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Reactive oxygen species target specific tryptophan site in the mitochondrial ATP synthase

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ABSTRACT

The release of reactive oxygen species (ROS) as side products of aerobic metabolism in the mitochondria is an unavoidable consequence. As the capacity of organisms to deal with this exposure declines with age, accumulation of molecular damage caused by ROS has been defined as one of the central events during the ageing process in biological systems as well as in numerous diseases such as Alzheimer's and Parkinson's Dementia. In the filamentous fungus *Podospora anserina*, an ageing model with a clear defined mitochondrial etiology of ageing, in addition to the mitochondrial aconitase the ATP synthase alpha subunit was defined recently as a hot spot for oxidative modifications induced by ROS. In this report we show, that this reactivity is not randomly distributed over the ATP Synthase, but is channeled to a single tryptophan residue 503. This residue serves as an intra-molecular quencher for oxidative species and might also be involved in the metabolic perception of oxidative stress or regulation of enzyme activity. A putative metal binding site in the proximity of this tryptophan residue appears to be crucial for the molecular mechanism for the selective targeting of oxidative damage.

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1. Introduction

Molecular damage caused by the exposure to ROS is a major cause of numerous diseases such as Alzheimer's and Parkinson's Dementia as well as a central factor of the ageing process by the 'free radical theory of ageing' (FRTA) [1] and its refined version, the 'mitochondrial free radical theory of ageing' (MFRTA) [2]. The latter theory identifies mitochondria as the major site of ROS generation and the predominant target of damage of all kinds of biomolecules. As a result, mitochondria become dysfunctional and biological systems degenerate and die. On the other hand, however, ROS are also employed in many life-sustaining mechanisms such as in signaling pathways and defense against pathogens.

In ageing research the conditions and effects of ROS generation, signaling, scavenging and damaging, are intensively studied in various systems including different model organisms. *Podospora anserina* is one of these organisms. This filamentous fungus is characterized by a short lifespan of a few weeks and is tractable to experimentation. It contains a small genome [3], of which the sequence has been completely determined [4]. Ageing of *P. anserina* has a clear mitochondrial etiology. A number of specific factors and pathways which are basically involved in keeping a functional population of mitochondria have been demonstrated to affect ageing and lifespan [3, 5–10]. Although ROS generation and scavenging do clearly contribute to the ageing process in *P. anserina* [11, 12], recently counter-intuitive data were obtained when the effect of the modulation of mitochondrial superoxide dismutase (PaSOD3) was studied [13]. In contrast to what is expected from the MFRTA, strains deleted for *PaSod3* were not short-lived and strains overexpressing *PaSod3* were not long- but short-lived. Intriguingly, the latter strains were characterized by a reduced abundance of a mitochondrial peroxiredoxin involved in scavenging of hydrogen peroxide, and in PaCLP protease, as a part of the mitochondrial protein quality control system. Taking these observations into account allowed the generation of a mathematical model that, at least partly, explains the unexpected results obtained from the study (manuscript in preparation). In particular, in addition to ROS metabolism mitochondrial protein quality control

Abbreviations: Desferal, Deferoxamine N′-{5-[acetyl(hydroxy)amino]pentyl}-N-[5-({4-[(5-aminopentyl)(hydroxy)amino]-4-oxobutanoyl}amino)pentyl]-N-hydroxysuccinamide; FRTA, Free radical theory of ageing; Kyn, kynurenine; MFRTA, mitochondrial free radical theory of ageing; MS, mass spectrometry; NFK, *N*-formyl-kynurenine; ROS, reactive oxygen species; SRM, single reaction monitoring; TRP-OH, hydroxytryptophan

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systems need to be included in networks of pathways involved in the control of ageing. Such systems may be triggered by and acting on specific posttranslational modifications. One of such modifications has also been identified in *P. anserina* mitochondria as the irreversible oxidation of tryptophan residues and the formation of *N*-formylkynurenine (NFK) [14, 15]. NFK is frequently observed in proteins by mass spectrometry along with the other oxidation intermediates displayed in Fig. 1, particularly in tissues with high metabolic rates and in proteins with long half lives [16]. Prominent is its presence in human and bovine heart mitochondrial proteins [15, 17], rat skeletal muscle proteins [18], bovine α-crystalline [19], as well as CP43 [20] and LHCII [21] in photosynthetic membranes.

Here we show that oxidative modifications are not—as intuitively accepted—randomly distributed to all tryptophan residues over the entire sequence of the alpha subunit of the mitochondrial ATPase, but selectively targeted to one specific tryptophan.

2. Materials and methods

2.1. P. anserina cultivation and isolation of crude mitochondria fraction

P. anserina is a filamentous ascomycete which is normally growing on solid medium. To obtain sufficient material for mitochondria isolation, after germination of monokaryotic ascospores on solid cornmeal medium with 60 mM ammonium acetate, small pieces of mycelium are first grown 2–3 days on solid PASM medium [22] under permanent light. To prevent transfer of solid medium into the subsequently used liquid medium, the solid medium was overlaid with a cellophane sheet. The mycelium from this solid medium was then transferred to liquid CM medium [23] and incubated for 2 days shaking under permanent light. Different age stages were obtained by pregrowth to the respective age on solid medium and subsequent culture in liquid medium. For instance, to isolate mitochondria from 6 days old cultures, ascospores were germinated for two days on solid cornmeal medium with ammonium acetate. Subsequently, pieces of mycelium from the growth front were transferred to solid medium with cellophane. After two days of growth, the mycelium was scratched from the plates and used to inoculate liquid medium. After additional 2 days of growth the mycelium was harvested, giving a total age of an individual of 6 days. Senescent cultures were isolated by growing the fungus on solid medium until it reaches senescence (= stop of growth, alteration of pigmentation). The solid medium with cellophane was now inoculated with mycelium 2–3 cm (4–5 days) behind the growth front. To prevent rejuvenation, cultivation of senescent mycelium in liquid medium was performed without shaking in Fernbach flasks (permanent light, 5–7 days). The isolation of mitochondria from these different mycelial samples was modified according to Gredilla [12]: After high-speed centrifugation (15,000 *g*, 20 min) the mitochondria were directly resuspended in isolation buffer without BSA, additional wash steps were omitted. In total crude mitochondrial extracts of three independent wild-type isolates of the age stages 6 days and senescent (14–15 days) were prepared. In control experiments 100 μM Desferal was added to all buffers used during isolation.

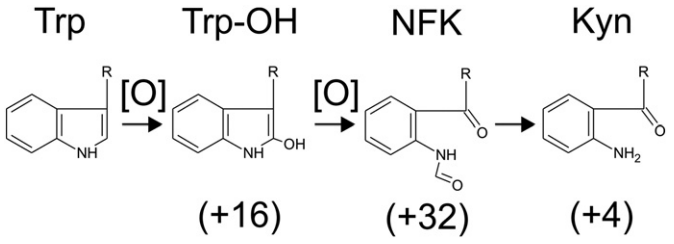


Fig. 1. Chemical structure of hydoxytryptophan (Trp-OH), N-formlykynurenine (NFK), and kynurenine (Kyn)—products and intermediates of the tryptophan oxidation [17]. Alternative structures with hydroxyl substitution at the benzene ring are plausible for Trp-OH and cannot be distinguished based on the mass shift. The mass shifts observed by MS relative to Trp are noted beneath the formulas.

2.2. 2-D Blue-native/tricine-SDS-PAGE and colorless-native PAGE

Isolated crude mitochondrial fractions were resuspended in a medium containing 50 mM imidazol, pH 7.0, 50 mM NaCl, 5 mM ε-aminocaproic acid, and 10% glycerol. The membranes were solubilized on ice for 30 min with the non-ionic detergent digitonin at a final concentration of 1% (4 g digitonin/g protein). Insoluble material was removed by centrifugation (21,000 *g*, 4 °C, 10 min). The supernatant was loaded directly onto blue-native gradient gels. Blue-native PAGE was performed using the Hoefer SE 600 system ($18\times16\times0.15$ cm^3, 10 lanes) as described previously [24, 25]. Stacking gels with a total acrylamide concentration of 3.5% and separating gels with linear acrylamide gradients 5–13% were prepared. 250 μg solubilized protein (as determined before solubilization) was applied per lane. Bovine heart mitochondria prepared from tissue and stored at −80 °C (4 g digitonin/g protein, 70 μg of protein before solubilization) served as molecular mass standard.

After electrophoresis, gels were stained with Coomassie R-250 or lanes of the BN-gel were cut out and incubated in a solution of 1% (w/v) SDS and 1% (w/v) β-mercaptoethanol at 20 °C for 30 min. Subsequently, lanes were analyzed by Tricine-SDS-PAGE in second dimension on a gel with two stacking gels, one native and one denaturing, with a total acrylamide concentration of 10% and a separating gel with 16.5% [26].

2.3. Protein analysis

After electrophoresis, gels were silver stained [27, 28]. Protein bands of interest were excised inside a laminar flow hood. Silver stained spots were destained [29], and in-gel digestion was performed with trypsin [30]. After extraction of the peptides with 50% (v/v) acetonitrile / 0.5% (v/v) formic acid the solvent was removed completely by lyophilization. Previous to LC-ESI–MS/MS analysis peptides were resuspended in 2% acetonitrile with 0.1% formic acid.

2.4. LC–MS analysis

LC–MS analysis was performed as described previously [31] with slight modifications. For reverse phase chromatography, a gradient of solvent A (95% water, 5% acetonitrile, 0.1% formic acid) and solvent B (10% water, 85% acetonitrile, 5% isopropanol, 0.1% formic acid) was used. For MS analysis, a Thermo LTQ Orbitrap mass spectrometer was operated in a duty cycle consisting of one 400–2000 *m/z* FT-MS and four MS/MS LTQ scans. For detection of low quantities of tryptophan oxidation for all three tryptophan sites in the ATP synthase alpha subunit an inclusion list was defined for the doubly and triply charged ions of the peptides ILQWEADFLSHLK, YSIVVAATASEAAPLQYLAPFTGASIGEWFR, and WNSGNDETK and their respective variants with oxidized tryptophan residues (Table 1).

2.5. Data analysis

Analysis of the LC–MS/MS data was performed using the Sequest algorithm [32] implemented in the Bioworks 3.3.1 software (Thermo scientific) for peptide identification versus a database [4] consisting of all *P. anserina* proteins with a tolerance of 10 ppm for the precursor mass accuracy and 1 u for the fragment mass accuracy. For detection of oxidized peptides for tryptophan residues modifications of 15.9949, 31.9898, and 3.9949 u were defined as parameters during the search for hydroxy-tryptophan, *N*-formyl kynurenin and kynurenin, respectively. False discovery rates were estimated by the number

Table 1
Peptide masses defined in the inclusion list for LC–MS analysis.

Peptide variant	Modification	$[M+2H]^{2+}$	$[M+2H]^{3+}$
lLQWEADFLSHLK	Unmodified	800.4301	533.9558
ILQW(OH)EADFLSHLK	Hydroxy-Trp	808.4272	539.2875
ILQW(NFK)EADFLSHLK	NFK	816.4250	544.6191
ILQW(Kyn)EADFLSHLK	Kyn	802.4276	535.2875
YSIVVAATASEAAPLQYLAPFTGASIGEWFR	Unmodified	1643.8427	1096.2309
YSIVVAATASEAAPLQYLAPFTGASIGEW(OH)FR	Hydroxy-Trp	1651.8402	1101.5626
YSIVVAATASEAAPLQYLAPFTGASIGEW(NFK)FR	NFK	1659.8377	1106.8942
YSIVVAATASEAAPLQYLAPFTGASIGEW(Kyn)FR	Kyn	1645.8402	1097.5626
WNSGNDETK	Unmodified	533.7254	356.1527
W(OH)NSGNDETK	Hydroxy-Trp	541.7229	361.4844
W(NFK)NSGNDETK	NFK	527.7254	352.1527
W(Kyn)NSGNDETK	Kyn	533.7254	356.1527

of spectral matches to a decoy database [33]. Acceptance criteria and filters were set to achieve a false positive rate of 5%.

2.6. Absolute peptide quantification

For quantitative analysis of the oxidative modification, single reaction monitoring (SRM) was applied on a triple quadrupole mass spectrometer [34]. Custom peptides were synthesized for Trp and Kyn variants of the peptide I*LQWEADFLSHLK by Thermo Scientific using isoleucine residue with stable isotopes to induce a mass shift of 7 Da to the peptides. For analysis tryptic digests were spiked with peptide standards and analyzed on a Thermo TSQ Vantage coupled to the reversed phase chromatography described in Section 2.4. The SRM transitions used for the analysis are listed in Supplemental Table 1. For quantification of the Trp-OH and NFK variants the Trp reference peptide was applied as standard, as only Trp and Kyn variants of the peptide could be obtained commercially. As judged by the similar ionization efficiencies observed for the Trp and Kyn variant, a systematic error of less than 20% can be expected by this approximation.

3. Results

While indications for oxidative modification of tryptophan residues in the ATP synthase in mitochondrial samples from *P. anserina* and other species have been described earlier [14], in this work focus was put on the detailed characterization, quantification, and localization of these oxidative modifications within the protein complex. Crude mitochondria from juvenile (6 days) and senescent *P. anserina* cultures were isolated by differential centrifugation. For the investigation three independent cultures from each age stage were used. To determine the role of free iron as a factor for ROS generation, mitochondria were isolated from three independent *P. anserina* individuals in the presence and absence of the strong iron chelating agent Desferal.

3.1. Separation of ATP synthase complexes

Fig. 2 displays the separation of solubilized mitochondrial membrane proteins by 2D-BN/SDS-PAGE. BN-PAGE in the first dimension separates native monomers and dimers of the ATP synthase, while SDS-PAGE in the second dimension separates the protein subunits constituting these protein complexes. To estimate the apparent mass of the protein complexes in the blue-native gel digitonin-solubilized bovine heart mitochondria served as reference [14], as these are well characterized and are often used to estimate the mass of membrane protein complexes in blue-native gels [35, 36]. In contrast to other species, e.g., rats [37], a separated F_1 subcomplex is hardly observed in the gels from *P. anserina* mitochondria. The gel system resolves the alpha subunits very well into two bands originating from monomeric and dimeric ATP synthase complexes. The alpha subunit contains three tryptophan residues. Apart from the gamma subunit, which contains a single tryptophan, subunit alpha is the only subunit of the F_1 part containing tryptophan in its mature protein sequence.

3.2. Identification of tryptophan oxidation site

For localization and structural characterization of tryptophan oxidation sites in the primary structure of the alpha subunit, bands corresponding to the dimeric and monomeric ATP synthase in the blue-native gel (Fig. 2: V_2, V_1) were digested with trypsin and the resulting tryptic peptides were applied to LC–MS/MS analysis. On average about 6500 MS/MS spectra could be assigned to peptides of ATP synthase subunits, yielding excellent sequence coverage of 30–80% for all proteins of the F_1 part. Although all subunits of the membrane integral F_O part were also detected, the number of distinct peptides observed was significantly lower due to the reduced number of tryptic cleavage sites in these proteins [38]. For alpha and beta subunits sequence coverage of 50% and 72% respectively, was obtained with

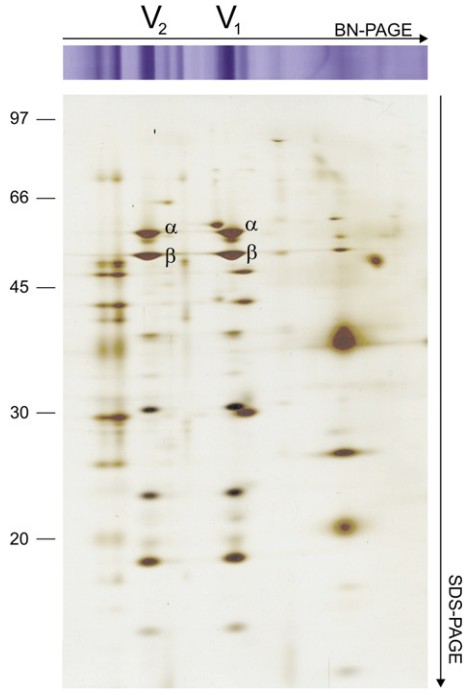
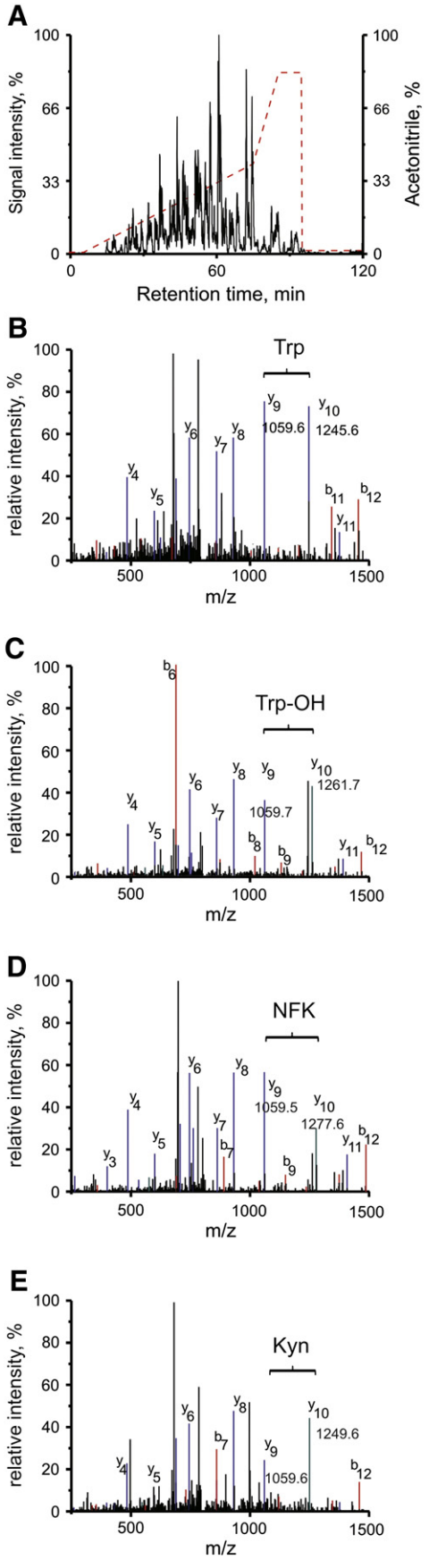


Fig. 2. Monomers and dimers of ATP synthase from *P. anserina* mitochondria are efficiently resolved in 2D-BN/SDS-gels. In contrast to mitochondrial samples from other species, significant amounts of F_1 subcomplex are not observed in the gels.



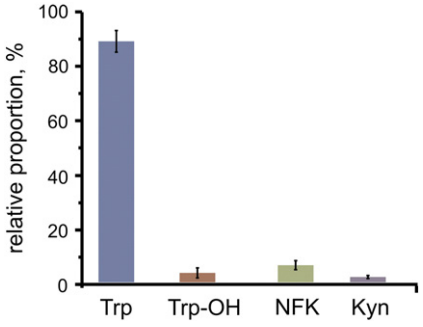


Fig. 4. Relative proportion of different variants of the peptide ILQ**W**EADFLSHLK quantified by the use of isotope-labeled reference peptides.

more than 2500 spectra assigned to peptides of each subunit. Fig. 3A displays a basepeak chromatogram of the analysis. All three Trp sites in the alpha subunit (Table 1) were sequenced by LC–MS/MS spectra from the corresponding tryptic peptides. Trp oxidation, however, could only be observed for Trp^{503} in the peptide ILQWEADFLSHLK. Species of this peptide with three intermediates of tryptophan oxidation—hydroxyltryptophan (Trp—OH), N-formlykynurenine (NFK), and kynurenine (Kyn) (Fig. 1)—were identified with p-values of $2 \cdot 10^{-6}$ and better. The assignment of these intermediates is unambiguous due to the high quality of the obtained MS/MS spectra (Fig. 3B–E). While the oxidation of the peptide could be demonstrated merely by the mass shift of the precursor and an incomplete ion series in the fragment spectra, the presence of y_9 and y_{10} ions unambiguously proves the oxidation of the Trp residue. The lower retention times as compared to the unmodified peptide (Trp: 68 min, Trp-OH: 51 min, NFK: 63 min, Kyn: 45 min) depict the reduced hydrophobicities of the oxidized Trp species.

Two other peptides of the alpha subunit containing Trp residues (Table 1) were observed, however, any corresponding peptides with oxidized Trp species could not be detected even when an inclusion list (Table 1) was applied for the LC–MS analysis.

3.3. Quantitation of tryptophan oxidation

For a quantitative analysis of the different oxidation intermediates of Trp^{503} observed in the LC–MS analysis isotope-labeled reference peptides were applied [34]. Fig. 4 depicts the relative proportion of the different oxidation intermediates of the peptide ILQWEADFLSHLK. Under all investigated conditions the NFK form was the most abundant oxidized species.

While the location of the oxidized tryptophan was well defined in all analyzed replicates, and also the amount of oxidized Trp was reproducible in technical replicates, as judged by peak areas of the respective peptides, a significant variation in the amount of oxidized Trp was observed between different biological replicates. In respect to the biological variation (for juvenile and senescent cultures), as well as monomeric and dimeric ATP synthase complexes, no statistically significant difference in the degree of oxidation was detected in the analysis in juvenile versus senescent cultures nor in monomeric versus dimeric ATP synthase. The degree of oxidation was, however, significantly reduced by more than 30%, when the strong iron

Fig. 3. A) Basepeak chromotogram of a tryptic digest from ATP synthase isolated from BN-gels analysed by LC–MS/MS. The four variants of the peptide ILQWEADFLSHLK are eluted with retention times of 68 min (Trp), 51 min (Trp-OH), 63 min (NFK), and 45 min (Kyn). Oxidized species from the two other tryptophan residues in the ATP synthase alpha subunit are not observed in the analysis. B–E) MS/MS spectra of the four variants of the peptide. The fragment ions y_9 and y_{10} representing the transition from EADFLSHLK to **W***EADFLSHLK allow the differentiation of oxidation intermediates.

chelator Desferal was present during the isolation of mitochondria suggesting a potential involvement of iron or another transition metal in the oxidation process.

4. Discussion

The ATP synthase is one of the most abundant proteins in the inner mitochondrial membrane generating most of the approximately 70 kg ATP utilized in humans every day. It is devoid of redox-active prosthetic groups and does not conduct any redox functions. However, the regulation of the chloroplast ATP synthase by the redox state is well known [39] and also for the mitochondrial enzyme indications for a redox regulation have been shown recently [40]. Due to its spatial proximity to the mitochondrial electron transport chain, the ATP synthase is a primary target of ROS.

4.1. Selectivity of tryptophan oxidation

Using an LC–MS/MS approach the selective targeting of Trp^{503} of the ATP synthase alpha subunit for oxidative modification can be unambiguously displayed, while no oxidation can be observed for the other Trp residues in the protein complex. Although oxidative stress appears as an unstructured and random process, channeling of oxidative damage to defined locations within protein complexes and protein subunits appears as a common characteristic of proteins frequently exposed to ROS. This selectivity has been observed for mitochondrial proteins—like the aconitase from bovine mitochondria displaying a predominant oxidation of Trp^{373} [15], as well as for proteins from other systems and organelles as the selective N-formylkynurenylation of Trp^{365} identified in subunit CP43 of the photosynthetic PSII complex under high-light stress [20]. Recently, a targeting of oxidative modifications to specific tryptophan residues was also recognized in pharmaceutical formulations [41] and a detailed analysis could attribute selectivity to a metal binding site in the proximity of the Trp residue.

4.2. Susceptibility of tryptophan towards oxidation

Although the oxidation of tryptophan is regularly observed in isolated proteins, in contrast to cysteine and methionine the reactivity towards superoxide anion radicals and hydrogen peroxide is quite low. Taylor and coworkers observed the different susceptibility of tryptophan and methionine towards oxidation, when analyzing peptides from the 18 kDa subunit of complex I [17], and suggested different oxidation mechanisms for the two amino acids. In pharmaceutical products the low reactivity of H_2O_2 and peroxides with tryptophan [42] is documented, while the reactivity of Trp with singlet oxygen [43] and hydroxyl radicals released by the Fenton reaction is high and is only surpassed by cysteine and methionine [44].

4.3. Localization within the ATP synthase structure

Fig. 5A displays a structural representation of the F_1 portion of the ATP synthase. Due to the high homology with a sequence identity of 80%, based on the ATP synthase structure from *S. cerevisiae* [45] a structure of subunit alpha from *P. anserina* was generated by homology modeling using Swiss-Model [46–48]. In the resulting structure, it is evident that all three tryptophans of subunit alpha are exposed to the surface and potentially accessible from the surrounding. Trp^{503}, which is the sole target for oxidative modification, is located in the peripheral region of the alpha subunit at a remote distance to catalytic and regulatory nucleotide binding sites or inter-subunit interfaces. A feature distinguishing Trp^{503} from the other two Trp sites is the location of three amino acid residues—Glu^{504}, Asp^{506} and His^{510} in its proximity (Fig. 5B). These residues are able to constitute a binding site for transition metal ion and induce an increased local concentration of these ions in the proximity of Trp^{503}. While this sequence pattern is well conserved in the subphylum Pezizomycotina and present in 55 of 59 sequences available for subunit alpha from this subdivision in the NCBI database, it appears to be absent in unicellular fungi in the subdivision Saccharomycotina and higher eukaryotes, in which Trp^{503} is replaced by a phenylalanine in all known sequences.

4.4. Biological implications

As removal of damaged protein is a critical function for the maintenance of mitochondria [49] and an impairment of protein degradation and repair has been connected to age-associated accumulation of oxidized proteins, for cellular homeostasis efficient means for the perception of oxidative stress are required. While oxidized cysteine and methionine can be efficiently reduced by repair mechanisms [9], tryptophan oxidation products are irreversible and have the potential to form markers visible for the mitochondrial quality control system. While an effect of the oxidation of Trp^{503} on the enzymatic activity of the ATP synthase is unlikely due to the distance to inter-subunit contact sites and catalytical as well as regulatory nucleotide binding sites, the disturbance of its structural integrity might be sufficient to trigger recognition by the protein quality control system. The LON protease, as an important component in the degradation of oxidized proteins in the mitochondrial matrix [50, 51], is induced by the presence of oxidized proteins both in activity and expression level [34, 39]. Although not shown yet, the quality of the mitochondrial ATP synthase may be controlled by proteases like iAAA protease or mAAA protease inserted in the inner mitochondrial membrane.

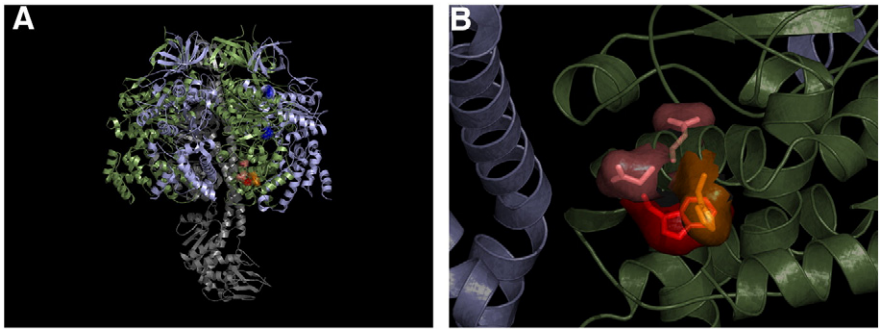


Fig. 5. A) Trp residue 503 (red) is surface exposed and located in the peripheral region of the alpha subunit with a distance of more than 30 Å from both the catalytical and regulatory nucleotide binding sites. The two other Trp residues (blue) are also exposed to the surface of the protein and are also accessible from the solvent. For the generation of a structural model of the alpha subunit of *P. anserina* a Swiss modeller [46–48] was used based on the structure published for *S. cerevisiae* [45] (PDB ID: 2XOK). B) The residues Glu^{504}, Asp^{506} and His^{510} located in the proximity of Trp^{503} at a distance of 5–9 Å are highlighted in a close-up of the structure. These residues are able to constitute a metal ion binding site. No such site can be defined for the two other Trp residues in the protein.

With its reactivity towards hydroxyl radicals and singlet oxygen, tryptophan oxidation might mediate a sensory function for strong ROS or in connection with the putative metal binding site could be involved in the perception of metal catalyzed protein oxidation. Due to its abundance and its location in the inner mitochondrial membrane the ATP synthase might be able to perceive compounds with diffusion ranges limited by short half-lives [52] and mediate an up-regulation of the protein turnover before more sensible components are affected.

The channeling of oxidative damage to specific subunits and domains is a common feature observed in different enzymes evolved under the evolutionary pressure imposed by oxidative stress, like the D1 protein of the photosystem II as a prominent example [53] which accounts for up to 50% of protein synthesis [54, 55] in chloroplasts under high-light conditions. This focuses the effort for maintenance of protein complexes to the degradation and replacement of a single protein subunit [56] and, thereby transforms the random and untargeted process releasing ROS into a unidirectional signal that can be perceived by the quality control system.

Supplementary materials related to this article can be found online at doi:10.1016/j.bbabio.2011.11.006.

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