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PHYTOCHEMISTRY
Phytochemistry 65 (2004) 1839–1851
www.elsevier.com/locate/phytochem

Identification of oxidised proteins in the matrix of rice leaf mitochondria by immunoprecipitation and two-dimensional liquid chromatography-tandem mass spectrometry

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Received 14 January 2004; received in revised form 5 April 2004
Available online 10 May 2004

Abstract

Highly purified mitochondria were isolated from green 7-day-old rice leaves. The mitochondria were sonicated and the matrix fraction isolated as the 100,000*g* supernatant. Part of the matrix fraction was left untreated while the other part was subjected to a mild oxidative treatment (0.5 mM H_2O_2 + 0.2 mM $CuSO_4$ for 10 min at room temperature). The oxidised proteins in both samples were tagged with dinitrophenylhydrazine (DNP), which forms a covalent bond with carbonyl groups. The DNP-tagged proteins were immunoprecipitated using anti-DNP antibodies and digested with trypsin. The mixture of peptides was analysed by nano-HPLC coupled online to an ESI-Quad-TOF mass spectrometer. The peptides were separated by stepwise ion exchange chromatography followed by reverse phase chromatography (2D-LC), and analysed by MS/MS. Proteins were identified by un-interpreted fragment ion database searches. Using this approach we identified 20 oxidised proteins in the control sample and a further 32 in the oxidised sample. Western blots of 2D-gels of the same samples prior to immunoprecipitation verified that the oxidation treatment increases protein oxidation also for specific proteins. Likewise Western blots showed that neither the isolation of mitochondria nor their subfractionation introduced carbonyl groups. We therefore conclude that a number of proteins are oxidised in the matrix of rice leaf mitochondria in vivo and further identify a group of proteins that are particularly susceptible to mild oxidation in vitro.
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Keywords: Rice (*Oryza sativa*); Gramineae; Monocotyledon; Proteomics; Oxidised proteins; Mass spectrometry; Mitochondrion; Matrix

1. Introduction

In plants all stress phenomena – biotic and abiotic – are accompanied by an increased production of reactive oxygen species (ROS) and this can lead to damage to proteins, lipids and DNA. In green plant cells in the light the chloroplasts and peroxisomes are probably the major sites of ROS production. In contrast, in non-green plant cells and in green plant cells in darkness the electron transport chain in the mitochondria may well be the major ROS producer like it is in mammalian cells (Møller, 2001; Foyer and Noctor, 2000). There are three levels in the defence strategy against ROS damage – avoidance, detoxification and repair. Avoiding or minimising ROS production generally involves preventing the electron transport chain from becoming too reduced particularly the sites of ROS production in complexes I and III. Once formed, ROS can be detoxified by superoxide dismutase and by several other enzymes or enzyme systems present in the mitochondrial

Abbreviations: CCO, cytochrome *c* oxidase; DNP, dinitrophenylhydrazine; ESI, electrospray ionisation; IP, immunoprecipitation; MDH, malate dehydrogenase; MS, mass spectrometry; ROS, reactive oxygen species; SMP, submitochondrial particle; TOF, time-of-flight.

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doi:10.1016/j.phytochem.2004.04.007

matrix. Finally, if the first two strategies fail, ROS will accumulate and damage will occur that should somehow be repaired (Møller, 2001).

In mammals ROS damage to proteins is implicated in a number of pathologies and ageing, but it is often not clear if protein damage by ROS is the reason for or a consequence of physiological malfunction (Dean et al., 1997). However, mitochondrial dysfunction leading to increased oxidative stress is clearly involved in a range of neuropathies and here the primary targets for oxidation may well be inside the mitochondrion (Carelli et al., 2002; Arbuzova et al., 2002). By comparison to mammalian mitochondria, plant and fungal mitochondria contain unique enzymes that either have been shown to be involved in – the alternative oxidase (Maxwell et al., 2002) – or may be involved in – the rotenone-insensitive NAD(P)H dehydrogenases – limiting the production of ROS in the electron transport chain (ETC) (Møller, 2001).

The interaction between ROS and proteins is complex and can result in a large variety of modifications from oxidation of single side groups to chain breakage (Dean et al., 1997; Berlett and Stadtman, 1997; Marnett et al., 2003; Ghezzi and Bonetto, 2003). The formation of carbonyl groups is considered to be an irreversible modification unlike the redox cycling of cysteine and methionine residues (Berlett and Stadtman, 1997; Ghezzi and Bonetto, 2003). Production of carbonyl groups may require more stringent oxidation conditions (Berlett and Stadtman, 1997) and as such be a valuable marker for oxidative stress. Protein carbonyl groups are one of the most studied modifications probably because a relatively simple method is available – conjugation with dinitrophenylhydrazine (DNP) and detection of the resulting DNP-tagged protein by a specific peroxidase-linked antibody. The amino acid residues normally considered to form DNP-reactive carbonyl groups by oxidation are arginine, lysine, proline and threonine (Berlett and Stadtman, 1997). However, several other residues can form carbonyl groups and carbonyls can also be formed by addition of lipid oxidation products or sugars (Dean et al., 1997; Berlett and Stadtman, 1997). Thus, the potential complexity of protein oxidation in vivo or in vitro is substantial.

Taylor et al. (2003a) searched more than 800,000 tandem MS spectra from an extensive study of the human heart mitochondrial proteome (Taylor et al., 2003b) for the presence of oxidised tryptophan residues and found 39 proteins containing this modification. To date this is the most comprehensive study of mitochondrial protein oxidation.

We are developing methods for the identification of oxidised proteins in plant mitochondria to understand the impact of oxidative stress on this important organelle. In the present study we have used immunoprecipitation (IP) of DNP-tagged proteins, followed by 2D-LC MS/MS of tryptic peptides to identify oxidised proteins in the soluble matrix fraction of mitochondria from green leaves. By in vitro oxidation, we have further identified a number of potentially oxidation-prone proteins in these mitochondria.

2. Materials and methods

2.1. Plant material

Rice (*Oryza sativa* L. ssp. *japonica*) (cv. Arborio) was sown in trays with peat and grown in a controlled-environment chamber at 29 °C, 70–80% RH in constant light (ca. 200 $\mu mol\, m^{-2}\, s^{-1}$) for 7 days and then in constant darkness for 24 h. Seedlings were watered with distilled water daily to keep the soil surface wet.

2.2. Isolation of total leaf protein for testing introduction of carbonyl groups

Rice leaves were homogenized in six different ways to test whether the procedure introduces protein oxidation. Homogenization was done either with an UltraTurrax (IKA Werke, Germany), a Warring blender or grinding in a mortar. In the mortar it was done either under liquid nitrogen or with the mortar ice-cold (−20 °C). The ice-cold homogenisation medium containing 0.3 M mannitol, 5 mM EDTA, 30 mM K-MOPS, 1% (w/v) BSA, 5 mM DTT, 1% PVPP, pH 7.3 was either untreated or purged with nitrogen gas to exclude atmospheric oxygen. After filtering through nylon cloth (120 μm), the homogenate was saved at −20 °C until analysed by Western blotting.

2.3. Isolation and purification of mitochondria

Leaves from 8-day-old rice seedlings were harvested, cut into 3–5 mm pieces and homogenized with an UltraTurrax by 2×10 s bursts in the homogenisation medium (30 g fresh weight/200 ml medium). The homogenate was strained through nylon cloth (120 μm) and centrifuged at 1000*g* for 5 min. The supernatant was centrifuged at 20,000*g*×25 min and the pellets (crude mitochondria) were resuspended in wash medium (0.3 M mannitol, 1 mM EDTA, 10 mM MOPS, pH 7,2). The crude mitochondria were layered on top of a step Percoll gradient −8 ml 40% Percoll, 24 ml 20% Percoll both in Percoll buffer (0.4 M mannitol, 5 mM Mops, 1 mM EDTA, 0.4% PVP, pH 7.2) – and centrifuged at 40,000*g* × 30 min. The mitochondrial band was taken out, diluted >10 times with wash medium and the mitochondria pelleted at 20,000*g* × 20 min. The pellets were resuspended in wash medium, diluted, pelleted and resuspended in wash medium. To remove the last thylakoid membranes (chlorophyll) the partially purified

mitochondria were layered on top of a self-generating Percoll gradient consisting of 33% Percoll in Percoll buffer. The gradient was developed by spinning at $40{,}000g \times 30$ min, the mitochondrial band was taken out, Percoll removed by repeated dilution and pelletation as above and the final pellet resuspended in a small volume of wash medium.

2.4. Subfractionation of mitochondria

The purified mitochondria were ruptured by 10×5 s bursts from an sonicator (U50, IKA Werke, Germany, Power setting cycle 1, 60% amplitude) in a medium containing 0.4 M sucrose, 5 mM MOPS, 20 mM $MgCl_2$, 1 mM PMSF, pH 7.0. Three ml of wash medium without EDTA was added and undamaged mitochondria and large fragments pelleted at $20{,}000g \times 15$ min. The pellets were resuspended and subjected to a further round of sonication and pelletation. The combined supernatants were centrifuged at $100{,}000g \times 60$ min and the pellets containing inside-out submitochondrial particles (Møller et al., 1987; Rasmusson and Møller, 1991) resuspended in wash medium + 5% dimethylsulfoxide and frozen together with the supernatant (the matrix fraction) in liquid nitrogen and stored at −80 °C until used.

2.5. Testing subfractionation procedure

Isolated mitochondria were subfractionated basically as described above but with slight changes to evaluate if the procedure introduces protein oxidation. Sonication was done either with 2×5 bursts, each with 5 s or with 2×10 bursts, each of 5 s, both with 10 s cooling on ice between each burst. The ice-cooled tube used for sonication was either untreated or purged with nitrogen gas to exclude atmospheric oxygen. Purging of the buffer was done for 5 min before and during sonication.

2.6. Determination of enzyme activities and protein concentration

Cytochrome *c* oxidase (CCO) and malate dehydrogenase (MDH) activities were measured essentially as described by Møller et al. (1987) and Rasmusson and Møller (1991).

Protein concentrations were estimated using the Lowry reagent (Bio-Rad) as described by the producer.

2.7. In vitro oxidation of matrix protein

Mitochondrial matrix protein (2.0 mg) was oxidised by treatment with metal-catalysed oxidation reagent, 0.2 mM $CuSO_4$ and 0.5 mM H_2O_2 in a total volume of 2.26 ml (0.4 M sucrose, 5 mM K-MOPS, 20 mM $MgCl_2$, 1 mM PMSF, pH 7.0) for 10 min at room temperature (22–23 °C). In the control sample (2.0 mg) $CuSO_4$ and H_2O_2 were omitted. Adding EDTA, pH 7.0 to a final concentration of 10 mM and freezing in liquid nitrogen stopped the reaction.

2.8. Labelling of carbonyls and immunoprecipitation

Based on Talent et al. (1998) protein samples to be immunoprecipitated or analysed by SDS–PAGE were labelled with dinitrophenylhydrazine by adding HCl and DNP to final concentrations of 1 M and 13 mM, respectively, at a final protein concentration of 0.6 mg/ml. The sample was incubated at room temperature with gentle agitation for 30 min. The labelling reaction was stopped by adding ice-cold TCA to 20% w/v and incubating on ice for 30 min. The protein precipitate was centrifuged ($20{,}000g$) for 20 min at 4 °C. Pellets were washed three times in 1:1 (v/v) ethanol:ethylacetate and air-dried. Pellets were solubilised in 8 M urea and diluted with PBS to 200 mM urea to allow antibody interaction. Dynabeads M-280 tosyl-activated (Dynal, NO) were covalently coated with rabbit anti-DNP antibodies (Dakocytomation P 0401) and blocked with bovine serum albumin as described by the manufacturer. Coated Dynabeads were added to the DNP-labelled protein solutions in amounts to ensure surplus of binding capacity and then incubated for 16 h at room temperature with agitation. The unbound proteins were removed and the beads with immuno-complexes were washed at room temperature in PBS for 15 min, and twice for 15 min in 10 mM sodium-phosphate buffer (pH 7.2). Immunoprecipitated proteins were eluted from the Dynabeads by washing three times with 1% formic acid, 50% acetonitrile. The combined eluates were dried in a speed-vac.

For two-dimensional (2D) electrophoresis, proteins with carbonyl groups were labelled with DNP in the IPG strip as described below.

2.9. Electrophoresis and immunoblotting

SDS–PAGE was done using standard Laemmli (1970) denaturing Tris–glycine gels (10 or 12% acrylamide). Gels were stained with Coomassie R-350 (Amersham) or silver as described by Shevchenko et al. (1996).

For analysis by 2D electrophoresis 1.0 mg of oxidised and of control matrix protein was precipitated by adding TCA to 12% w/v, incubating on ice for 30 min and centrifuging ($20{,}000g$) for 20 min at 4 °C. Pellets were washed twice in 70% ice-cold acetone and air-dried. Pellets were solubilised in 7 M urea, 2 M thiourea, 2% Chaps, 2% dodecylmaltoside, 20 mM DTT and a trace of bromphenol blue by agitating for 30 min at room temperature. Samples were centrifuged ($50{,}000g$) for 30 min at 20 °C and 1% v/v IPG buffer (Amersham) was

added to the supernatant. Immobilised pH gradient (IPG) isoelectric focussing pH 5–8, 17 cm gel strips (Bio-Rad) were rehydrated overnight in the solubilised protein supernatant and focussed for a total of 110 kV h on a IPGphor (Amersham) as described by the manufacturers.

Protein carbonyls were labelled with DNP in-strip as described by Choi et al. (2002). Second dimension Tricine SDS–PAGE was carried out as described by Schägger and von Jagow (1987). Semi-dry transfer of protein to PVDF (Millipore) membranes was done using the conditions and buffer system described by Kyhse-Andersen (1984). After transfer, membranes were incubated in 2% Tween 20 in TBS for 30 min and washed three times in TBS. Immunolabelling was done by incubating the membranes in HRP conjugated anti-DNP antibody (Dakocytomation P 0402) diluted 1:4000 in TBS, 0.05% (v/v) Tween 20, 2% BSA (Sigma A-7906, (fraction V)) for 3 h at room temperature, washed three times in TBS and developed using home-made chemiluminescence reagents (1.25 mM 5-amino-2,3-1,4-phthalazinedione (luminol), 2.7 mM H_2O_2, 0.07 mM *p*-coumaric acid in TBS) and exposing the membrane to Kodak X-Omat AR film. Subsequently the membrane was stripped from antibodies by washing two times for 5 min in 5 mM KOH, 5 min in Milli-Q water followed by two washes in TBS. Protein was stained using the BLOT-FastStain (Chemicon, CA, USA) as described by the manufacturer. Developed films, stained gels and membranes were scanned on a flatbed scanner using Amersham ImageMaster Labscan v3.00 software and processed using Amersham ImageMaster 2D Elite v3.1 and Adobe Photoshop v5.0 software.

2.10. Tryptic digestion of proteins

Eluted immunoprecipitated proteins were solubilised in 8 M urea, 0.4 M ammonium hydrogen carbonate, reduced by adding DTT to 7.5 mM and incubating at 56 °C for 15 min, alkylated by adding iodoacetamide to 15 mM and incubating in the dark at room temperature for 15 min. The solution was diluted with Milli-Q water to four times the original volume and 0.3 μg trypsin (modified porcine, Promega) was added. The digestion was incubated at 37 °C for 18 h and frozen until MS analysis. Protein spots cut from PVDF membranes was destained with water and processed as described (anonymous: http://pcl.tamu.edu/data/pdf/md.pdf). Peptide extracts were dried in a speed-vac and frozen until analysis.

2.11. Mass spectrometry and data treatment

Two-dimensional LC-MS/MS was used to identify tryptic peptides from the immunoprecipitate. A nanoflow capillary high-pressure liquid chromatography system (Famos, Switchos, Ultimate (LC-Packings, The Netherlands)) equipped with a strong cation exchange column (500 μm ID × 15 mm, BioX-SCX, LC-Packings) for the first dimension, a RP trap column (300 μm ID × 5 mm, PepMap C18, 5 μm, LC-Packings) and a nanoscale second dimension RP capillary column (75 μm ID × 15 cm, PepMap C18, 3 μm, LC-Packings) was set up for automated 2D-LC as described by the manufacturer. The nano-HPLC system was interfaced directly to a Q-TOF tandem mass spectrometer (Q-TOF Ultima Global, Micromass, UK) using the nano Z-spray electrospray source. Each sample was resolved by a 9-cycle fully automated chromatographic run. In the first cycle, the peptide mixture was injected onto the cation exchange column, unbound peptides washed off and captured on the trap column. The trap column was washed and switched on-line with the second dimension RP nano-scale column and peptides were eluted by a 55 min gradient of 2–50% acetonitrile in 1% acetic acid, 1% formic acid. The trap and second dimension RP column was then washed in 90% acetonitrile in 1% acetic acid, 1% formic acid for 10 min and equilibrated for 10 min. The flow rate in the second dimension was 400 nl min^{-1}. In the second to ninth cycle salt solutions in increasing concentrations (0.04, 0.05, 0.06, 0.08, 0.1, 0.5, 1 and 2 M NaCl) were injected onto the cation exchange column, eluting peptides to the trap column where the peptides were captured and salts washed off prior to second dimension RP separation in the same manner as for the first cycle.

Automated LC-MS/MS was done on a nanoflow capillary high-pressure liquid chromatography system (Famos, Switchos, Ultimate, LC-Packings, NL) equipped with a RP trap column (300 μm ID × 5 mm, PepMap C18, 5 μm, LC-Packings), and a nanoscale analytical RP capillary column (75 μm ID × 15 cm, PepMap C18, 3 μm, LC-Packings) that was interfaced directly to a Q-TOF tandem mass spectrometer (Q-TOF Ultima Global, Micromass UK). Peptides were eluted by a 55 min gradient of 2–50% acetonitrile in 1% acetic acid, 1% formic acid. The flow rate was 400 nl min^{-1}. The system was washed and equilibrated as above.

The mass spectrometer was operated in the data-dependent acquisition mode and was calibrated using Glu-fibrinopeptide B (Sigma) in the MS/MS mode.

Data reduction was performed using MassLynx and ProteinLynx software, and the resulting MS/MS data set was searched against the NCBI database using the Mascot search engine (ver. 1.9.0.5, Matrix Science Ltd., London, UK). The MS/MS data was first searched with carbamidomethylation of cysteine as a fixed modification and deamidation of Asn and Gln (mass difference +1) and oxidation of Met, His, Trp (+16) as variable modifications. Tolerances for Mascot searches were 1 Da for the MS and 0.4 Da for the MS/MS matching. Subsequently the data was searched four additional

times with different variable modifications. The allowed modifications were (i) formation of kynurenin (+4), (ii) formylkynurenin (+32), (iii) hydroxykynurenin (+20), from Trp and (iv) addition of DNP to oxidised Arg, Pro, Lys and Thr (+180).

Proteins were considered identified if at least one matching peptides had a score above the significance threshold of the search (Mowse probability better than 0.05) and a peptide mass tolerance less than 0.2. The resulting protein hit-list was filtered for redundant entries (non-rice or proteins with a lower score than the best score for that protein). This filtered hit-list was re-searched using the Mascot error-tolerant algorithm to match peptides with unsuspected modifications.

3. Results

3.1. The matrix fraction of rice leaf mitochondria is free from inner membrane contamination

Purification of rice leaf mitochondria on a self-generating Percoll gradient improved the specific activity of the mitochondrial inner membrane marker cytochrome *c* oxidase (CCO) and the matrix marker malate dehydrogenase (MDH) by 6- and 7-fold, respectively (Table 1), and removed all chlorophyll present in the crude mitochondria (not shown).

If intact mitochondria are treated with metal-catalysed oxidation reagent (Cu^{2+}/H_2O_2) it is difficult to know to what extent the proteins inside the selectively permeable inner membrane are exposed to the ROS generated in the external medium. Furthermore, if membranes are present, carbonylated proteins that will react with DNP can arise, not only from direct oxidation, but also from conjugation with aldehydes formed when polyunsaturated fatty acids in the membrane phospholipids are peroxidised. To have a simpler model system, we therefore used the matrix fraction isolated from purified rice leaf mitochondria by sonication and differential centrifugation (Table 1). The matrix fraction contained very little total CCO activity and the specific CCO activity was >100 times higher in the SMP fraction indicating that the matrix fraction was <1% contaminated by inner membrane. The yield of matrix fraction was >80% as judged by total MDH activity. This matrix fraction was used in subsequent experiments.

3.2. Twenty oxidised proteins are detected in the untreated matrix fraction by 2D-LC-MS/MS

Twenty oxidised proteins were identified in the immunoprecipitate (IP) of the matrix fraction by 2D-LC followed by MS/MS (Table 2). Of these, 10 are dehydrogenases or oxidases i.e. redox-active enzymes – four of the Krebs cycle or associated enzymes (citrate synthase is not a redox enzyme) and six in the group ''other redox enzymes''. One oxidised protein, superoxide dismutase, is involved in ROS detoxification and two proteins, hsp60 and hsp70, are chaperones. The last six oxidised mitochondrial proteins have assorted functions. Two of these, the voltage-dependent anion channel and cytochrome b_5 reductase, belong in the outer membrane indicating that there is a (small) outer membrane contamination in the matrix fraction. Finally, oxalate oxidase, presumably from the apoplast, and the small subunit of Rubisco from the chloroplast stroma are contaminants and were probably adsorbed to the outer surface of the outer membrane during homogenisation.

Control experiments showed that homogenising the tissue in the absence of oxygen (under liquid nitrogen) neither reduced the amount of DNP-reactive proteins in the tissue homogenate nor changed their pattern on 1D-gels (results not shown). This indicates that the oxidised proteins were present in the tissue before homogenisation. Likewise, subfractionation of the mitochondria was done under various conditions including sonication

Table 1
Purification and subfractionation of rice leaf mitochondria

Fraction	Protein (mg)	CCO		MDH	
		Total	Specific	Total	Specific
Purification of mitochondria					
Crude mitochondria	305	367	1.20	2690	8.82
Purified mitochondria	6.20	49.6	7.99	392	63.2
Subfractionation of purified mitochondria					
First pellet	2.24	19.8	8.84	7.5	3.4
First supernatant (= matrix + SMP)	5.40	9.7	1.80	239	44.3
SMP	1.19	11.1	9.35	6.4	5.3
Matrix	3.74	0.3	0.09	340	90.9

The results are from one preparation but similar trends were observed with other preparations. Total activity is given as $\mu mol\ min^{-1}$ and specific activity as $\mu mol\ min^{-1}\ mg^{-1}$ protein. SMP, inside-out submitochondrial particles = inner membrane vesicles.

CCO, cytochrome *c* oxidase; MDH malate dehydrogenase.

Table 2
Oxidised proteins identified by 2D-LC-MS/MS in the untreated matrix fraction

Identity (EC No.)	Acc. No.	Species	kDa	Cov.%	Pep#	Score	Localization
Krebs cycle and associated enzymes							
Pyruvate dehydrogenase E1β-subunit (1.2.4.1)	gi|3850999	ZEAMA	40	4	1	52	Mit. matrix
Dihydrolipoamide dehydrogenase (1.8.1.4)	gi|13365781	ORYSA	53	16	4	258	Mit. matrix
Citrate synthase (2.3.3.1)	gi|11066954	ORYSA	55	3	1	83	Mit. matrix
NAD^+-isocitrate dehydrogenase (1.1.1.41)	gi|15237075	ARATH	40	5	1	74	Mit. matrix
Malate dehydrogenase (1.1.1.137)	gi|14164433	ORYSA	36	50	10	889	Mit. matrix
Respiratory complexes							
ATP synthase β-subunit (3.6.3.14)	gi|231587	ORYSA	60	5	2	118	Mit. matrix
ROS-detoxifying enzymes							
Superoxide dismutase (Mn) (EC 1.15.1.1)	gi|7433347	ORYSA	25	25	5	375	Mit. matrix
Chaperones							
Hsp60	gi|283035	ZEAMA	61	5	2	56	Mit. matrix
Hsp70	gi|399940	PHAVU	73	8	3	117	Mit. matrix
Other redox enzymes							
Formate dehydrogenase (1.2.1.2)	gi|21263611	ORYSA	41	14	4	233	Mit. matrix
Aldehyde dehydrogenase (1.2.1.3)	gi|11691926	ORYSA	60	13	4	304	Mit. matrix
Methylmalonate-semialdehyde dehydrogenase (1.2.1.27)	gi|7431455	ORYSA	58	7	2	111	Mit. matrix*
Glutamate dehydrogenase (1.4.1.2)	gi|7431775	ZEAMA	44	10	2	105	Mit. matrix
(*S*)-2-Hydroxy-acid oxidase (1.1.3.15)	gi|7431424	ORYSA	40	15	3	89	Peroxisome
Cytochrome b_5 reductase ((1.6.2.2)	gi|15128231	ORYSA	32	6	1	70	Mit. OMM
Other enzymes							
Glycine decarboxylase T protein	gi|1346121	FLAPR	45	10	2	105	Mit. matrix
Other proteins							
Voltage-dependent anion channel	gi|18076158	ORYSA	30	21	3	121	Mit. OMM
Translational elongation factor TuM	gi|18001149	ORYSA	49	6	2	96	Mit. matrix
Contaminants							
Oxalate oxidase (1.2.3.4)	gi|6996619	TRIAE	24	9	2	105	Apoplast*
RuBisCO small subunit A (4.1.1.39)	gi|132096	ORYSA	20	8	1	73	Chloroplast

Acc. No., GenBank Accession Number; species, e.g., *Oryza sativa*; kDa, mass of preprotein; Cov.%, coverage based on preprotein; Pep#, number of peptides identified; score, Mascot score, scores above 50 for individual peptides indicate identity; localization, if known or *, based on TargetP prediction (http://www.cbs.dtu.dk/services/TargetP/).

Mit., mitochondrion; OMM, outer mitochondrial membrane; Perox., peroxisome.

in an oxygen-depleted medium under nitrogen gas. Again there was no effect on the amount or pattern of DNP-reactive proteins (results not shown). Thus, any oxidised proteins found in the matrix fraction before the oxidative treatment represent the in vivo level of protein oxidation and this gives us information about which proteins are particularly prone to oxidation in vivo.

3.3. A further 32 oxidised proteins are detected in the oxidised matrix fraction by 2D-LC-MS/MS

Many more DNP-conjugated proteins were identified in the matrix fraction after exposure to metal-catalyzed oxidation reagent. These have been placed in the same groups in Table 3 as used for the control matrix in Table 2. Out of the 20 oxidised proteins in the control matrix 19 were also found in the oxidised matrix, the only exception being the contaminant oxalate oxidase. This gives confidence in the 2D-LC-MS/MS method.

The proteins oxidised by metal-catalysed oxidation reagent include five additional Krebs cycle or associated enzyme such that all the Krebs cycle enzymes present in the matrix are oxidised in the oxidised sample. The enzyme complex glycine decarboxylase appears to be particular susceptible to oxidation as all four component enzymes are oxidised (Table 3). Otherwise the list of oxidised proteins in the oxidised matrix fraction includes an additional four redox enzymes bringing the total to ten. Two more ROS-detoxifying enzymes, catalase and ascorbate peroxidase, are also oxidised. Ascorbate peroxidase is one of the four enzymes in the ascorbate/glutathione cycle well known to be involved in ROS detoxification in the chloroplast (Foyer et al., 1994, and references therein) but recently also shown to be present in plant mitochondria (Jimenez et al., 1997; Chew et al., 2004). Concerning catalase, the question is whether it is of mitochondrial origin. Catalase activity is always detected in even highly purified plant mitochondria (e.g.

Table 3
Oxidised proteins identified by 2D-LC-MS/MS in the oxidised matrix fraction

Identity (EC No.)	Acc. No.	Species	kDa	Cov.%	Pep#	Score	Localization
Krebs cycle and associated enzymes							
Pyruvate dehydrogenase, E1α-subunit (1.2.4.1)	gi|3851005	ZEAMA	43	17	4	127	Mit. matrix
Pyruvate dehydrogenase, E1β-subunit (1.2.4.1)	gi|3850999	ZEAMA	40	17	6	336	Mit. matrix
Dihydrolipoamide dehydrogenase (1.8.1.4)[a]	gi|13365781	ORYSA	53	29	9	435	Mit. matrix
Dihydrolipoamide S-acetyltransferase (2.3.1.12)	gi|5669871	ZEAMA	59	6	2	59	Mit. matrix
Citrate synthase (2.3.3.1)	gi|11066954	ORYSA	52	17	4	140	Mit. matrix
Aconitase (4.2.1.3)	gi|15224580	ARATH	99	3	2	136	Mit. matrix
NAD^+-isocitrate dehydrogenase (1.1.1.41)	gi|6539562	ORYSA	40	17	4	200	Mit. matrix
Succinyl-CoA-ligase, α-subunit (6.2.1.5)	gi|15241592	ARATH	37	13	3	161	Mit. matrix
Succinyl-CoA-ligase, β-subunit (6.2.1.5)	gi|15225353	ARATH	46	16	5	133	Mit. matrix
Fumarase (4.2.1.2)	gi|7436831	SOLTU	54	4	1	62	Mit. matrix
Malate dehydrogenase (1.1.1.37)[a]	gi|14164433	ORYSA	36	72	14	1186	Mit. matrix
NAD^+-malic enzyme 59 kDa iso form (1.1.1.39)	gi|585452	ORYSA	67	2	1	43	Mit. matrix
Respiratory complexes							
ATPase α-subunit (3.6.3.14)	gi|9408184	SORBI	48	11	3	184	Mit. matrix
ATPase β-subunit (3.6.3.14)	gi|231587	ORYSA	59	23	8	430	Mit. matrix
Cytochrome *c*	gi|2394300	ORYSA	12	29	3	155	Mit. IMS
ROS-detoxifying enzymes							
Ascorbate peroxidase (1.11.1.11)	gi|15808779	HORVU	28	14	2	148	Unclear*
Catalase (1.11.1.6)	gi|20192	ORYSA	57	18	5	286	Peroxisome*
Peroxiredoxin (/unnamed protein product)	gi|6630684	ORYSA	21	23	2	57	Mit. matrix*
Superoxide dismutase (1.15.1.1)	gi|7433347	ORYSA	25	51	10	572	Mit. matrix
Chaperones							
Hsp60	gi|283035	ZEAMA	61	24	10	561	Mit. matrix
Hsp70	gi|399940	PHAVU	73	12	3	189	Mit. matrix
Other redox enzymes							
Formate dehydrogenase (1.2.1.2)[a]	gi|21263611	ORYSA	41	63	14	703	Mit. matrix
Aldehyde dehydrogenase (1.2.1.3)	gi|11691926	ORYSA	60	36	12	624	Mit. matrix
Methylmalonate-semialdehyde dehydrogenase (1.2.1.27)	gi|7431455	ORYSA	58	14	5	351	Mit. matrix
Glutamate dehydrogenase (1.4.1.2)	gi|7431775	ZEAMA	44	27	7	414	Mit. matrix
(*S*)-2-Hydroxy-acid oxidase (1.1.3.15)	gi|7431424	ORYSA	40	21	6	361	Peroxisome
Cytochrome b_5 reductase (1.6.2.2)	gi|15128231	ORYSA	32	29	5	155	Mit. matrix
Glycolate oxidase (1.1.3.15)	gi|15231850	ARATH	40	21	5	292	Peroxisome*
Ferric leghemoglobin reductase (1.6.2.6)	gi|5823556	VIGUN	56	6	3	162	Mit. matrix*
Putative NADPH:quinone oxidoreductase	gi|15528813	ORYSA	22	25	2	114	Mit. matrix*
3-Isopropylmalate dehydrogenase-like protein	gi|11251553	ARATH	41	9	2	108	Mit. matrix*
Other enzymes							
Glycine decarboxylase T-protein	gi|1346121	FLAPR	45	18	5	189	Mit. matrix
Glycine decarboxylase P-protein (1.4.4.2)	gi|13366201	ORYSA	112	42	24	1518	Mit. matrix
Glycine decarboxylase H-protein	gi|13786469	ORYSA	17	28	3	132	Mit. matrix
Serine hydroxymethyltransferase (2.1.2.1)	gi|1707998	SOLTU	57	20	8	418	Mit. matrix
Cysteine synthase	gi|10241630	ORYSA	40	9	3	125	Mit. matrix*

(*continued on next page*)

Table 3 (*continued*)

Identity (EC No.)	Acc. No.	Species	kDa	Cov.%	Pep#	Score	Localization
Alanine-glyoxylate aminotransferase (2.6.1.44)	gi|15225026	ARATH	44	5	1	114	Peroxisome*
Peptidylprolyl isomerase (5.2.1.8) / cyclophilin	gi|1084454	ORYSA	19	7	1	61	Mit./Cyt./ Mic.
Thiosulfate sulfurtransferase	gi|4406372	DATGL	42	10	2	53	Mit. matrix*
Prolyl tRNA synthetase (6.1.1.15)	gi|15237183	ARATH	61	2	1	47	Chloroplast*
Other proteins							
Voltage-dependent anion channel	gi|18076158	ORYSA	29	30	4	86	Mit. OMM
Translational elongation factor EF-TuM	gi|18001149	ORYSA	49	30	8	388	Mit. matrix
Unnamed protein product	gi|9294558	ARATH	15	9	1	48	Chloroplast*
Putative aminotransferase	gi|32492077	ORYSA	105	5	3	169	Chloroplast*
Calmodulin	gi|16225	ARATH	16	26	1	92	ER*
Ribosomal protein S2	gi|11466379	MESVI	26	4	1	45	Chloroplast
Contaminants							
RuBisCO small subunit A (4.1.1.39)	gi|132096	ORYSA	20	8	1	56	Chloroplast
Rubisco subunit binding-protein β-subunit	gi|2506277	PISSA	63	2	1	56	Chloroplast
Fructose-bisphosphate aldolase (4.1.2.13)	gi|14090214	ORYSA	42	13	3	181	Mit. matrix*
Putative transketolase	gi|11875175	ORYSA	81	12	5	168	Chloroplast*
Chitinase	gi|18146827	TRIAE	35	8	1	63	Vac./Apopl.

Acc. No., GenBank Accession Number; species, e.g., *Oryza sativa*; kDa, mass of preprotein; Cov.%, coverage based on preprotein; Pep#, number of peptides identified; Score, Mascot score, scores above 42 for individual peptides indicate identity, only proteins with at least one peptide with a score above 42 was included in the list; localization, if known or *, based on TargetP prediction (http://www.cbs.dtu.dk/services/TargetP/).

Mit.mitochondrion; OMM, outer mitochondrial membrane; Perox., peroxisome; ER, endoplasmatic reticulum associated; Vac., vacuole; Apopl., apoplast; Mic., microbodies; Mit. IMS mitochondrial intermembrane space; Cyt., cytosol.

[a] These proteins were also identified on the 2D-IEF/PAGE gels (see Fig. 1(d) and Table 4).

Neuburger et al., 1982; Struglics et al., 1993), but it might represent contamination by a very small percentage peroxisomes or be due to adsorbed enzyme molecules.

For 17 out of the 19 oxidised proteins found in both IP-samples a larger, and often much larger, number of peptides was identified in the oxidised sample than in the control sample (Tables 2 and 3). The two exceptions are hsp70 and Rubisco, a chloroplast contaminant. Although the mass spectrometry method used in the present paper is not quantitative, a larger number of peptides identified is indicative of a significantly higher amount of protein (Washburn et al., 2001; Taylor et al., 2003b). Thus, the pool of proteins partly oxidised in the control sample is apparently oxidised to a greater extent by the treatment with the Cu^{2+}/H_2O_2 reagent.

Many of the peptides matching to the proteins listed in Tables 2 and 3 were identified in more than one form. The most common modifications were deamidation of glutamine and asparagine, oxidation of methionine and N-terminal carbamidomethylation. Deamidation of formate dehydrogenase has previously been reported as potentially affecting the ability of the cognate kinase to phosphorylate a nearby threonine (Bykova et al., 2003). In general deamidation may act as a molecular clock that mark proteins for turn-over (Robinson and Robinson, 2001 and references therein). Protein deamidation in the untreated control and the in vitro-oxidised matrix samples did not differ significantly in this study. While N-terminal carbamidomethylation most likely has occurred during the in-solution digestion step of the sample preparation, oxidation of methionine may have happened in vivo. However, we have no means of discriminating between the in vitro or in vivo event. No oxidised tryptophan residues or DNP-tagged peptides were detected in any sample. This is somewhat surprising since oxidation of tryptophan have been reported in both in vivo- and in vitro-oxidised proteins (Finley et al., 1998; MacCoss et al., 2002; Taylor et al., 2003a,b).

The resolution power of the 2D-LC/MS/MS method was tested using a control matrix sample that had not been subjected to DNP-labelling and immunoprecipitation. By applying the same protocol the 2D-LC/MS/MS method resolved more than 1800 peptides and more than 100 proteins were identified (data not shown). This clearly demonstrated that the resolution power of the method was not the limiting factor in this study.

3.4. 2D-IEF-SDS–PAGE of the matrix fraction confirms the trends observed by 2D-LC/MS/MS

Most of the identified oxidised proteins are relatively abundant household proteins, e.g., the Krebs cycle enzymes, which could indicate that mitochondrial ROS

strikes "at random" both in vivo (the untreated matrix fraction; Table 2) and in vitro (the matrix fraction exposed to metal-catalysed oxidation reagent; Table 3). To check this we compared protein staining and carbonyl content on 2D-IEF/SDS–PAGE gels and blots, respectively, for each of our two matrix fractions (Fig. 1, Table 4).

The 2D protein pattern is almost unaffected by the oxidation of the matrix fraction (Figs. 1(a) and (b)) indicating that little chain breakage had taken place. The amount of DNP-reactive proteins clearly increases after reaction of the matrix fraction with metal-catalysed oxidation reagent (Figs. 1(c) and (d)). Although most abundant proteins are labelled, there is little correlation between the amount of protein in a spot and the intensity of DNP-labelling. For instance, the prominent protein spots 15 and 16 (FDH) are relatively weakly labelled whereas there is very little protein in the strongly labelled spot 9 (Fig. 1(d)).

The specific oxidation (DNP-labelling) on immunoblots from control and oxidised matrix (Table 4) was calculated as the ratio between the anti-DNP spot volume (Figs. 1(c) and (d)) and the protein spot volume (Figs. 1(a) and (b)). The change in protein oxidation between the control and the oxidised matrix was expressed as the ratio between the specific DNP-labelling values for the two samples (Table 4). The specific DNP-labelling for many spots increased about 2-fold (spots 2, 3, 4, 5, 11, 14, 16, 17, 18, 19, 20), while in a few spots the increase was more marked (spots 1, 6, 7, 12). Finally, the specific DNP-labelling of some spots remained unaltered by the in vitro oxidation treatment (spots 8, 9, 10, 13, 15). The specific labelling for control matrix sample spots 9 and 12 has values of 198 and 4, respectively (Figs. 1(a), (c) or (b), (d)). In the oxidised matrix sample the value for spot 9 is only slightly increased (to 235), whereas the value for spot 12 is increased about 10-fold (to 38). The protein in spot 9 has the highest specific labelling and may be close to carbonyl saturation under the given conditions.

There is a very good agreement between the results in Fig. 1 and Table 4 and those in Tables 2 and 3. For example, the P-protein of glycine decarboxylase (GDC) was identified as an oxidised protein in the oxidised sample in Table 3 and is found in spot 1 (Fig. 1(d); identification by position on gels and blots). The GDC P-protein bound DNP very weakly in the control matrix fraction but much stronger after oxidation and consequently had the largest increase in specific labelling. This is consistent with the results in the control IP-sample where GDC-P was not detected, probably due to low amounts in the immunoprecipitate, and in the oxidised sample where the GDC P-protein was detected with a very high coverage, 24 peptides covering 42% of the protein (Table 3). Dihydrolipoamide dehydrogenase was identified in spot 7 (Fig. 1(d)) by LC-MS/MS (results not shown) and the specific labelling increased about 5-fold in response to in vitro oxidation (Table 4). Dihydrolipoamide dehydrogenase was also found in both the control and in the oxidised IP-matrix sample (Tables 2 and 3), but more peptides were found in the latter. Formate dehydrogenase and malate dehydrogenase were identified by LC-MS/MS in the prominent spots 16 and 19, respectively (Fig. 1(d)) and their labelling

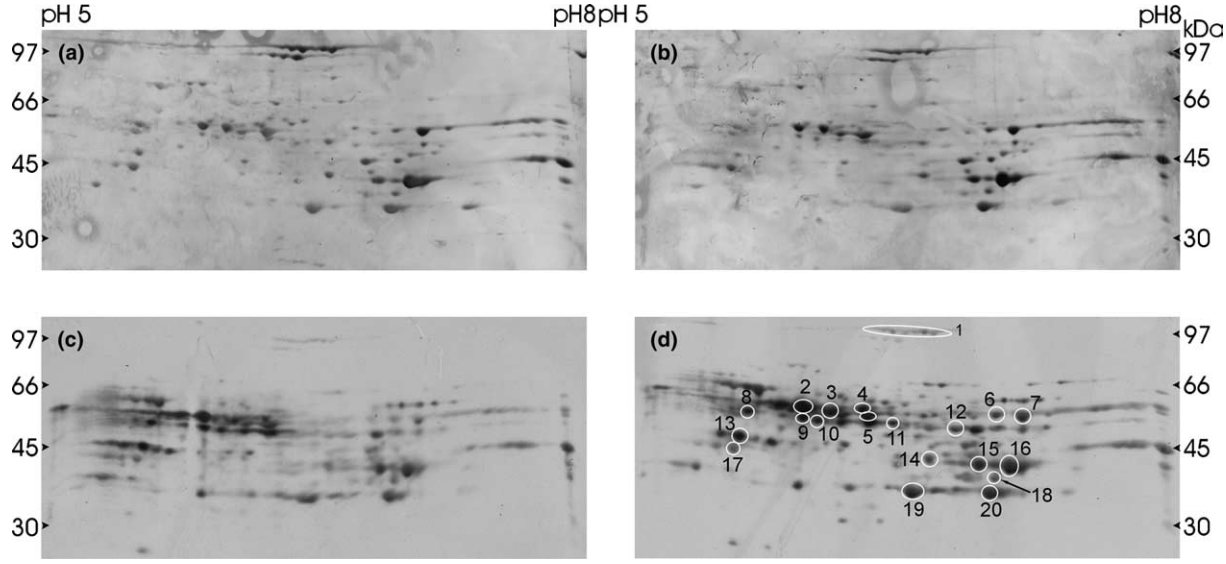

Fig. 1. Western-blotted 2D gels of oxidised proteins in the matrix of rice leaf mitochondria. Total protein pattern on the membrane of the control matrix fraction (a) and the oxidised matrix fraction (b). Immunodetected protein carbonyls conjugated with DNP in the control matrix fraction (c) and in the oxidised matrix fraction (d). Photographic film exposed to the anti-DNP signal for 30 s was developed and scanned. Scanning was done on a density-calibrated scanner to provide intensity information. The resulting images were processed using Imagemaster 2D Elite software to match spots and provide spot volumes. The spots circled in (d) were cut out, digested and identified by MS/MS (see Tables 3 and 4).

Table 4
Specific DNP-labelling of matrix proteins resolved by immunoblotting of 2D-gels spot numbers refer to Fig. 1(d)

Spot No. (Fig. 1)	Specific DNP-labelling		
	Control (vol/vol)	ROS (vol/vol)	Ratio
1 (GDC-P)	0.01	0.64	59.35
2	32.92	64.78	1.97
3	19.10	32.73	1.71
4	16.23	33.26	2.05
5	11.98	20.24	1.69
6	1.92	12.08	6.30
7 (DHLA-DH)	2.19	10.29	4.71
8	21.30	20.80	0.98
9	197.72	235.14	1.19
10	63.62	56.82	0.89
11	17.67	46.00	2.60
12	3.81	37.60	9.87
13	32.25	34.63	1.07
14	4.22	8.62	2.04
15	13.89	18.14	1.31
16 (FDH)	9.84	16.14	1.64
17	2.63	5.00	1.90
18	6.39	9.88	1.55
19 (MDH)	9.35	17.85	1.91
20	9.55	14.73	1.54

The specific DNP-labelling was calculated as the ratio between the anti-DNP and the protein spot volumes. The change in specific DNP-labelling between the control and the oxidised matrix was expressed as the ratio between the specific DNP-labelling values. GDC-P, glycine decarboxylase P-subunit, DHLA-DH, dihydrolipoamide dehydrogenase, FDH, formate dehydrogenase, MDH, malate dehydrogenase.

intensities increased moderately (Table 4). Both formate dehydrogenase and malate dehydrogenase were found in both the control and in the oxidised IP-matrix sample (Tables 2 and 3), with more peptides in the latter.

4. Discussion

This study shows that a group of 20 proteins is oxidised in the soluble matrix fraction from isolated plant mitochondria (Table 2) and that these proteins likely were oxidised in vivo. A further group of 32 oxidised proteins were identified when the matrix fraction was exposed to an oxidative treatment (Table 3). Considering that the pattern of oxidised proteins was very similar before and after oxidation of the matrix fraction (Figs. 1(c) and (d)), it is possible that the in vitro treatment simply brought the oxidation level of a number of already oxidised proteins above the detection limit of our method. The specific DNP-labelling varied more than 100-fold between different proteins in the control sample (Table 4) indicating that protein oxidation in vivo is a selective process. Likewise, the in vitro oxidation treatment increased oxidation of some proteins very markedly while leaving others unaffected (Table 4) indicating that also this oxidation is relatively selective. Whether the identified oxidised proteins were oxidised in vivo or in vitro, they are clearly more susceptible to oxidative modification than many of the other 2000–3000 proteins suggested to be present in this cellular compartment (Heazlewood et al., 2003). These susceptible proteins include all of the soluble Krebs cycle enzymes as well as a number of ROS-detoxifying enzymes, chaperones and several redox enzymes, like aldehyde dehydrogenase, that could all be termed stress proteins.

The oxidised proteins identified in the control sample were apparently further oxidised by the in vitro oxidation as judged by greater number of peptides identified (Tables 2 and 3) and increased specific DNP labelling on the 2D-gels (Fig. 1, Table 4). At this point we cannot convert the specific labelling in a particular spot into percentage of the protein molecules oxidised, but this would be desirable, as it would give us an idea of the size of the steady-state oxidation level of each protein. One would expect a rapid turnover of the oxidised forms as they are often more susceptible to protease attack (see Dean et al., 1997; Møller and Kristensen, 2004).

We identified a number of oxidised proteins in the mitochondrial matrix fraction that are normally considered to belong in other compartments (Tables 2 and 3). While some of these are no doubt bona fide contaminants, e.g., small subunit of Rubisco, others may have a functional association with the mitochondria. Such an association was recently reported for glycolytic enzymes and mitochondria isolated from *Arabidopsis* cells (Giegé et al., 2003).

Apart from the observation that several ROS-detoxifying enzymes were oxidised, the most interesting observation in this study is perhaps that the four component enzymes in glycine decarboxylase are susceptible to oxidation (Tables 2 and 3). During photorespiration, which for instance occurs in green leaves under low-CO_2 conditions, a massive flow of carbon passes from the chloroplasts through the peroxisomes to the mitochondria where two glycine molecules are converted into one serine molecule while NADH is produced. This gives rise to an increased NADH reduction level in the matrix (Wigge et al., 1993), which in turn would lead to an increased reduction of the electron transport chain and therefore to increased ROS production (Møller, 2001). It was recently reported that the H-protein in GDC is inhibited during abiotic stress by conjugation of its lipoic acid residue with a lipid peroxidation product (Taylor et al., 2002), a modification not detectable by the DNP method. It will be interesting to see how the carbonylation level of glycine decarboxylase varies with environmental conditions and how this affects the activity of the enzyme complex.

The present study shows that mitochondria in vivo contain a certain number of oxidised proteins, which is consistent with the results of Taylor et al. (2003a). They analysed intact cardiac mitochondria and detected a total of 39 proteins containing oxidised tryptophan

residues, a modification not detectable by the DNP method. Three of the proteins with oxidised tryptophan, citrate synthase, isocitrate dehydrogenase and voltage-dependent anion channel, were also found to be oxidised in the control matrix sample (Table 2) and a further two, aconitase and dehydrolipoamide *S*-acetyltransferase, were identified in the oxidised matrix (Table 3). More than half the remaining proteins with the oxidised tryptophans belong in the inner membrane, which is absent from our matrix fraction. Thus, there is a considerable overlap in the presence of oxidised tryptophan in a protein and its susceptibility to carbonylation.

Sweetlove et al. (2002) used quite a different approach to identify mitochondrial proteins that are affected by oxidative stress. They produced oxidatively stressed cells by treating *Arabidopsis* cell cultures for 16 h with H_2O_2 or menadione. Mitochondria were isolated from the cells, the proteins separated on 2D gels and protein spots identified that either increased or decreased markedly in size as a result of the oxidative stress. This approach would only identify oxidised proteins whose position on the 2D gel was changed by the oxidation. In addition, proteins with up- or down-regulated expression would be detected in their study but not in ours. In spite of this, as many as 11 spots out of a potential 28 spots (we here ignore the spots that increased in abundance in Sweetlove et al. (2002) were identified in both studies). Sweetlove et al. (2002) found breakdown products of seven proteins – malate dehydrogenase, succinyl-CoA synthase α- and β-subunits, aconitase, ATPase α- and β-subunits, and voltage-dependent anion channel – also identified by us as susceptible to oxidation. The breakdown products could either be the result of protease attack or chain breakage caused by ROS attack (yielding carbonyl groups). A further four proteins that decreased in abundance – fumarase, superoxide dismutase, methylmalonate-semialdehyde dehydrogenase, and aldolase (Sweetlove et al., 2002) – were identified by us as being oxidised (Tables 2 and 3).

Sakai et al. (2003) studied proteomic changes after reperfusion of ischemic rat hearts, a treatment that is known to cause oxidative damage to the tissue. An analysis of the whole organ proteome by 2D gel electrophoresis gave eight spots that increased in amount and three proteins were identified. Two of these proteins, mitochondrial HSP60 and elongation factor Tu, were found to be oxidised in both of our samples (Tables 2 and 3). The increased expression of these proteins as a result of oxidative stress in rat hearts might therefore indicate that the tissue compensates for their oxidative inactivation by de novo synthesis.

It is important to recognise that we have not identified all oxidised proteins in the matrix fraction, only those with a free carbonyl group that can react with DNP. Thus, neither oxidised methionine nor oxidised tryptophan is detected. For most of the oxidised proteins the specific modification is unknown; in fact we do not even know whether a given protein is modified consistently on one (or several) specific amino acid residues. Cysteine and methionine residues are easily oxidised and this modification is often detected by mass spectrometric analyses of proteins. In contrast, reports of the detection of other oxidised amino acids are scarce e.g. (Requena et al., 2001; Roberts et al., 2001) and, with a few exceptions (e.g. Taylor et al., 2003a; MacCoss et al., 2002) these oxidation products have been generated by in vitro oxidation of single proteins or simple protein mixtures. Model studies on Cu,Zu-SOD, a ROS-detoxifying enzyme in the cytosol and chloroplast, have shown that a histidine in its active site is susceptible to oxidation by H_2O_2. This oxidation leads to inactivation of the enzyme, but no inactivation is observed in vivo possibly because the in vivo H_2O_2 concentration is much lower than the concentration employed in the model study (Uchida and Kawakishi, 1994, and references therein). This immediately raises the question of how the level of protein oxidation caused by treatment of isolated mitochondrial matrix with metal-catalysed oxidation reagent compares with the level of protein oxidation in the intact plant cell under unstressed and stressed conditions. This will be the subject of further studies.

One important question in the study of protein oxidation is whether oxidation occurs at specific sites similar to modification by glycosylation or phosphorylation, or if oxidation occurs at random all over the protein. Several observations indicate that this is a specific process (Uchida and Kawakishi, 1994). While oxidation sites at the amino acid residue tryptophan can be identified by mass spectrometry by virtue of its mass difference (Taylor et al., 2003a), specific sites of carbonylation have never been identified by mass spectrometry. We attempted to identify the site(s) of oxidation by utilizing DNP as a mass tag and allowing this modification in the database searches of the mass spectrometry data. No DNP tagged peptides were identified in either sample, despite the fact that DNP clearly tagged a number of proteins in the samples (Fig. 1, Tables 2 and 3). The reason for this is unknown but might be caused by clustering of multiple modifications in the individual peptides, a low stoichiometry of the individual oxidation site or due to unexpected fragmentation patterns of DNP and the tagged peptides.

In summary, the 2D-LC/MS/MS results and the 2D immunoblot patterns of oxidised proteins were very similar, consistent with the view that oxidative stress both in vivo and in vitro has a specific effect on a group of particularly susceptible proteins. The visualisation and identification of the same proteins on the immunoblots as by the gel-free method suggest that the anti-DNP immunoprecipitation procedure is robust. Even the identification of a number of oxidised contaminants

in the mitochondrial fraction supports the specificity and robustness of the method – the contaminants mostly derive from the chloroplast or peroxisome, two organelles that also have a marked production of ROS in vivo.

Acknowledgements

We are grateful to Ina Blom for excellent technical assistance, to Dr. Gerhard Saalbach for helpful discussions and to Dr. Søren K. Rasmussen for continuous support. Economic support from the Danish Agricultural and Veterinary Research Council and the Danish Natural Science Research Council is gratefully acknowledged.

References

Arbuzova, S., Hutchin, T., Cuckle, H., 2002. Mitochondrial dysfunction and Down's syndrome. BioEssays 24, 681–684.

Berlett, B.S., Stadtman, E.R., 1997. Protein oxidation in aging, disease, and oxidative stress. J. Biol. Chem. 272, 20313–20316.

Bykova, N.V., Stensballe, A., Egsgaard, H., Jensen, O.N., Møller, I.M., 2003. Phosphorylation of formate dehydrogenase in potato tuber mitochondria. J. Biol. Chem. 278, 26021–26030.

Carelli, V., Ross-Cisneros, F.N., Sadun, A.A., 2002. Optic nerve degeneration and mitochondrial dysfunction: genetic and acquired optic neuropathies. Neurochem. Int. 40, 573–584.

Chew, O., Whelan, J., Millar, A.H., 2004. Molecular definition of the ascorbate–glutathione cycle in *Arabidopsis* mitochondria reveals dual targeting of antioxidant defenses in plants. J. Biol. Chem. 278, 46869–46877.

Choi, J.G., Malakowsky, C.A., Talent, J.M., Conrad, C.C., Gracy, R.W., 2002. Identification of oxidized plasma proteins in Alzheimer's disease. Biochem. Biophys. Res. Commun. 293, 1566–1570.

Dean, R.T., Fu, S.L., Stocker, R., Davies, M.J., 1997. Biochemistry and pathology of radical-mediated protein oxidation. Biochem. J. 324, 1–18.

Finley, E.L., Dillon, J., Crouch, R.K., Schey, K.L., 1998. Identification of tryptophan oxidation products in bovine alpha-crystallin. Protein Sci. 7, 2391–2397.

Foyer, C.H., Lelandais, M., Kunert, K.J., 1994. Photooxidative stress in plants. Physiol. Plant 92, 696–717.

Foyer, C.H., Noctor, G., 2000. Oxygen processing in photosynthesis: regulation and signalling. New Phytol. 146, 359–388.

Ghezzi, P., Bonetto, V., 2003. Redox proteomics: identification of oxidatively, modified proteins. Proteomics 3, 1145–1153.

Giegé, P., Heazlewood, J.L., Roessner-Tunali, U., Millar, A.H., Fernie, A.R., Leaver, C.J., Sweetlove, L.J., 2003. Enzymes of glycolysis are functionally associated with the mitochondrion in *Arabidopsis* cells. Plant Cell 15, 2140–2151.

Heazlewood, J.L., Millar, A.H., Day, D.A., Whelan, J., 2003. What makes a mitochondrion? Genome Biol., 4.

Jimenez, A., Hernandez, J.A., delRio, L.A., Sevilla, F., 1997. Evidence for the presence of the ascorbate–glutathione cycle in mitochondria and peroxisomes of pea leaves. Plant Physiol. 114, 275–284.

Kyhse-Andersen, J., 1984. Electroblotting of multiple gels – a simple apparatus without buffer tank for rapid transfer of proteins from polyacrylamide to nitrocellulose. J. Biochem. Biophys. Methods 10, 203–209.

Laemmli, U.K., 1970. Cleavage of structural proteins during assembly of head of bacteriophage-T4. Nature 227, 680–685.

MacCoss, M.J., McDonald, W.H., Saraf, A., Sadygov, R., Clark, J.M., Tasto, J.J., Gould, K.L., Wolters, D., Washburn, M., Weiss, A., Clark, J.I., Yates, J.R., 2002. Shotgun identification of protein modifications from protein complexes and lens tissue. Proc. Natl. Acad. Sci. USA 99, 7900–7905.

Marnett, L.J., Riggins, J.N., West, J.D., 2003. Endogenous generation of reactive oxidants and electrophiles and their reactions with DNA and protein. J. Clin. Invest. 111, 583–593.

Maxwell, D.P., Nickels, R., McIntosh, L., 2002. Evidence of mitochondrial involvement in the transduction of signals required for the induction of genes associated with pathogen attack and senescence. Plant J. 29, 269–279.

Møller, I.M., 2001. Plant mitochondria and oxidative stress: electron transport, NADPH turnover, and metabolism of reactive oxygen species. Annu. Rev. Plant Physiol. Plant Mol. Biol. 52, 561–591.

Møller, I.M., Kristensen, B.K., 2004. Protein oxidation in plant mitochondria as a stress indicator. Photochem. Photobiol. Sci. (in press).

Møller, I.M., Liden, A.C., Ericson, I., Gardeström, P., 1987. Isolation of submitochondrial particles with different polarities. Methods Enzymol. 148, 442–453.

Neuburger, M., Journet, E.P., Bligny, R., Carde, J.P., Douce, R., 1982. Purification of plant mitochondria by isopycnic centrifugation in density gradients of percoll. Arch. Biochem. Biophys. 217, 312–323.

Rasmusson, A.G., Møller, I.M., 1991. NAD(P)H dehydrogenases on the inner surface of the inner mitochondrial membrane studied using inside-out submitochondrial particles. Physiol. Plant 83, 357–365.

Requena, J.R., Groth, D., Legname, G., Stadtman, E.R., Prusiner, S.B., Levine, R.L., 2001. Copper-catalyzed oxidation of the recombinant SHa(29-231) prion protein. Proc. Natl. Acad. Sci. USA 98, 7170–7175.

Roberts, J.E., Finley, E.L., Patat, S.A., Schey, K.L., 2001. Photooxidation of lens proteins with xanthurenic acid: a putative chromophore for cataractogenesis. Photochem. Photobiol. 74, 740–744.

Robinson, N.E., Robinson, A.B., 2001. Molecular clocks. Proc. Natl. Acad. Sci. USA 98, 944–949.

Sakai, J., Ishikawa, H., Kojima, S., Satoh, H., Yamamoto, S., Kanaoka, M., 2003. Proteomic analysis of rat heart in ischemia and ischemia–reperfusion using fluorescence two-dimensional difference gel electrophoresis. Proteomics 3, 1318–1324.

Schägger, H., von Jagow, G., 1987. Tricine sodium dodecyl-sulfate polyacrylamide gel electrophoresis for the separation of proteins in the range from 1-kDa to 100-kDa. Anal. Biochem. 166, 368–379.

Shevchenko, A., Wilm, M., Vorm, O., Mann, M., 1996. Mass spectrometric sequencing of proteins from silver stained polyacrylamide gels. Anal. Chem. 68, 850–858.

Struglics, A., Fredlund, K.M., Rasmusson, A.G., Møller, I.M., 1993. The presence of a short redox chain in the membrane of intact potato tuber peroxisomes and the association of malate dehydrogenase with the peroxisomal membrane. Physiol. Plant 88, 19–28.

Sweetlove, L.J., Heazlewood, J.L., Herald, V., Holtzapffel, R., Day, D.A., Leaver, C.J., Millar, A.H., 2002. The impact of oxidative stress on *Arabidopsis* mitochondria. Plant J. 32, 891–904.

Talent, J.M., Kong, Y.L., Gracy, R.W., 1998. A double stain for total and oxidized proteins from two-dimensional fingerprints. Anal. Biochem. 263, 31–38.

Taylor, N.L., Day, D.A., Millar, A.H., 2002. Environmental stress causes oxidative damage to plant mitochondria leading to inhibition of glycine decarboxylase. J. Biol. Chem. 277, 42663–42668.

Taylor, S.W., Fahy, E., Murray, J., Capaldi, R.A., Ghosh, S.S., 2003a. Oxidative post-translational modification of tryptophan residues in cardiac mitochondrial proteins. J. Biol. Chem. 278, 19587–19590.

Taylor, S.W., Fahy, E., Zhang, B., Glenn, G.M., Warnock, D.E., Wiley, S., Murphy, A.N., Gaucher, S.P., Capaldi, R.A., Gibson, B.W., Ghosh, S.S., 2003b. Characterization of the human heart mitochondrial proteome. Nat. Biotechnol. 21, 281–286.

Uchida, K., Kawakishi, S., 1994. Identification of oxidized histidine generated at the active site of Cu,Zn-superoxide dismutase exposed to H_2O_2 – selective generation of 2-oxo-histidine at the histidine 118. J. Biol. Chem. 269, 2405–2410.

Washburn, M.P., Wolters, D., Yates, J.R., 2001. Large-scale analysis of the yeast proteome by multidimensional protein identification technology. Nat. Biotechnol. 19, 241–247.

Wigge, B., Krömer, S., Gardeström, P., 1993. The redox levels and subcellular distribution of pyridine nucleotides in illuminated barley leaf protoplasts studied by rapid fractionation. Physiol. Plant 88, 10–18.