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Protein:protein aggregation induced by protein oxidation

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ABSTRACT

When the level of reactive oxygen species (ROS) in cells exceeds a genetically coded defense capacity, the cells experience damage to vital components such as DNA, proteins and lipids that leads to non-specific interactions and the production of a series of high molecular weight protein aggregates. The dynamics of oxidative stress induced aggregation were studied here using model proteins and yeast. Model proteins were oxidized at increasing ROS concentrations and analyzed using size exclusion chromatography (SEC). Changes in the SEC elution profile showed that aggregation happens in stages and protein fragments produced as a result of oxidation also give rise to aggregates. Yeast cells were stressed with hydrogen peroxide to investigate *in vivo* aggregation. Equal amounts from control and oxidized lysates were chromatographed on a size exclusion column and proteins of molecular weight exceeding 700 kDa were collected from both samples which were then differentially labeled using light and heavy isotope coded *N*-acetoxysuccinamide and mixed in a 1:1 ratio. The coded mixture was analyzed using LC/MS and peptides that appeared as singlets representing the proteins that aggregated with higher molecular mass protein complexes were identified. Twenty-five proteins were identified to be of this type. Fifteen members in this group were found to have been carbonylated. These proteins are part of the proteome known as the aggresome. The protein content of the aggresome may provide vital information for mechanistic studies targeting disease and aging.

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1. Introduction

Protein complexes and their formation are an important part of regulating biological systems. Clearly the formation and participation of these protein clusters in biological processes is a natural, genetically coded process of vital importance to cells. There are, however, other forms of protein aggregation as in the case of neurodegenerative diseases and physiological aging [1] that appear not to be an inherent part of cellular regulation. These aggregates can negatively impact metabolism and protein turnover [2] even causing cell death. It is not clear why or how these negative forms of protein aggregation occur, but irreparable damage to proteins from oxidative stress is clearly involved in physiological aging [3] and neurological degeneration [4].

Central components of oxidative stress are (1) the generation of reactive oxygen species (ROS) through both enzymatic and non-enzymatic processes and (2) the participation of ROS in DNA, RNA, and protein oxidation. Protein oxidation occurs as a result of either direct attack by ROS or indirectly through peroxidation of lipids that further degrade and attack proteins. As a result of this oxidation, carbonyls are introduced into proteins either by direct oxidation of amino acids or indirectly by attachment of a carbonyl-containing moiety such as 4-hydroxynonenal [5–8]. Carbonyl formation often alters protein conformation as well. This in turn can increase protein hydrophobicity and enhances non-specific protein–protein interactions [5,6,9–11] that compromise cell viability and impair protein turnover [10,11]. These aggregates are generally composed of a single protein in the case of neurological diseases such as Alzheimer's [12] and Parkinson's disease [13]. Much less is known about oxidative stress induced aggregation in other cases. An objective of the work described here was to examine other forms of aggregation in greater detail.

Several different methods have been developed to study protein–protein interactions. Affinity selection of associated proteins using immobilized target proteins is one of the methods [14]. Immobilized proteins are also used in combination with reflectometry for the detection of protein–protein interactions [15]. Peptide tagging is another method used for the detection of specific proteins interacting with a target protein [16]. One common feature of all these methods is the need for a target, or bait protein. Basically, in all these cases a target protein has to be identified and purified before its interaction with other proteins can be detected. Another limitation of these methods is the need for a target protein to be in native form. This means these methods are not very effective for

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detecting new, non-specific protein–protein interactions caused by oxidative stress.

A common feature of oxidative stress induced protein aggregates is that they should be of higher molecular weight and size than the individual protein components of which they are composed. Size exclusion chromatography (SEC) was used in this work for differentiation on the basis of size in recognizing and isolating protein complexes. Considering the fact that many non-specific protein–protein interactions are often hydrophobic, non-denaturing conditions should be used in the separation method selected to avoid disturbing the interactions that hold the aggregate together. Size exclusion chromatography has all the specifications required for size-based fractionation of complex protein mixtures without denaturation. Aggregates fractionated by SEC can also be analyzed by a wide variety of standard proteomics platforms that will detect and identify protein components.

Even though the general understanding is that oxidative stress induced protein aggregation compromises cell viability, the mechanism by which this occurs is poorly understood. Both model proteins and yeast were used in this study to investigate protein aggregation *in vitro* and *in vivo* under oxidative stress conditions. *In vitro* oxidation studies were used to address several important questions. One was how easily proteins aggregate when oxidized. Another was the degree to which self-aggregation occurs to form homogeneous clusters, as in neurological diseases, versus random aggregation to form heterogeneous aggregates. The extent to which these model system studies applied in a complex samples was then examined in yeast using 5 mM hydrogen peroxide to induce oxidative stress. The utility of *Saccharomyces cerevisiae* as a model system for investigating the impact of oxidative stress and protein aggregation on cell death is well documented [17–20].

2. Materials

Biotin hydrazide, sodium cyanoborohydride ($NaCNBH_3$), trifluoroacetic acid (TFA) were purchased from Pierce (Rockford, IL, USA). Cytochrome *c* (equine heart), thyroglobulin (bovine), iodoacetamide, dithiothreitol (DTT), *N*-hydroxysuccinimide, acetic anhydride, trypsin, yeast extract–peptone–dextrose (YPD) medium (1% yeast extract, 2% peptone, 2% glucose), ethylenediaminetetraacetic acid (EDTA), *N*-α-tosyl-L-lysine chloromethyl ketone (TLCK), HEPES and Tris buffers were obtained from Sigma–Aldrich Chemical Co. (St. Louis, MO, USA). Heavy isotope coded acetic anhydride was purchased from C/D/N Isotopes Inc. (Pointe-Claire, Quebec, Canada). Sodium phosphate, urea, sodium chloride, hydrogen peroxide, magnesium chloride and calcium chloride were purchased from Mallinkrodt (St. Louis, MO, USA). Protease inhibitor cocktail was purchased from Roche Diagnostics (Indianapolis, IN, USA). The Vydac 208TP54 reversed-phase C_8 column was purchased from W.R. Grace & Co. (Columbia, MD, USA). The DE44G00621 Zorbax 300SB-C18 reversed-phase column (75 μm × 150 mm) was purchased from Agilent Technologies, Inc. (Palo Alto, CA, USA). Superdex 200 10/300 GL column was purchased from GE Healthcare (Bjorkgatan, Uppsala, Sweden). The reversed-phase chromatography (RPC) analyses were done on an Integral Micro-Analytical Workstation (Framingham, MA, USA). The LC system used in conjunction with mass spectrometer was an Agilent 1100 series instrument purchased from Agilent Technologies, Inc. (Palo Alto, CA, USA). Mass spectral analyses were done using a PE Sciex QSTAR™ hybrid LC/MS/MS Quadrupole TOF mass spectrometer (Framingham, MA, USA). All spectra were obtained in the positive ion mode.

3. Methods

3.1. Differential oxidation of model proteins

Metal-catalyzed oxidation of cytochrome *c* was accomplished according to Stadtman and co-workers [8]. The chemical reaction leading to the formation of ROS is shown below.

$$2Fe^{3+} + H_2A \rightarrow 2Fe^{2+} + A + 2H^+ \quad (1)$$

$$O_2 + 2Fe^{2+} + 2H^+ \rightarrow 2Fe^{3+} + H_2O_2 \quad (2)$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^\bullet + OH^- \quad (3)$$

In a series of reactions shown above the formation of Fe^{2+} leads to generation of hydroxyl radicals which in turn are responsible for oxidation of proteins (Fenton reaction).

Cytochrome *c* was solubilized at a concentration of 10 mg/mL (in the case of cytochrome *c* and thyroglobulin mixture they were mixed in an equal ratio to make a final protein concentration of 10 mg/mL) in oxidation buffer (50 mM HEPES buffer; pH 7.4, 100 mM KCl; 10 mM $MgCl_2$), and dialyzed against the same buffer at 4 °C to remove any impurities that might be present and could interfere with oxidation reaction. Differential oxidation of cytochrome *c* and cytochrome *c*/thyroglobulin mixture was accomplished by the addition of a freshly prepared mixture of neutral ascorbic acid (25 mM) and ascending concentrations of $FeCl_3$. $FeCl_3$ was added in a series of experiments to the final concentration of 10 μM, 100 μM, 200 μM, 400 μM, 800 μM, 1 mM, 5 mM, 10 mM and 100 mM to 100 μL aliquots of the model protein solutions. The control sample was prepared by the addition of EDTA to the final concentration of 100 mM prior to the addition of a freshly prepared mixture of neutral ascorbic acid (25 mM) and $FeCl_3$ (10 μM). After 24 h of oxidation at 37 °C in a shaking bath the reaction was quenched with the addition of 100 mM EDTA. The concentration was then adjusted to 2.5 mg/mL and the reaction mixture was filtered.

3.2. Yeast strain and culture conditions

The method of Yoo and Regnier [21] was followed in growing *S. cerevisiae* wild-type strain SM1058 [22]. The cells were grown at 37 °C in YPD medium using a shaking incubator at 200 rpm. Cell growth was determined by optical density (OD) measurements at 600 nm. For processing a mid-log phase culture, the overnight-cultured cells were inoculated in fresh medium to a cell density of 0.2–0.3 OD_{600}.

3.3. Preparation of total protein from yeast treated with hydrogen peroxide

Exponentially growing cells at a density of 2.4 OD_{600} were treated with 5 mM hydrogen peroxide to induce oxidative stress. Cells from 500 mL cultures were harvested 1 h after the addition of hydrogen peroxide and then washed twice with cold water by centrifugation at 3000 rpm for 10 min at 4 °C. The pellet was resuspended in lysis buffer (pH 7.4) containing 0.3 M sorbitol, 0.1 M NaCl, 5 mM $MgCl_2$, 10 mM Tris, 5 mM biotin hydrazide, 1 tablet Complete-Mini protease inhibitor and 1% aprotinine (EMD Biosciences, San Diego, CA). Cells were broken by repeated vortexing at 4 °C for 10 min with an equivalent volume of glass beads (0.6 mm diameter; Sigma, G-8772). Supernatant was collected by centrifugation at 14 000 rpm for 10 min at 4 °C. The lysate was incubated at room temperature for 2 h to complete the Schiff base formation between protein carbonyls and biotin hydrazide. After 2 h an equal volume of 30 mM $NaCNBH_3$ in lysis buffer was added to reduce C=N bonds. Protein concentration was measured by the Bradford method using

a Coomassie protein assay kit (Pierce). Control sample was prepared in the absence of hydrogen peroxide treatment.

3.4. Size exclusion chromatography of oxidized proteins

One hundred microliters of each solution was separated on a Superdex 200 10/300 GL column using 3.8 mM PBS as the elution buffer. One hundred microliters of oxidized and control yeast lysate (final concentration of 2.5 mg/mL) were chromatographed using same gel-filtration column and PBS as the elution buffer. Separations were conducted using an Agilent 1100 series instrument and the flow rate of 0.5 mL/min. UV absorbance at 280 nm was used to monitor proteins retention time. During lysate elution from the SEC column, a fraction was collected to isolate yeast proteins of molecular weight larger than 700 kDa. Calibration curves generated using model proteins of known molecular weight were used to determine time intervals for fraction collection.

3.5. Proteolysis

Six molar of urea and 10 mM DTT were added to protein fractions collected from the SEC column. After a 1-h incubation at 65 °C, iodoacetamide was added to a final concentration of 10 mM and the reaction allowed to proceed for an additional 30 min at 4 °C. Samples were then diluted sixfold by the addition of 50 mM HEPES (pH 8.0) in 10 mM $CaCl_2$. Sequence grade trypsin (2%) was added and the reaction mixture incubated at 37 °C for at least 8 h. Proteolysis was stopped by the addition of TLCK (trypsin:TLCK ratio of 1:1 (w/w)).

3.6. Preparation of the isotopic labeling agent

The initial step in the synthesis of *N*-acetoxysuccinamide was to add 3 g (26.8 mmol) of *N*-hydroxysuccinimide to 1 g (8.9 mmol) of acetic anhydride and stir for 15 h at room temperature. White product crystals appeared gradually. Excess solvent was removed by rotary evaporation at room temperature. The white crystalline residue was washed with hexane and dried in a vacuum. The product melting point was 136–138 °C. The heavy isotope coded reagent was prepared using heavy isotope coded acetic anhydride ($^{13}CD_3O^{13}CO^{13}CO^{13}CD_3$). The final product is 5 mass units heavier than the non-coded reagent. The synthesis procedure followed was same as above.

3.7. Differential labeling of the digested yeast lysate fractions

Digested fraction (proteins with MW ≥ 700 kDa) from control yeast lysate was labeled using light isotope coded *N*-acetoxysuccinamide while corresponding fraction from oxidized yeast lysate was labeled using heavy isotope coded *N*-acetoxysuccinamide [23]. Fiftyfold molar excess of *N*-acetoxysuccinamide (light or heavy depending on sample) was added to each digest and incubated at room temperature for 8 h. Excess hydroxylamine was added to each sample and pH was adjusted to 11–12 using sodium hydroxide. Mixtures were incubated at room temperature for 10 min and then pH was adjusted back to 6.0–7.0 using acetic acid. Equal volumes of light and heavy isotope coded digests of corresponding fractions were mixed in a 1:1 ratio and analyzed using LC/MS for the detection of peptides appeared as singlets.

3.8. LC/MS analysis

Corresponding light and heavy isotope coded fractions from control and oxidized samples, respectively, were mixed in a 1:1 ratio. The multiplexed mixtures were then separated on an Agilent zorbax C_{18} column (0.5 mm × 150 mm) using an Agilent 1100 series instrument (Agilent Technologies, Inc., Palo Alto, CA) at 4 μL/min. Solvent A was 0.01% TFA in deionized H_2O (dI H_2O) and solvent B was 95% CH_3CN/0.01% TFA in dI H_2O. The flow from the column was directed to the Q-STAR workstation (Applied Biosystems, Framingham, MA) equipped with an ESI source. Peptides were separated in a 60 min linear gradient (from 0% B to 60% B). MS spectra were obtained in the positive ion mode at a sampling rate of one spectrum per second. The LC/MS spectra were then analyzed using Gistools for the detection of features appeared as doublets or singlets [24]. Singlet features were subjected to MS/MS analysis for the identification of proteins present only in the control or oxidized lysate.

3.9. LC/MS/MS analysis of singlet peptides

The list of singlet peptides reported by Gistools was used as an inclusion list for targeted MS/MS. Features with mass within 50 ppm of the targeted precursor mass window and retention time within 5 min of targeted precursor's retention time window were subjected to CID fragmentation.

3.10. Mascot search

Aquired MS/MS data were submitted to Mascot for database searches and identification of corresponding peptides and proteins. Analyst software Version 1.0 was used to create the peak list from raw data. No smoothing of the data, signal-to-noise ratio or peak de-isotoping was applied. Charge states were determined automatically using isotope distribution. The following search parameters were used for peptide identification—database: NCBInr; taxonomy: *S. cerevisiae* (72 412 entries); enzyme: trypsin; missed cleavages: 2; fixed modifications: carbamidomethylation of cysteine, acetylation of N-terminal amino group and lysine residues; variable modifications: biotinylation of lysine, arginine, thereonine and proline [25,26]. Peptide tolerance ±0.8 Da, MS/MS tolerance ±0.8 Da, monoisotopic peaks were used for identification. The Mascot Mudpit scoring system was used as a measure of identification certainty. (This scoring scheme removes the proteins with high scores due to a large number of low score peptides and is a default choice for datasets with more than 1000 queries.) Proteins with scores exceeding the identity threshold (less than 5% rate of false positive) were accepted as hits.

4. Results

4.1. Analytical strategy

Among the many ways that oxidative stress can impact protein structure two of the most easily recognized are cleavage of the polypeptide backbone and aggregation with other proteins [27]. Both events change the apparent molecular weight of the protein and are easily detected by size discriminating separation methods. Although reversed-phase chromatography, SDS polyacrylamide gel electrophoresis (SDS-PAGE), and 2D gel electrophoresis (2-DGE) can all be used in the separation of aggregates, size exclusion chromatography is by far the best in terms of minimizing structural perturbations during the separation. Mobile phase additives impact hydrophobic association with RPC, SDS-PAGE, and 2-DGE. It is for this reason that SEC was the principal protein separation method used in this work.

One of the questions examined in this study was the degree to which proteins varied in oxidative damage. There are so many components in a yeast lysate that changes in the molecular weight of

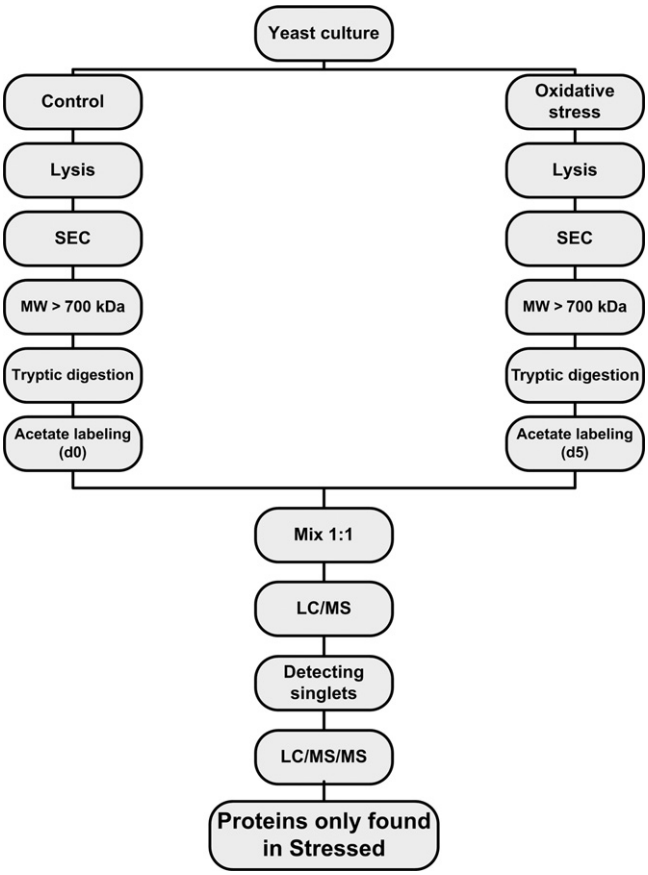

Fig. 1.

individual proteins cannot be observed by SEC alone. This issue was addressed using the global internal standard quantification protocol outlined in Fig. 1 and proteomic identification methods to identify proteins that changed in concentration or structure with oxidative stress. Cultured yeast was divided into two equal fractions and one of them stressed with 5 mM H_2O_2. Equal amounts from each sample were separated on an SEC column and proteins with molecular weights of 700 kDa and above were collected. The MW cutoff for collection was set to 700 kDa to assure that proteins in the fractions were members of an aggregate. Fractions collected from control and oxidized samples were separately digested and differentially labeled. Fractions collected from the control sample were labeled with light isotope coded *N*-acetoxysuccinimide while those from the stressed sample were labeled with heavy isotope coded *N*-acetoxysuccinimide. Digested fractions from each sample were then mixed in a 1:1 ratio and analyzed by LC/MS. LC/MS spectra were analyzed using Gistools software [24] for the detection of doublet peak clusters of co-eluting peptides with a mass difference matching the mass difference of heavy and light isotope coded labeling reagents. Relative differences in peptide abundance between samples were calculated based on the isotope ratio of these doublet clusters.

Theoretically, 3 classes of peptides are expected from these experiments. The first class would be those that appear as singlet clusters in the mixture. These singlets either were not present in control fraction or totally disappeared from the treated fraction. The second class would include peptides that increased in concentration in a fraction and the third would be the class of peptides that decreased in concentration. Since the focus of this study was to identify proteins that aggregated as a result of oxidative stress, only newly appearing proteins in the high molecular aggregate fraction were identified. Proteins and peptides that simply increased and decreased in concentration were not analyzed and reported. All singlet peptides were analyzed using MS/MS for protein identification.

4.2. Oxidation of cytochrome c and thyroglobulin to study the extent of heterogeneous versus homogeneous intermolecular aggregation

Although numerous studies have been carried out on the formation of homo-polymers during oxidation of pure proteins *in vitro*, there are few reports on cross-aggregation of proteins in model systems [28]. This is important because the complexity of the cellular milieu suggests that heterogeneous clusters could be formed *in vivo*. The relative tendency of proteins to form hetero versus homogeneous complexes was investigated *in vitro* by co-oxidizing cytochrome *c* (MW 12.4 kDa) and thyroglobulin (700 kDa) using a series of increasing concentrations of ferric chloride (as explained in methods). Cytochrome *c* was chosen because it has a visible absorption spectrum with a maximum at 415 nm that allows it to be traced in complex mixtures and it is easily separated from thyroglobulin by size exclusion chromatography.

The overlaid SEC chromatograms for oxidized cytochrome *c* at increasing concentration of ferric chloride in reaction mixtures are shown from bottom to top (Fig. 2). Three cytochrome *c* species (monomer, dimer and tetramer) are seen in the control sample. The fact the sample was heated to 37 °C is the reason for the high levels of dimer and tetramer. Increasing concentrations of ROS decreased the concentration of dimer and tetramer while increasing fragmentation. This trend continued to 5 mM $FeCl_3$ at which point the dimer peak became broader and skewed toward higher molecular weight. A possible explanation for this is that lower molecular weight fragments of cytochrome *c* aggregate with the dimer. At 100 mM $FeCl_3$, cytochrome *c* aggregates of 2000 kDa and heavier were seen.

Cytochrome *c* and thyroglobulin (700 kDa) were solubilized in oxidation buffer to a final protein concentration of 10 mg/mL in a 1:1 molar ratio and then oxidized. Protein complex formation was again monitored by SEC. Fig. 3a shows an overlaid 280 nm UV trace of the oxidized cytochrome *c* and thyroglobulin mixture using different oxidation concentrations. This figure indicates that even at low oxidant levels protein polymers are formed of sufficiently high molecular weight to produce a visual precipitate. Fig. 3b provides more detail of how this occurred. The first chromatogram at the bottom is from cytochrome *c* solubilized in oxidation buffer and immediately separated on the SEC column while the second is from the cytochrome *c* and thyroglobulin mixture incubated at 37 °C for 12 h. A protein with an absorbance of 415 nm is seen to elute with nearly the same retention time as thyroglobulin. Since absorbance at 415 nm is from cytochrome *c* and a protein of this retention time is not formed with cytochrome *c* alone, the protein of 415 nm absorbance must be from a complex of cytochrome *c* and thyroglobulin. Moreover, the absence of oxidant in the incubation mixture suggests this complex is not due to covalent bond formation. It was subsequently found that this complex could be dissociated (data not shown). Since the complex formed slowly during the course of a long incubation at 37 °C, it is likely due to a structural change in one or both of the proteins allowing a weak hydrophobic or electrostatic interaction between the partners. With the introduction of 10 μM and 100 μM ferric chloride into the incubation mixture still higher molecular weight aggregates were formed, probably through the association of multiple moles of cytochrome *c* with single molecules of thyroglobulin.

Several important conclusions can be reached from the SEC analysis of oxidized model proteins. One is that oxidative cleav-

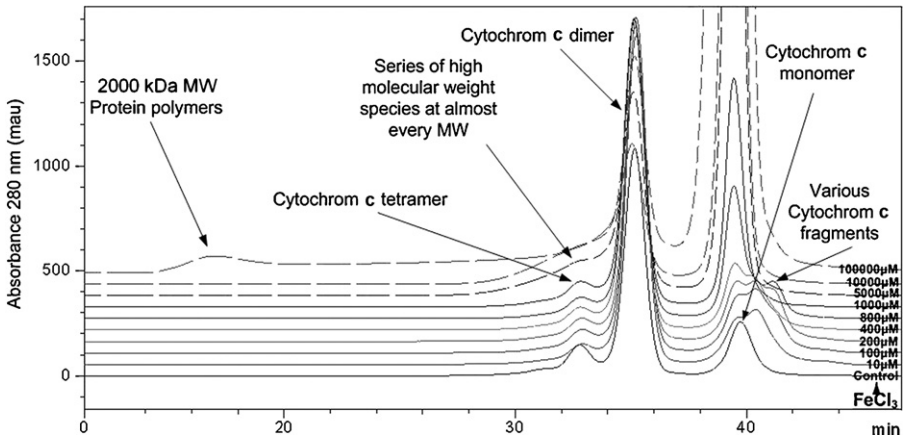

Fig. 2.

age can occur at least as easily as protein aggregation. Moreover, these cleavage fragments then readily participate in aggregation. A second is that incubating proteins for a long period of time at 37 °C can induce aggregation to the dimer and tetramer level, even in the absence of strong oxidative stress. This is probably due to non-oxidative conformational changes. Third, it is concluded that heterogeneous association of proteins occurs at least as readily as homogeneous aggregation in some cases. This is important because so little is known about the aggregation process in this form of aggregation.

4.3. Protein aggregation caused by oxidative stress in yeast

S. cerevisiae has proven to be an excellent model to explore the oxidative stress hypothesis of aging for several reasons [18,19,29]. One is that yeast is a eukaryotic cell with a short life cycle. The problem with mammals is that they age so slowly; it is hard to carry out a large number of experiments. A second is that yeast has a relatively small and well-defined genome. A third is that yeast expresses numerous proteins orthologous to mammalian proteins.

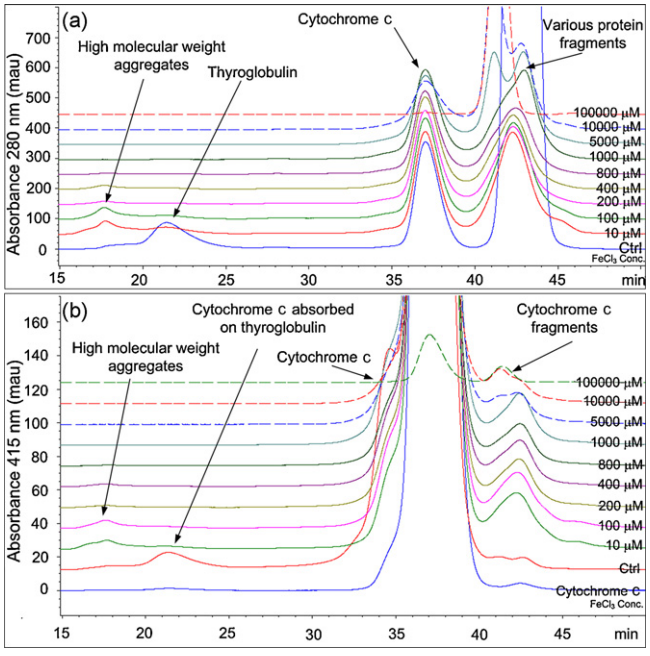

Fig. 3.

Data derived from protein aggregation induced by oxidative stress in yeast is very likely to be of value in the study of mammalian neurological diseases and aging.

Based on the free radical theory of aging, formation of protein carbonyls plays an integral role in loss of protein tertiary structure and aggregation. Other oxidative modifications at amino acid side chains may also play a role. To investigate the extent of protein carbonyl involvement in oxidized protein aggregation along with the identity of those proteins prone to aggregation, yeast cells were stressed with hydrogen peroxide for 1 h. A control culture was prepared in the absence of hydrogen peroxide. Biotin hydrazide was added to the lysis buffer to tag the protein carbonyls and prevent protein cross-linking via carbonyls after lysis. Schiff bases formed by biotinylation were then reduced using $NaCNBH_3$. After measuring protein concentration in control and oxidized yeast lysates, equal amounts of lysate were examined by SEC. Proteins and protein complexes with a molecular weight exceeding 700 kDa were collected from the SEC column (Fig. 4). Because the average molecular weight of proteins expressed by yeast is in the range of 50 kDa with a small number at 100 kDa, protein complexes of >700 kDa are likely to have 10–15 or more proteins.

It will be noted that in the treated sample there is substantially greater absorbance at 280 nm in the >700 kDa fraction than the control sample (Fig. 4). This increase in protein concentration in the treated sample could have occurred in several ways. One possibility is that as expected proteins became non-specifically associated with high molecular weight complexes after oxidative stress. Another explanation is that there is a large difference in protein degradation between the control and treated samples. It is known that protein half-life in normal yeast is roughly 30 min

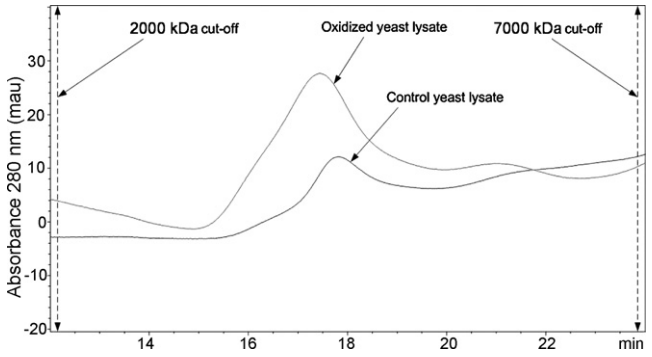

Fig. 4.

Table 1
List of all yeast proteins that were found solely in oxidized fraction

#	prot_acc	prot_desc	Score	Coverage	MW	Number of peptides
1	gi|433836	Unnamed protein product	243	70.1	24 853	14
2	gi|297485	GP38	226	14.7	38 322	6
3	gi|32693297	Translation elongation factor 1-alpha	224	8.2	43 286	4
4	gi|51013305	YPR080W	224	5.9	52 752	5
5	gi|32693293	Translation elongation factor 1-alpha	205	15.3	42 260	13
6	gi|3366	Unnamed protein product	77	18.4	59 152	8
7	gi|1360375	PDC1	71	16.3	63 378	8
8	gi|4109	Pyruvate decarboxylase	71	16.9	62 024	8
9	gi|172140	Phosphofructokinase, beta subunit	70	9.2	107 490	9
10	gi|4388536	Actin	69	48.8	42 785	13
11	gi|3328	Actin	65	42.4	42 801	15
12	gi|4627	Alpha, alpha-trehalose-phosphate synthase (UDP-forming)	51	16	57 940	5
13	gi|1323435	PFK1	45	3.6	111 598	3
14	gi|3694	Fatty acid synthetase subunit beta	43	4.4	211 731	9
15	gi|798945	Adh2p	41	9.8	38 341	3
16	gi|600808	Unknown	38	1.6	102 369	3
17	gi|123579	Heat shock protein 60, mitochondrial precursor (stimulator factor I 66 kDa component) (P66) (CPN60)	37	14	63 116	9
18	gi|4114	Unnamed protein product	37	6.9	63 870	4
19	gi|4614	Unnamed protein product	36	3.1	39 688	1
20	gi|1149544	N2650	34	8.2	18 038	1
21	gi|312258	PUP2	34	7.7	29 509	2
22	gi|3724	Unnamed protein product	32	17.8	37 202	5
23	gi|1323341	TDH3	32	15.7	37 108	5
24	gi|1431189	CDC48	32	12.7	94 519	10
25	gi|230909	Phosphoglycerate kinase	31	10.1	46 680	5

List of all proteins as well as their scores and coverage is listed. For complete list of peptides and sites of carbonylation please refer to Supplementary Table 1.

and that oxidative stress drastically reduces the ability of proteosomes and lysosomes to degrade protein complexes [30]. Reducing the degradation rate of protein complexes in oxidatively stressed cells without reducing protein synthesis would cause the observed phenomenon.

Differentiation between these two possibilities was achieved using the protocol in Fig. 1. Control and oxidatively stressed samples were tryptic digested separately and then differentially acetylated using light and heavy isotope coded forms of *N*-acetoxysuccinamide, respectively. The Gistools software [24] was used to identify doublet and singlet peaks. According to the labeling strategy seen in Fig. 1, the lighter of these peaks is from the control sample while the heavier is from the stressed sample. The heavy form of *N*-acetoxysuccinamide is 5 mass units heavier than the light.

Large numbers of singlets were detected. After MS/MS analysis of all singlet peptides, they were subjected to a database search. Light acetate tagging was selected as a constant variable while heavy acetate tagging was not specified. As a result, all peptides that originated from the control sample were identified with a high score. Other peptides and their corresponding parent proteins remained unidentified. By reversing this procedure all peptides that carried heavy isotope tags and their parent proteins were identified. A list of all proteins identified from heavy labeled singlet peptides is seen in Table 1.

The 25 proteins listed in this table are those hits with highest scores and the most reliable identification. Numbers in the first column of the table are the NCBInr accession number while the second column contains the protein name and the third column the Mascot total score. Amino acid coverage and molecular weight are in the next two columns. Carbonylated peptides were identified for 15 proteins in this list (for more detail on these proteins see Supplementary Table 1). Even though the score for biotinylated peptides is normally low, the fact that these peptides were found in a pool of other singlet peptides from the same protein makes the identification more reliable. Identifying carbonylated peptides further confirms the hypothesis that the changes observed were the result of protein oxidation to the carbonyl level.

The results in Fig. 4 and Table 1 are interpreted to mean that the substantial increase in protein concentration in the >700 kDa fraction of oxidatively stressed yeast is due to proteins of lower molecular weight associating with preexisting protein complexes of the yeast interactome. Many more proteins than the 25 identified above are likely to be responsible for this increase. The fact that the proteins identified originated from singlets means that the increase in absorbance seen in the oxidatively stressed sample is due primarily to changes in the molecular weight of proteins, not reduced degradation.

5. Discussion

Mitochondria give eukaryotes the huge advantage of having a dedicated system to harvest energy from the environment through aerobic oxidation of metabolites. This is achieved by transferring electrons to oxygen through a series of interconnected, energy capturing metalloproteins. The price eukaryotes pay for this great asset is that during the course of electron transport a small percentage of electrons leak from the electron transport chain to form harmful ROS. ROS at low levels is generally destroyed, producing no adverse effects. But at high metabolic loading, with aging, or in the case of degenerative diseases that cause aberrations in the mitochondrial electron transport chain, ROS formation can be excessive. Under conditions of extreme or prolonged oxidative stress, cell survival is threatened.

The most important observation coming from the data presented above was that oxidative stress in yeast can cause the incorporation of stress-damaged proteins into the interactome. Based on the fact that protein complexes in the interactome are involved in everything from capturing metabolic energy to gene and protein expression, cell signaling, and recycling damaged proteins, this is very significant. Recent studies have shown that unidentified oxidized proteins bind to the surface of 20S proteosomes where they act as irreversible inhibitors of protein degradation [31].

Because the 20S proteosome is responsible for destroying oxidatively damaged proteins [32], the inhibition of oxidized protein degradation creates a negative feedback loop. The probable net effect is that the ability of oxidatively stressed systems to degrade oxidized proteins decreases slowly with an accompanying increase in the level of oxidatively damaged proteins. Diminishing the activity of this system at a time when it is critically needed is a blow to the cell, but compromising the activity of many protein complexes by random adsorption of oxidized proteins onto the surfaces of multiple complexes would be even more serious. Based on the results from this study it seems that indeed, many protein complexes are attached by oxidized proteins.

Moreover, the aggregation process seems to be very different in this case than the polymerization process commonly associated with Alzheimer's and Parkinson's disease. Results from this study and the 20S proteosome studies [31] show that adsorption of damaged proteins onto the surface of existing, native protein complexes is an alternative to polymerization in sequestering oxidized proteins. The biological impact seems to be different as well. Whereas protein fibrils and amyloid structures resulting from polymerization are broadly toxic to cells [33], protein adsorption onto the surface of protein complexes only compromises a particular complex or organelle.

Given that protein complexes can form in several ways, in which biological systems do these various mechanisms apply? Oxidative stress induced polymerization is associated with slow growing neurological tissue where a small number of disease-specific proteins can undergo a very unique type of conformational conversion to a steric zipper-like, β-sheet structure [34]. With α-synuclein fibrils for example this cross-β spine region is arranged in a parallel, in-register structure wherein the same residues from different molecules are stacked on top of each other [35]. This parallel, in-register zipper-like structure appears to be a common feature shared by multiple amyloid fibrils. Clearly, polymerization to form fibrils only occurs when these very unique proteins are present in cells.

With the hundreds of other oxidatively damaged proteins in stressed cells [36] these studies suggest that when they reach a level where aggregation occurs it will probably be by a mechanism similar to the one described here. The first step in the process is likely to involve conformational change(s) that will increase local hydrophobicity or ionic character at the oxidized protein surface. This in turn leads to a pairing of these newly created surface domains with complimentary hydrophobic or charged surface domains in protein complexes. As we know from reversed-phase chromatography and ion exchange chromatography systems, adsorption of denatured proteins at surfaces almost never goes beyond a monolayer. Moreover, adsorption is reversible. Both of these characteristics of surface adsorption are very different than in fibril formation where very high binding affinities are involved.

6. Conclusions

Oxidative stress induced aggregation of proteins occurs widely in nature. It is concluded from these studies that beyond the homo- and cross-polymerization mechanisms so widely referenced in the literature, oxidized proteins also participate in non-genetically coded complexation by adsorption to the surface of existing, native complexes. Moreover, our model protein study suggests that oxidized proteins are less likely to associate with themselves than the large number of cytosolic proteins found in complexes. This simple, random adsorption process seems to be more similar to what happens in aging than in neurological diseases where fibril formation is the norm. Combination of SEC and mass spectrometry based proteomics has proven effective in isolation and characterization of protein aggregates in yeast cells.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jchromb.2008.04.025.

References

[1] T.C. Squier, Exp. Gerontol. 36 (2001) 1539.
[2] T. Grune, T. Jung, K. Merker, K.J.A. Davies, Int. J. Biochem. Cell Biol. 36 (2004) 2519.
[3] A.E. Kabakov, V.L. Gabai, Experientia 49 (1993) 706.
[4] C.A. Ross, M.A. Poirier, Nat. Rev. Mol. Cell Biol. 6 (2005) 891.
[5] K.J.A. Davies, Free Radicals and Oxidative Stress: Environment, Drugs and Food Additives, 1995, p. 1.
[6] K.B. Beckman, B.N. Ames, Physiol. Rev. 78 (1998) 547.
[7] J.A. Cohn, L. Tsai, B. Friguet, L.I. Szweda, Arch. Biochem. Biophys. 328 (1996) 158.
[8] J.R. Requena, C.C. Chao, R.L. Levine, E.R. Stadtman, Proc. Natl. Acad. Sci. U.S.A. 98 (2001) 69.
[9] T. Grune, R. Shringarpure, N. Sitte, K. Davies, J. Gerontol. 56 (2001) B459.
[10] D. Harman, Ann. N. Y. Acad. Sci. (2001) 1.
[11] R.S. Sohal, Free Radic. Biol. Med. 33 (2002) 37.
[12] A.J. Brookes, D. St Clair, Trends Neurosci. 17 (1994) 404.
[13] H. Liu, X. Wang, Neural Regener. Res. 2 (2007) 239.
[14] G. MacBeath, S.L. Schreiber, Science 289 (2000) 1760.
[15] C. Campagnolo, T. Ryan, R. Atkinson, C. Batt, Mol. Biol. Cell 11 (2000) 131A.
[16] G. Rigaut, A. Shevchenko, B. Rutz, M. Wilm, M. Mann, B. Seraphin, Nat. Biotechnol. 17 (1999) 1030.
[17] K.J. Bitterman, O. Medvedik, D.A. Sinclair, Microbiol. Mol. Biol. Rev. 67 (2003) 376.
[18] V.D. Longo, P. Fabrizio, Cell. Mol. Life Sci. 59 (2002) 903.
[19] M. MacLean, N. Harris, P.W. Piper, Yeast 18 (2001) 499.
[20] D. Sinclair, K. Mills, L. Guarente, Annu. Rev. Microbiol. 52 (1998) 533.
[21] B.-S. Yoo, F.E. Regnier, Electrophoresis 25 (2004) 1334.
[22] S. Michaelis, I. Herskowitz, Mol. Cell. Biol. 8 (1988) 1309.
[23] F.E. Regnier, L. Riggs, R. Zhang, X. Li, P. Liu, A. Chakraborty, E. Seeley, C. Sioma, R.A. Thompson, J. Mass Spectrom. 37 (2002) 133.
[24] X. Zhang, W. Hines, J. Adamec, M. Asara John, S. Naylor, E. Regnier Fred, J. Am. Soc. Mass Spectrom. 16 (2005) 1181.
[25] H. Mirzaei, F. Regnier, J. Chromatogr. A 1141 (2007) 22.
[26] H. Mirzaei, F. Regnier, Anal. Chem. 77 (2005) 2386.
[27] K.J.A. Davies, M.E. Delsignore, S.W. Lin, J. Biol. Chem. 262 (1987) 9902.
[28] H. Mirzaei, F. Regnier, J. Proteome Res. 5 (2006) 3249.
[29] Q. Chen, Q. Ding, J.N. Keller, Biogerontology 6 (2005) 1.
[30] N. Bader, T. Grune, Biol. Chem. 387 (2006) 1351.
[31] S.R. Powell, P. Wang, A. Divald, S. Teichberg, V. Haridas, T.W. McCloskey, K.J.A. Davies, H. Katzeff, Free Radic. Biol. Med. 38 (2005) 1093.
[32] K. Aquilano, G. Rotilio, M.R. Ciriolo, Meet. Soc. Free Radical Res.—Eur. Sect.: Free Radicals Oxid. Stress: Chem., Biochem. Pathophysiol. Implic. Ioannina, Greece, 26–29 June, 2003, p. 85.
[33] A.D. Roth, G. Ramirez, R. Alarcon, R. Von Bernhardi, Biol. Res. 38 (2005) 381.
[34] R. Sawaya Michael, S. Sambashivan, R. Nelson, I. Ivanova Magdalena, A. Sievers Stuart, I. Apostol Marcin, J. Thompson Michael, M. Balbirnie, J.W. Wiltzius Jed, T. McFarlane Heather, O. Madsen Anders, C. Riekel, D. Eisenberg, Nature 447 (2007) 453.
[35] M. Chen, M. Margittai, J. Chen, R. Langen, J. Biol. Chem. 282 (2007) 24970.
[36] H. Mirzaei, F. Regnier, J. Proteome Res. 5 (2006) 2159.