM to β-subunit) of nitrotyrosine formation and ATPase activity

Wild type β-subunit was treated with different excess molar ratios of TNM to β-subunit. The resulting TNM-treated subunits were analysed spectrophotometrically to determine the amount of nitrotyrosine (A), and the activity of reconstituted F1 with these β-subunits (B) was evaluated as described in the Experimental section. The experimental data obtained at [TNM] > 50 (Figure 2A, closed circles) was extrapolated to intersect the y-axis. The values at each [TNM] were subtracted from all experimental data, resulting in two dose–response curves: one highly reactive Tyr nitrated at low TNM concentrations (molar ratios < 25 TNM/β-subunit; Figure 2A, gray circles) and a less reactive one nitrated at molar ratios higher than 50 (Figure 2A, open circles).

Analysis of nitrated recombinant WT β-subunit with TNM

Nitration of recombinant β-subunits was accomplished using different concentrations of TNM as indicated above. To identify nitrate Tyr residues (and any other putative modification) in the WT protein, the TNM-treated β-subunit was analysed by LC-MS/MS. Treatment with molar ratios of 2.5 or 5 (TNM/β-subunit) did not nitrate Tyr, but resulted in the oxidation of eight methionine residues (out of 12) and deamidation of six asparagine residues (6 residues out of 25; Figure 3A). The identification of nitrotyrosine was performed by analysing the spectra obtained by MS/MS (Figure 3C). Each spectrum was analysed as described in detail in the Experimental section. The representative spectrum shown in Figure 3(C) has an appropriate signal-to-noise ratio, long and contiguous ladders, with overlapping b and y ion sets, where prominent peaks were constituted by b and y ions with low mass errors (Figure 3D).

One of the most commonly observed residue modifications, when performing SDS/PAGE of proteins followed by in-gel digestion for MS analyses, is the oxidation of methionine. This modification is achieved by residual persulfate in the polyacrylamide gel [47–49], and during and after protein digestion [50]. Thus, horse heart myoglobin (a non-related protein) and recombinant, purified β-subunit were used to quantify the number of artifactual methionine sulfoxides introduced into our system. Our experimental values obtained
Table 1 Effect of site-directed mutagenesis of \( \beta \)-subunit and nitrogen on ATPase activity

The activity was expressed as the mean of triplicate experiments ± S.D. Activity of reconstituted ATPase was evaluated as described in the Experimental section. The specific activity of WT with no TNM treatment was 40 ± 4 μmol/min per mg of protein (when tested with 2.5 mM MgCl\(_2\) and 52 ± 2 μmol/min per mg of protein with 5 mM MgCl\(_2\)) when corrected by the yield of reconstitution and F1 protein content. This specific activity was comparable with that published for rat liver F1 (24 and 55 ± 5 μmol/min per mg of protein at 25°C with 4.8 mM MgCl\(_2\) in Tris/HCl and Tris/citrate buffers respectively [83] and 58 and 120 ± 10 μmol/min per mg of protein for bovine heart F1 when assayed at 22°C and 30°C with 2.2 mM magnesium acetate. The letters a, b, c, d and e indicate a significant difference for each treatment (no treatment (no TNM), a molar ratio of 2.5 (TNM/β-subunit, mol/mol) as a control treatment, and a molar ratio of 0.25) (\( P < 0.05 \) compared with no TNM WT). aP was 0.05 compared with WT treated with a molar ratio of 2.5; bP < 0.05 and bP < 0.01 compared with WT treated with a molar ratio of 25; cP < 0.01 compared with WT treated with a molar ratio of 2.5; and dP < 0.01 compared with Y368F treated with a molar ratio of 2.5. The symbols α, †, § and ‡ indicate a significant difference among β-subunits (WT, Y345F, Y368F and Y345,368F). †P < 0.01 compared with no TNM WT; †P < 0.01 compared with WT treated with a molar ratio of 0.03; §P < 0.01 compared with no TNM Y345F; §P < 0.01 compared with Y345F treated with a molar ratio of 0.03; ‡P < 0.05 compared with no TNM Y368F.

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<tr>
<th>Protein</th>
<th>ATPase activity (nmol NADH/min · mg)</th>
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<tr>
<td></td>
<td>No TNM</td>
</tr>
<tr>
<td>WT</td>
<td>2402 ± 25</td>
</tr>
<tr>
<td>Y345F</td>
<td>2149 ± 29</td>
</tr>
<tr>
<td>Y368F</td>
<td>2017 ± 21</td>
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<tr>
<td>Y345,368F</td>
<td>1770 ± 35(^\dagger)</td>
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by MS analyses of both of these proteins indicated that 15 ± 2 % of all methionine residues were oxidized as a consequence of the procedure, and not as a consequence of the TNM treatment. Thus the number of oxidized methionine residues indicated above reflected the number of oxidized methionine residues corrected by those artificially introduced by the procedure.

When the ratio of TNM/β-subunit was increased to 25, the nitration of eight Tyr residues (out of 12) was observed (Figure 3B). A ratio of 250 did not alter the pattern of Tyr nitration (results not shown). Spectral counting of LC-MS/MS of the β-subunits treated with molar ratios of 25 and 250 indicated that nitration of residues Tyr\(^{261}\), Tyr\(^{282}\) and Tyr\(^{419}\) was not significantly different between these treatments, excluding these residues as critical for protein inactivation despite being modified by TNM.

Number of essential Tyr residues required for ATPase inactivation

The number of essential Tyr residues required for ATPase activity loss was determined by evaluating the ATP hydrolysis of F1 reconstituted with the nitrated WT β-subunit and recombinant (unmodified) α- and γ-subunits. The number of essential Tyr residues modified by TNM that were required for ATPase inactivation was determined by inactivation kinetics. Under conditions of excess TNM, the pseudo-first-order rate constant for ATPase inactivation is given by the equation \( k' = k[N \text{TNM}]_0 \), and \( k' = k + n \log[\text{TNM}] \). This last equation can be used to determine the value of \( n \), which indicates the inactivation stoichiometry. The slope of the line obtained from the log–log plot of \( k' \) against the TNM concentration was found to be 0.91, indicating that a molar ratio of 0.91 TNM/F1 was required for the inactivation of the ATPase (Figure 4A). Using data obtained earlier, it can be concluded that inactivation of ATPase was accomplished by nitration of 1.34 Tyr residue/F1 (0.91 TNM/F1, from Figure 4A × β-subunit/1.36 nitroformate or TNM from Figure 2 × 2 nitrotyrosines/β-subunit for 100 % inactivation from Figure 2).

Using the above equation and the data of ATPase activity, a single second-order rate constant \( k \) for the inactivation of ATPase was calculated from the experimental \( k' \). The \( k \) was equal to 89 ± 4 M\(^{-1}\) · min\(^{-1}\) (means ± S.E.M.) at 22°C. This value was within those obtained for TNM-mediated inactivation of holotryptophanase (56 M\(^{-1}\) · min\(^{-1}\) [51]), arginine kinase (195 M\(^{-1}\) · min\(^{-1}\); recalculated from Figure 2 in [52]) and carboxylic ester hydrolase (420 M\(^{-1}\) · min\(^{-1}\) [53]). Based on other studies [51,52], a value of \( k \) for the nitration of free Tyr with TNM was calculated as 4.6 × 10\(^3\) M\(^{-1}\) · min\(^{-1}\) at 30–37°C. Comparing this rate constant with the one obtained in the present study (corrected by the 8% reactivity of TNM with β-subunit; 1113 M\(^{-1}\) · min\(^{-1}\)), it could be concluded that the Tyr residue(s) in the β-subunit have a reactivity towards TNM 4-fold lower than that of free Tyr, suggesting that steric hindrance contributes more to the reaction than the difference in the phenolic \( pK_a \) between free Tyr (\( pK_a \) 10.46) and Tyr in the β-subunit (\( pK_a \) between 7–8; see below). Effect of site-directed mutagenesis of \( \beta \)-subunit Tyr residues on ATPase activity

To indicate the direct role of these Tyr residues (Tyr\(^{245}\) and Tyr\(^{358}\)), site-specific mutagenesis was performed. Three kinds of mutants of β-subunit were obtained, in which Tyr\(^{245}\) and/or Tyr\(^{358}\) were substituted for phenylalanine (Y345F, Y368F and Y345,368F). Given that benzene (as a model compound for phenylalanine) is 10000-fold less reactive with TNM than phenol (as a model compound for Tyr), it is expected that phenylalanine would be relatively more resistant to nitration than Tyr [55]. To confirm the suitability of these substitutions, the ATPase activity of reconstituted F1, with each β-subunit mutant was examined, resulting in no significant change in the ATPase \( V_{max} \) activity (only 26 % activity loss was recorded with Y345,368F; \( P < 0.05 \); Table 1). These results are in agreement with other studies that performed the same substitutions of Tyr\(^{245}\) with phenylalanine [56,57] or Tyr\(^{358}\) with phenylalanine [58,59] of the β-subunit of F1-ATPase in E. coli, resulting in minor impairment of ATPase activity. Double substitutions (Y345,368F) have not been reported before, and we have confirmed that this protein had a minor impact on ATPase activity, indicating its suitability for our study.

Reactivity of \( \beta \)-subunit Tyr\(^{245}\) and Tyr\(^{358}\) to nitration

From the rest of the nitrated Tyr residues (5 residues), we focused on testing the role of Tyr\(^{245}\) and Tyr\(^{358}\), because they were found to be mainly nitrated in liver mitochondria under in vivo conditions [32], and chemical modification with nucleotide
Tyrosine nitration and ATPase activity loss

Figure 3 LC-MS/MS analysis for nitrated WT β-subunit

β-subunits were treated with a molar ratio of 2.5 (Control, A) or 25 (B) TNM/β-subunit. Residues covered by MS were indicated in light grey, modified residues in dark grey, and circles in (B) indicate Tyr345 and Tyr368. (C) A representative MS/MS spectrum of a peptide obtained with trypsin with a nitroTyr residue, in this case, for Tyr345. (D) The delta mass error of each peak obtained in (C). AMU, atomic mass unit.

analogue of these and other residues resulted in F1-ATPase activity loss [30,31]. A TNM-to-β-subunit molar ratio of 25 was selected as the optimal treatment to nitrate Tyr345 and Tyr368, minimizing other protein modifications. This molar ratio is at the lower end of the wide range of TNM concentrations used to nitrate Tyr residues (from 2 to 1000 molar excess ratios of TNM/protein [18,26,34,60]). Under different conditions (pH, TNM concentrations), in addition to Tyr, modifications on histidine [61], methionine [61], tryptophan [61] and cysteine [61,62] had also been reported. As for our study, the fact that β-subunits do not have cysteine residues excludes this possibility. Furthermore, there was no significant difference in modified methionine and asparagine among controls, molar ratio of 2.5 and 25, even when a significant activity change occurred between 2.5 and 25 (Table 1) concomitant with a significant change in Tyr nitration.

A molar ratio of 2.5 was chosen as a mock treatment to account for any unforeseen, artifactual effect that the treatment of TNM might have induced (e.g. putative contaminants present in TNM). This selection was supported by the lack of Tyr nitration observed by Western blotting using antibodies to nitrotyrosine (results not shown), and on the maintenance of ATPase activity (Table 1).

The nitration of β-subunits Y345F, Y368F and Y345,368F was evaluated by spectrophotometry after incubation with a molar ratio of 25 TNM/β-subunit (Table 2). Tyr nitration was still significant in Y345F, less in Y368F, and almost prevented in Y345,368F. From the data presented in Table 2, the nitration at each of these residues/β-subunit can be calculated. Nitrotyrosine...
concentrations from Table 2 were expressed as the molar ratio of nitrotyrosine per β-subunit. The number of total nitrotyrosines/β-subunit was calculated as 1.344 (0.112 nitroTyr/WT β-subunit), 0.869 (0.079 nitroTyr/WT β-subunit), 0.308 (0.028 nitroTyr/WT β-subunit), and 0.14 (0.014 nitroTyr/WT β-subunit). The individual concentration of nitrotyrosine per β-subunit was calculated as 0.87 for Tyr345 and 0.31 for Tyr368, assuming that nitration in other Tyr residues is negligible. Indeed, the addition of nitrated Tyr368 and Tyr345 is close to the nitroTyr/WT β-subunit (0.87 ± 0.31 = 1.18 compared with 1.3), indicating that when Y345,368F is exposed to TNM, Tyr residues other than 345 and 368 have a higher nitration (approx. 20%) than when present in the WT (0.12 compared with 0.14). Thus the concentration of nitrated Tyr residues was 0.87 Tyr345, 0.31 Tyr368, and 0.14 for all other nitrated Tyr residues. These results indicate, that despite LC-MS/MS analysis indicating that eight Tyr residues (out of 12 in the β-subunit) were nitrated, the quantification of nitration evaluated by spectrophotometry unequivocally indicated that there was a significant Tyr nitration at residues 345 and 368 (indicated by circles on Figure 3B), whereas all other residues were not significantly modified. This apparent discrepancy can be explained by the different sensitivity of techniques used (spectrophotometry vs. LC-MS/MS). The yield of nitration calculated from these data was 43.5%, 15.5%, and 7% for Tyr345, Tyr345, and all others respectively. [Using the amount of nitrotyrosine/β-subunit (calculated as described above), and the amount of TNM that reacted with the β-subunit (from the molar ratio of 25 TNM/β-subunit, only 8% of the TNM was recovered as nitroformate ion, yielding 2 TNM/β-subunit), the yield of nitration was calculated as 43.5% (0.87/2 × 100%), 15.5% (0.31/2 × 100%), and 7% (0.14/2 × 100%), for Tyr345, Tyr368, and all others respectively. Note that the 7% of Y345,368F represents a higher limit for nitration of this subunit].

These results were consistent with those obtained with the dose–response curve of TNM to β-subunit (Figure 2A), in which two Tyr residues with very different reactivities were identified. If it is assumed that the more reactive residue is Tyr345, to obtain one nitrated Tyr/β-subunit, 28 and 100 TNM/β-subunit would be required for Tyr345 and Tyr368 respectively, indicating that residue 368 would be 3.6-fold more nitrated than residue 345.

Reactivity of Tyr to nitration is dependent on several factors, including a certain consensus of amino acid sequence [9], pK_a, the absence of steric hindrance, closeness to a loop structure, close proximity to negatively charged residues (aspartate and glutamate) and/or the absence of adjacent cysteine and methionine residues [28,63]. The basis for the difference in reactivity of these two Tyr residues might be found in the mechanism of the reaction between Tyr and TNM. If it is assumed that the reaction proceeds only with the phenolate form of Tyr, the reaction of Tyr nitration by TNM could be understood to be a radical collapse type (Figure 5). The reaction proceeds via the formation of a charge transfer complex, followed by an ion radical pair and finally through enone intermediates, and it can be speculated that the number of these intermediates would be restricted by the protein structure. Then Tyr with the more acidic pK_a should be more reactive (or nitrated).

By using the Henderson–Hasselbach equation, the corresponding pK_a of these residues was estimated, resulting in pK_a values of 7.2, 8.3 and 8.8 for Tyr345 and 7.34 for Tyr368 respectively, indicating that residue 368 would be 3.6-fold more nitrated than residue 345.

Figure 4  Inactivation of ATPase with TNM treatment

(A) The log of the pseudo-first order rate constant for the inactivation of ATPase (K) was plotted against the log [TNM] (K = kβ /kα). Wild-type β-subunits were treated with different molar ratios of TNM-β-subunit at pH 8.0 and at 22°C. At various time points (maximum 30 min), the activity was evaluated and from each molar ratio, the K was calculated. The equation for the line was log K = 1.55 + 0.91 (log [TNM]) with r^2 = 0.97. (B) Stoichiometry for inhibition of ATPase activity with nitrated Tyr345 (block-filled circles) and Tyr368 (open circles). Each β-subunit (WT, Y345F, Y368F and Y345,368F) was treated with a molar ratio of 0, 2.5 and 25 TNM/β-subunit. After reconstituting β with α- and γ-subunits, the ATPase activity was determined with an ATP-regenerating system. The ratio of nitrotyrosine/β-subunit was calculated using spectrophotometric analysis of each recombinant β-subunit treated with a molar ratio of 25 TNM/β-subunit.
Tyrosine nitration and ATPase activity loss

Evaluation of nitrated Tyr345 or Tyr368 on ATPase activity

The functional consequence of nitration of these residues was assessed by evaluating the ATPase activity of reconstituted F1, with each TNM-treated β-subunit (with a molar ratio of 25), or control-treated (with a molar ratio of 2.5; Table 1). Reconstituted F1 with TNM-treated Y345F β-subunit showed a smaller, but still significant, loss of ATPase activity (50 %) when compared with TNM-treated WT β-subunit (66 %). Reconstituted F1 with TNM-treated Y368F β-subunit abrogated most of the activity loss (19 % loss and 28 % loss compared with F1 with control-treated Y368F β-subunit and with no treatment respectively). However, reconstituted F1 with TNM-treated Y345,368F β-subunit completely abrogated the ATPase inactivation, resulting in an activity comparable with controls (112 % and 98 % compared with F1, with control-treated Y345,368F β-subunit, and with no treatment respectively). These findings indicated a clear role for the nitration of Tyr345 and Tyr368 on ATPase inactivation.

Stoichiometry for inhibition of ATPase activity due to nitration of Tyr345 and Tyr368

It has been shown that, of the six nucleotide-binding sites present on the bovine heart mitochondrial F1, only three sites exchange bound nucleotide rapidly during hydrolysis of ATP and participate sequentially in catalysis at high substrate concentration. The three non-exchangeable sites, referred to as noncatalytic sites, have been suggested to play a structural or regulatory role [64–67].

The use of single and double mutants allowed the evaluation of the extent of the association between nitration at each of these Tyr residues, believed to be located at catalytic (Tyr345) and noncatalytic (Tyr368) sites [65], and inhibition of catalysis (Figure 4B). Extrapolation indicated that modification of 0.7 Tyr345/F1 (or 1 Tyr345/F1) or 1.45 Tyr368/F1, was sufficient to stop nearly all the catalytic activity. Considering that the inactivation stoichiometry of WT β-subunit was 1.33 nitrotyrosine/F1, it can be calculated that 84 % of F1 has approximately equal numbers of F1, with one (55 %) and two (45 %) copies of nitrated Tyr345, whereas the rest (16 %) has a copy with a nitrated Tyr345.

Computer simulation of the ATPase structure using molecular visualization provided an illustration of the intermolecular interactions between Tyr residues and ATP (or its analogue ANP) that could explain the role of Tyr nitration in ATPase activity loss (Figure 6). The F-ATPases contain a P-loop or a ‘Walker A’ motif [68, 69], which acts as a nucleotide-binding site. In this loop, a conserved lysine residue is positioned close to the phosphate-binding site [70] (Figure 6A). The γ-phosphate of ATP in the catalytic site is usually surrounded by positively charged side chains [70, 71]. The ATP hydrolysis depends on a conserved catalytic (proton-abstracting) Glu, β-Glu[188], that primes a water molecule for the nucleophilic attack on the γ-phosphate group of ATP [70, 71] (Figure 6A). The 3D structure shows that the guanidino group of the ‘arginine finger residue’ α-Arg[376] is ~0.5 Å (1 Å = 0.1 nm) further away from the γ-phosphate in the nitroTyr345 structure than it is to the native Tyr345 (Figure 6B). α-Arg[376] in E. coli ATP synthase has been found not to participate directly in the catalysis, but it is required for promotion to the transition state stabilization [72, 73] and, in addition, a critical role in the α/β conformational signal transmission required for steady-state catalysis has also been inferred from point mutation studies [73]. Thus a longer distance between this residue and γ-phosphate may affect the stabilization of the transition state, and consequently, the Vmax of the reaction. The ε-amino group of the P-loop residue β-Lys[162] is also 0.3 Å further away from the γ-phosphate, consistent with a deficient stabilization of the phosphate in ANP (or ATP). Site-directed mutagenesis in E. coli F1 showed that β-Thr[205], β-Glu[188], and β-Asp[242] are all involved in Mg2+ co-ordination, forming an octahedral co-ordination around the ion that includes the oxygen atoms at the β- and γ-phosphate groups [74], similar to the coordination found in p21(H-Ras) protein [75]. Correct Mg2+ co-ordination had been shown to be required for catalytic activity at physiological rates, whereas elimination of any one of the Mg2+-co-ordinating residues led to complete loss of Mg2+-dependent nucleotide binding co-operativity of the catalytic sites [74]. The Mg ion in the nitroTyr345 structure had lost the electrostatic interaction to the β-phosphate, remaining interacted with the γ (Figure 6B). It could be expected that the looser binding of ATP in the structure with nitroTyr345 could affect the affinity of the site for ATP without necessarily affecting the Vmax. However, the nitration of Tyr affected the Vmax of the ATPase and not the Km (the present manuscript and the accompanying paper [32]). Thus the longer distance between the γ-phosphates and α-Arg[376] and β-Lys[162] [69, 71], in addition to the incorrect co-ordination of Mg2+ [74], should affect the stabilization of the transition state as well as the α/β conformational signal transmission required for hydrolysis of ATP, in agreement with our experimental results. Nitration of β-Tyr[368] – even though located further away from β-Tyr[345] – has a similar effect on the co-ordination of Mg and the distances of
Figure 6  Three-dimensional structures of β-subunits

Structures are based on PDB code 1bmf [71] and visualized and analysed using PyMOL (DeLano Scientific; http://www.pymol.org). In the reference structure of F1-ATPase [71], ADP and 5′-adenylyl-imidodiphosphate (AMP-PNP) were bound to the catalytic sites in the βDP- and βTP-subunits, respectively, and the βE-subunit had no bound nucleotide. The three conformations of the nucleotide-binding sites in these subunits correspond respectively to the ‘tight’, ‘loose’ and ‘empty’ states in the binding change mechanism [71]. Although the rat liver F1 crystal structure was available to perform this visualization (PDB code 1mab [84]), this structure was determined from an enzyme that lacked magnesium ion, and the three catalytic sites were all the same, thus it is unlikely that this structure represents a biologically relevant state. (A) Nucleotide-binding site in the β-subunit. All residues except α-Arg373 and α-Ser344 (shown in cyan) are in the β-subunit. Residues 159–164 are part of the P-loop. Yellow dashed lines represent electrostatic interactions between ATP (or its analogue phosphoaminophosphonic acid-adenylate ester; shown in red) and the protein. Magnesium is shown in green. (B) When Tyr345 is nitrated, several conformational effects were observed, the most profound of which are indicated with arrows in (A). (C) The nitration of Tyr368 results in a similar conformational changes as those observed in (B). (D) The nucleotide-binding site in the α-subunit is formed by amino acids within this subunit (shown in magenta), with the exception of Tyr368 and Arg372 (shown in grey) located in the β-subunit. There are no direct interactions between Tyr368 (bottom left-hand corner) and ATP. (E) When Tyr368 is nitrated, a cluster of amino acids is pushed out, resulting in the disruption of the Mg co-ordination and the electrostatic bond between Lys175 and the γ-phosphate (see arrows in D). (F) Similar conformational changes are observed as (E) in Tyr368 when Tyr368 is nitrated.

α-Arg73 and β-Lys162, resulting in similar conformational changes (see arrows in Figure 6A indicating broken bonds in Figures 6B and 6C).

The binding of adenine by the α-subunit involves several hydrogen bonds with amino acids present in the α-subunit, except for βDP-Arg72 and βDP-Tyr368 (Figure 6D). The former amino acid does not contribute to the αDP or αTP, and β-Tyr368 only contributes to the nucleotide binding in αDP [71]. This can be easily seen in Figure 6(D), where Tyr368 is located within 5–6 Å of the ATP binding site, not having a direct or indirect interaction with the substrate. Substituting this Tyr for phenylalanine did not result in significant conformational changes (results not shown). Indeed, substitutions of Tyr for phenylalanine may not be expected to have a relevant impact on activity. Substitutions of Tyr368 with phenylalanine [58,59] within the β-subunit of F1-ATPase in E. coli resulted in minor impairment of ATPase activity, consistent with our results obtained with Y368F-reconstituted F1 [58]. When Tyr368 is nitrated, the bulky nitro group changes the conformation...
of several amino acids in the α-subunit (Gln172, Ser177, Ser355, Met158, Arg359 and Arg362), changing several polar interactions between these and ATP, leaving ATP in a more ‘open’ pocket (Figure 6E). Nitration of this residue distorts not only the coordination of Mg between the β- and γ-phosphates, but also the electrostatic bond between the γ-phosphate with α-Lys175 (Figure 6E). A similar outcome can be observed in this site with the sole introduction of the nitration at β-Tyr345 (broken or changed bonds in Figures 6E and 6F where indicated with arrows in Figure 6D). This representation clearly underlies how the α/β conformational transmission is affected by the introduction of the nitration in these residues. Similar outcomes were observed when the 3D models were performed using aminotyrosine instead of nitrotyrosine (results not shown), structures in which the atoms have similar van der Waals radii, indicating that the steric effect of the nitro (or amino) group on neighbouring residues is more important for ATPase inactivation than charge effect, consistent with our results in the present study with dithionite.

### Concluding remarks

The goal of the present study was to address the stoichiometry and the role of nitration of two critical Tyr residues in the β-subunit of F1-ATPase, namely Tyr345 and Tyr368, in ATPase inactivation. This work unequivocally demonstrated the direct role of these two Tyr residues in ATPase inactivation. We focused on studying Tyr345 and Tyr368 in the β-subunit because these residues were mainly nitrated in rat liver mitochondria under *in vitro* and *in vivo* conditions [32].

Our results with site-directed mutagenesis clearly showed the direct role of nitration at Tyr345 and Tyr368 in the β-subunit in ATPase inactivation. It is inferred that to inactivate the ATPase, the nitration of either one-third of the catalytic (Tyr345) or at least one-third of the noncatalytic (Tyr368) site is required. Tyr368 plays a more relevant role (~4-fold) than Tyr345 in ATPase nitration under low nitrosative stress conditions because of its lower pK<sub>a</sub>. The higher reactivity of Tyr368 would be maintained in the mitochondrial matrix, for the pH is relatively basic, approx. 1 unit higher than that in the cytosol [77,78]. Under conditions of intensive nitrosative stress, when both Tyr residues would be nitrated, both Tyr residues will contribute equally to the inactivation of the ATPase because the higher stability of F1 particles with nitroTyr368 (2-fold; Table 3) cancels out the need for 2-fold more nitroTyr345 than nitroTyr368 required for ATPase inactivation (1.45 compared with 0.7).

These results from the present study are, in general, consistent with those obtained with 2-azido-ATP [65] with the difference that, in that study, one catalytic or two noncatalytic sites had to be modified to obtain inactivation. This difference might be explained by the different experimental setting. In Boyer and co-workers’ [65] studies, protein modification was performed by exposing mitochondrial F1 to 2-azido-ATP, whereas in the present study, the modification of the β-subunit was performed before assembling the F1 particle. The choice of this approach was based on testing the individual role of critical Tyr residues in β-subunits, excluding the participation or involvement of any other subunit and to evaluate if the reactivity of a free subunit was higher than that already assembled. In spite of these differences, it is interesting to draw a parallelism between Boyer and co-workers’ study [65] and the present study: one, the same residues were modified and found relevant for protein inactivation indicating that they are readily accessible and very reactive to chemical agents (or conditions of nitrosative stress), even when they are assembled.

### Number of nitrated β-subunits required for complete ATPase inactivation

The binding change mechanism for ATP hydrolysis originally proposed by Boyer [76] requires the active interaction of β- and α-subunits. To elucidate the number of copies of the β-subunit that must be modified for complete inactivation, the distribution of F1, with three β-subunits was calculated using the results from ATPase activity (Table 1) and the nitrotyrosine content of nitrated β-subunits (Table 2) assuming that all β-subunits (with or without nitration) have equal probability of forming a stable F1 (Table 3; column A shows normal distribution). However, this assumption did not fully conform to the experimental nitrotyrosine/F1, required for inactivation or the experimental activity loss, and it cannot be assumed that mutant/modified subunits will reconstitute as efficiently as WT ones. The F1 distribution was corrected by the inactivation stoichiometry and the distribution that matched the experimental activity loss is indicated in Table 3 (column B). It is clear that the percentages of F1 distribution with nitrated subunits were lower than those expected from a normal distribution. This indicated that the stability of F1 particles is challenged when modified copies are present. In this regard, comparing the values in columns A and B of Table 3, the stability of F1 with nitrated Tyr345 was 4-fold less than expected for a normal distribution, whereas that with nitrated Tyr368 was 2-fold lower, indicating that particles formed with nitrated Tyr368 will be twice as stable as those with nitrated Tyr345.

### Concluding remarks

The goal of the present study was to address the stoichiometry and the role of nitration of two critical Tyr residues in the β-subunit of F1-ATPase, namely Tyr345 and Tyr368, in ATPase inactivation. This work unequivocally demonstrated the direct role of these two Tyr residues in ATPase inactivation. We focused on studying Tyr345 and Tyr368 in the β-subunit because these residues were mainly nitrated in rat liver mitochondria under *in vitro* and *in vivo* conditions [32].

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These results from the present study are, in general, consistent with those obtained with 2-azido-ATP [65] with the difference that, in that study, one catalytic or two noncatalytic sites had to be modified to obtain inactivation. This difference might be explained by the different experimental setting. In Boyer and co-workers’ [65] studies, protein modification was performed by exposing mitochondrial F1 to 2-azido-ATP, whereas in the present study, the modification of the β-subunit was performed before assembling the F1 particle. The choice of this approach was based on testing the individual role of critical Tyr residues in β-subunits, excluding the participation or involvement of any other subunit and to evaluate if the reactivity of a free subunit was higher than that already assembled. In spite of these differences, it is interesting to draw a parallelism between Boyer and co-workers’ study [65] and the present study: one, the same residues were modified and found relevant for protein inactivation indicating that they are readily accessible and very reactive to chemical agents (or conditions of nitrosative stress), even when they are assembled.

### Table 3 β-subunit composition of F1, based on probability analysis and experimental data

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*At least one Tyr345 or Tyr368 had to be nitrated to center inactivation.
in F1; and two, steric hindrance is the main conformational change that drives the inactivation, for 2-azido-ATP should not change the phenolic pKₐ, and is a bulkier residue than the nitro. This latter conclusion is relevant, for it has been suggested that the reduction of nitrosotyrosine to aminotyrosine by a putative nitrotyrosine reductase (denitrase; [79-81]) can be viewed as a repair mechanism that restores protein activity. Our results do not support this concept and indicate that the modified residue has to be converted to Tyr [82] or removed by proteolysis [9] to regain the full activity.

**AUTHOR CONTRIBUTION**

Kazunobu Kato produced the recombinant proteins, MS analyses, and optimized assembly conditions. Yasuko Fujisawa performed experiments on the activity and quantification of nitrotyrosine. Cecilia Giulivi supervised the project, performed the 3D studies and provided biochemical support for the enzymatic analyses.

**ACKNOWLEDGEMENTS**

We thank Dr Tsuneo Imanaka for the gift of anti-tyrosinase against purified mitochondrial F1-ATPase. We are grateful to the excellent technical support of Ms. Catherine Ross-Inta.

**FUNDING**

This study was supported by funds provided by NIEHS (National Institute of Environmental Health Sciences) [grant number 012691 (to C.G.)] and Petroleum Research Fund [grant number PR 37470-B4].

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