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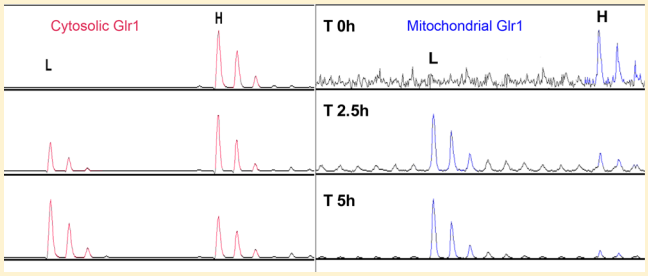
Partition and Turnover of Glutathione Reductase from *Saccharomyces cerevisiae*: A Proteomic Approach

Narciso Couto,[†,‡] Naglis Malys,[†,∥] Simon J. Gaskell,[†,§] and Jill Barber*[,†,⊥]

[†]Manchester Institute of Biotechnology, University of Manchester, 131 Princess Street, Manchester M1 7DN, United Kingdom
[⊥]School of Pharmacy and Pharmaceutical Sciences, University of Manchester, Manchester M13 9PT, United Kingdom

Supporting Information

ABSTRACT: Glutathione reductase (Glr1) is a low abundance protein involved in defense mechanisms against reactive oxygen species. Expressed on cytosolic ribosomes, the same gene, *GLR1*, uses alternative start codons to generate two forms of Glr1. Translation from the first AUG codon generates the mitochondrial form incorporating a presequence necessary for import; translation from the second AUG codon yields the cytosolic counterpart. Proteomic strategies were used to analyze the N-terminal sequences and the turnover of *Saccharomyces cerevisiae* Glr1. The N-terminus of cytosolic Glr1 was found normally to be *N*-acetylserine. When a Glr1-overproducing strain was employed, unprocessed mitochondrial Glr-1 with N-terminal acetylmethionine also accumulated in the cytosol. The processed mitochondrial Glr1 was surprisingly found to have three alternative N-termini, none of them acetylated. Mitochondrial Glr1 was turned over faster than the cytosolic form by a factor of about 2, consistent with the importance of redox homeostasis in the mitochondria. These experiments also allowed us to estimate the extent of "leaky scanning" in the synthesis of Glr1. Surprisingly, the second AUG appears to be responsible for most of the cellular Glr1. This is the first report of protein turnover measurements of a low-abundance protein distributed in different compartments of a eukaryotic cell.



KEYWORDS: *Saccharomyces cerevisiae, glutathione reductase, N-termini, protein turnover, proteomics, mass spectrometry*

INTRODUCTION

Glr1 (glutathione reductase, EC 1.8.1.7) is one of a small number of proteins known to be found in more than one compartment of eukaryotic cells. By converting oxidized glutathione to its reduced form, Glr1 helps to protect the cell against oxidative stress. It is well-known that high levels of reduced glutathione are necessary in order to maintain the normal function of the cells otherwise ROS (reactive oxygen species) start damaging critical biomolecules such as DNA and proteins.[1−7]

In yeast, Glr1 exists in both the cytosolic and the mitochondrial cellular compartments and both isoforms are produced by the same *GLR1* gene. The mitochondrial Glr1 is expressed from the first AUG start codon and is initially 16 amino acids longer, at the N-terminus, than the cytosolic counterpart expressed from the second in-frame AUG start codon.[8] The truncated form cannot migrate into the mitochondria because the recognition peptide, essential for import, is absent. The recognition peptide of the long Glr1 is cleaved during or after import, by peptidases located in the mitochondrial intermembrane space and/or the matrix,[9−12] leaving a functional protein in the mitochondria of a size similar to that of the cytosolic Glr1.[8−12]

This behavior is not unique to Glr1 and other examples of proteins with localization in different compartments of the cell and synthesized using a single gene have been reported in the literature.[13−16] An interesting case is DNA ligase I, an essential protein for completion of DNA synthesis. It exists in the nucleus and mitochondria of yeast and both forms are expressed by the same *CDC9* gene.[16] Similarly to glutathione reductase isoforms, these forms of DNA ligase I are expressed from consecutive in-frame AUG codons. The involvement of a single gene in the expression of several active isoforms is expected to be energetically favorable (one gene essentially does the work of two or more genes). The mechanisms behind this cellular phenomenon are not fully understood.

Outten and Culotta[8] showed in yeast cells that the cytosolic Glr1 is approximately 20 times more abundant than the mitochondrial form. This is a surprising result, insofar as the cytosolic form of the protein is produced by "leaky scanning" of the *GLR1* gene.[17−20] Two possible explanations present themselves: (1) that the context of the first AUG is so poor that "leaky scanning" is actually the dominant mechanism for translation of this gene or (2) that the mitochondrial form of the protein is synthesized preferentially but is in such rapid turnover that its steady-state concentration is far below that of the cytosolic form. More complex mechanisms could also be postulated, involving export of mitochondrial glutathione

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reductase to the cytosol or differential regulation of the cytosolic and mitochondrial proteins.

In order to understand the regulation of the two isoforms of yeast glutathione reductase, we therefore undertook the first systematic study of the turnover of a protein expressed in two different compartments of a eukaryotic cell. To satisfy this objective, we employed proteomics methodologies using mass spectrometry as the principal analytical technique. First, we undertook N-terminal analysis of Glr1 involving a modified tryptic digestion protocol. Second, we used a dynamic stable isotope labeling with amino acids in cell culture (SILAC) approach to study Glr1 turnover in both the cytosol and the mitochondria of yeast cells.

MATERIALS AND METHODS

Materials

Except where stated, reagents, chemicals, and high performance liquid chromatography (HPLC)-grade solvents were purchased from Sigma-Aldrich (Poole, UK) with the highest purity available.

Yeast Strains

Saccharomyces cerevisiae wild-type strain BY4742 (*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*) and GLR1-overexpressing strain Y258 (*MATapep4-3his4-580ura3-52leu2-3 112*), transformed with expression plasmid pBG1805-GLR1 were used in the present study. In the plasmid pBG1805-GLR1, glutathione reductase was fused in-frame at the C-terminal coding region of the *GLR1* gene with a 6H (6 histidine affinity tag), an HA epitope (hemagglutination), a protease 3C cleavage site, and a ZZ domain from *Staphylococcus aureus* Protein A, with high affinity to IgG matrix.[21] Gene expression was controlled by using *GAL1* inducible promoter. The GLR1-overexpressing strain Y258 was grown aerobically on a synthetic defined medium without uracil (S-URA medium). The wild-type strain was grown aerobically on a sterile lactate medium. Media preparations as well as the growth conditions are described in the Supporting Information (E1 and E2).

Preparation of Yeast Cytosol and Mitochondria

Cytosolic and mitochondrial fractions were prepared according the procedures described by Daumet al.,[22] Glick et al.,[23] and Meisinger et al.,[24,25] with minor modifications. The ratio of buffer vs cell pellet was maintained at 1 mL of buffer per 0.15 g of wet cells, unless otherwise stated. All the steps described were performed at 4 °C, and the buffers were prechilled before use. The cell pellet was resuspended in Tris-buffer (100 mM Tris-HCl (pH 9.4), 10 mM dithiothreitol (DTT)), and the suspension was incubated at 30 °C for 10 min with gentle shaking. The cells were repelleted, at 2500*g* for 10 min at 4 °C, washed, and resuspended with buffer A (1.2 M sorbitol, 20 mM potassium phosphate buffer (pH 6.5)). Spheroplast formation was achieved by adding lyticase (from *Arthrobacter luteus*), at the concentration of 2 units per OD (1 mg of wet cells is approximately 1000 OD), and the suspension was gently shaken at 30 °C for 30 min. Spheroplasts were washed three times with buffer A by resuspension and then centrifuged at 2500*g* for 10 min at 4 °C. Finally, spheroplasts were gently resuspended in breakage buffer (0.6 M mannitol, 10 mM Tris-HCl (pH 7.4), 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride (AEBSF)). One tablet of cOmplete, EDTA-free (Roche, Mannheim, Germany) protease inhibitor was added to the mixture, which was homogenized by 15 strokes in a Dounce homogenizer prior to centrifugation at 2500*g* for 10 min at 4 °C. The supernatant was collected in a clean falcon tube and kept at 4 °C. The pellet was further resuspended in breakage buffer (one-half of the volume previously used), rehomogenized, and recentrifuged as before, and the supernatant was combined with the previous one. After removing cell debris, the crude mitochondrial fraction was pelleted from the cytosolic fraction using polypropylene centrifuge tubes suitable for a fixed-angle JA-25.50 Beckman rotor suitable for the Avanti J-25 centrifuge (Beckman Coulter). The crude mitochondrial fraction was sedimented at 15000*g* for 10 min at 4 °C. The supernatant (the crude cytosolic fraction) was clarified by ultracentrifugation. Beckman polycarbonate bottles (10.4 mL, 16 mm × 76 mm) suitable for a fixed-angle type-70.1 Ti rotor were used to perform this ultracentrifugation which was carried out at 50,000 rpm (171500*g*) for 3 h at 4 °C on an Optima L-100 Ultracentrifuge (Beckman Instruments, Inc., Page Mill Road, U.S.A.).

The crude mitochondrial fraction was then resuspended in buffer B (0.6 M sorbitol, 10 mM Tris-HCl (pH 7.4), 1 mM ethylenediaminetetraacetic acid (EDTA), 0.25 M potassium chloride), and the suspension was incubated for 5 min at 4 °C with shaking. Debris was removed by centrifugation at 2500*g* for 10 min at 4 °C. The supernatant containing the mitochondrial fraction was repelleted by centrifugation at 15000*g* for 15 min at 4 °C as previously described. The mitochondria were washed and repelleted twice with buffer B to remove traces of cytosol, using the same conditions (15000*g* for 15 min at 4 °C). The mitochondria were then resuspended in buffer C (0.6 M sorbitol, 10 mM Tris-HCl (pH 7.4), 1 mM EDTA, 2 mM phenylmethanesulfonyl fluoride (PMSF)) at a ratio of 100 μL of buffer per 100 OD cells (as measured before cell lysis), and lysis of the mitochondrial membrane was achieved by combination of 10 strokes in a Dounce homogenizer followed by ultrasonication on ice (6 cycles of 10 s sonication and 10 s rest to prevent overheating of the sample). To remove debris, this fraction was centrifuged at 50,000 rpm (171500*g*) for 1 h at 4 °C using an Optima L-100 Ultracentrifuge (Beckman Instruments, Inc., Page Mill Road, U.S.A.).

Cytosolic and mitochondrial fractions were dialyzed by using SnakeSkin Pleated Dialysis Tubing, 3500 MWCO (Pierce Biotechnology, Rockford, lL, U.S.A.). These fractions were dialyzed overnight against 50 mM potassium phosphate buffer (pH 7.5). The dialysis medium was changed three times before collection of the purified fraction which was stored at −80 °C. Determination of the total protein concentration was performed using the Bradford assay as described in Supporting Information [SI] (E3).

Experimental Design for Glr1 Turnover Measurements

GLR1-overexpressing strain Y258 was used to perform turnover measurement of Glr1 in the cytosol and in the mitochondria. A synthetic defined medium (S-URA/raffinose) incorporating [$^2H_{10}$]leucine (60 mg L^{-1}) instead of light leucine, was prepared as described in the SI (E2). S-URA/raffinose sterile medium with [$^2H_{10}$]leucine (4 L, 1 L in each of four flasks of 2-L capacity) were inoculated with GLR1-overexpressing strain culture to an OD_{600} of 0.05. The culture was grown for 14−16 h with shaking (220 rpm) at 30 °C until the OD_{600} reached 0.6. Expression of *GLR1* was induced by addition of sterile galactose to a final concentration of 2% (v/v).The culture was grown for a further 5 h to an OD_{600} of 1.0−1.2. Yeast cells were collected

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by centrifugation at 4000*g*, 5 min at 30 °C in a Beckman Coulter Avanti J-6B centrifuge using a JS-4.2 rotor. The supernatant, containing the heavy leucine, was removed, and cells were resuspended in the same volume of prewarmed S-URA/raffinose/galactose medium incorporating light leucine (60 mg L^{-1}). This medium was used in all subsequent steps. At this point, defined as time zero, 125 mL of culture was removed from each flask and replaced by 125 mL of prewarmed S-URA/raffinose/galactose medium leading to a final OD_{600} of 0.87−0.90. After 2.5 h incubation (30 °C, 220 rpm), the optical density had returned to 1.1−1.2, and a further sample (125 mL per flask) was removed and the same volume of prewarmed medium added. This procedure was repeated six more times so that a total of eight fractions were obtained. After each fraction was collected, protein synthesis was inhibited by cycloheximide which was added at a final concentration of 10 μg mL^{-1} of culture. The cells were harvested by centrifugation in a Beckman Coulter Avanti J-6B centrifuge at 4000*g*, 10 min at 4 °C, the supernatant was removed and the pellet resuspended in cold water and transferred into preweighed falcon tubes. The cells were pelleted again by centrifugation at 2500*g* for 5 min at 4 °C and were kept at −80 °C until required.

Affinity Purification

Cytosolic and mitochondrial fractions recovered from the wild-type *S. cerevisiae* BY4742 strain were separately incubated with adenosine 2′,5′-diphosphate agarose, and enriched fractions were analyzed by SDS-PAGE, and protein concentrations were determined by Bradford assay.

Isolated cytosolic and mitochondrial fractions from the GLR1-overexpressing strain Y258 were submitted to affinity purification on IgG Sepharose 6 Fast Flow (GE Healthcare Life Sciences, Little Chalfont, UK) in order to obtain Glr1-enriched fractions. After purification, the fractions were analyzed by SDS-PAGE, Western-blot, and Bradford assay as described in the SI (E3−E7).

Mass Spectrometry and Data Analysis

Prior to mass spectrometry, protein samples were reduced, alkylated, and digested with trypsin. In some experiments, digestion at lysine residues was prevented by prior treatment of the proteins with [$^1H_6$]acetic anhydride or [$^2H_6$]acetic anhydride as described in the SI (E8). After enzymatic digestion, peptides were usually desalted using C_{18} ZipTip (Millipore, Billerica, MA, U.S.A.) before mass spectrometric analysis.

Matrix-Assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS) Analysis. Peptide solutions, 1 μL, were spotted on a massive target plate (MTP) 384 polished steel target (Bruker, Bremen, Germany) followed by 1 μL of matrix solution and were left to dry. As a MALDI matrix, a saturated solution of α-cyano-4-hydroxycinnamic acid in 50% (v/v) acetonitrile incorporating 0.1% (v/v) trifluoroacetic acid (TFA) was prepared. For the purpose of TOF calibration, a peptide mixture (Bruker, Bremen, Germany) was used.

MALDI MS and MALDI MS/MS experiments were performed on an Ultraflex II TOF/TOF mass spectrometer (Bruker, Bremen, Germany) in the reflectron mode for positive ions over a mass range of 700−4000 *m*/*z*. For MS analysis an average of 1000 laser shots was accumulated, and for product ion spectra operation an average of 2000 laser shots was accumulated per spectrum. LIFT technology was used to acquire MS/MS (tandem mass spectrometry) spectra. Data were acquired using FlexControl version 3.0 (Bruker, Bremen, Germany), and data analysis was performed using FlexAnalysis software (Bruker, Bremen, Germany).

Quadrupole Time-of Flight Mass Spectrometry (Q-TOF MS) Analysis. A Q-TOF global mass spectrometer (Waters, Manchester, UK) was calibrated using the product ion spectrum of the doubly charged peptide ion of [Glu^1]-fibrinopeptide. The MS data were acquired in continuum mode over the mass range of 350−1600 *m*/*z*. For MS/MS experiments, collision-induced dissociation (CID) was performed in the presence of argon with a collision cell pressure of 1−3 mbar. The collision offset voltage was set at 30 V, and the spectrum was acquired in the continuum mode over the range 50−1600 *m*/*z*.

An Ultimate 3000 capillary LC system (Dionex, Surrey, UK) was connected online to the Q-TOF instrument via a distal-coated fused silica PicoTip emitter (New Objective, Woburn, MA, U.S.A.) using a capillary voltage of 2.0−2.8 kV into a Z-spray ion source. The typical gradients of 65 and 90 min were used to separate peptides. Aqueous buffer (buffer A) consisted of 98% (v/v) HPLC water, 2% (v/v) HPLC acetonitrile with 0.1% (v/v) formic acid, and organic buffer (buffer B) was made with 95% (v/v) HPLC acetonitrile and 5% (v/v) HPLC water with 0.1% (v/v) formic acid.

Ion Trap Mass Spectrometry (IT-MS) Analysis. An Ultimate 3000 capillary LC system (Dionex, Surrey, UK) was interfaced with the Amazon ion trap instrument (Bruker, Bremen, Germany). Similar LC gradients were used here as in the Q-TOF Global. LC eluate was sprayed from a distal-coated fused silica PicoTip emitter (New Objective, Woburn, MA, U.S.A.) using a capillary voltage of 1700−2200 V. The typical settings for the mass spectrometer were the following: dry gas temperature was set to 150 °C, dry gas was set to 6 L min^{-1}, and the scan mode was set with a *m*/*z* range of 200−3000 with a scan speed of 8100 Th s^{-1}. When MS/MS was required, CID and electron transfer dissociation (ETD) were used.

Database Searching Using Mascot. All database searches were submitted to an in-house Mascot server (http://www.matrixscience.com/).

Peptide mass fingerprinting (PMF) was used with the MALDI-TOF MS data to assess the sequence coverage of proteins by matching the monoisotopic masses of peptides produced after enzymatic hydrolysis with those of theoretical peptides predicted. The following parameters were used for searching: taxonomy (*S. cerevisiae*); enzyme trypsin; number of missed cleavages (0−2); variable modifications: oxidation of methionine, deamidation of asparagine and glutamine and acetyl protein N-terminus. Data obtained from acetylated samples was also searched; light and heavy acetylated lysine and serine were included in the Mascot search and were incorporated as variable modifications when appropriate. A mass accuracy of 50 ppm was specified along with the use of monoisotopic masses of the peptides. Throughout this work, the data were run against the complete proteome databases: National Center for Biotechnological Information (NCBI) and yeast-ORF. Protein Prospector (http://prospector.ucsf.edu/) was also used to obtain the theoretical fragment ions that would result from a CID-type of reaction, and the mass spectra were annotated accordingly.

MS/MS ion search, Mascot search, was used for the LC−MS/MS data acquired on the Q-TOF and ion trap instruments. For the Q-TOF, the peptide tolerance was set to 100 ppm, and the MS/MS tolerance was set to 0.3 Da. For acetylated samples,

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	-1	1	2	3	4	5	6	7	8	9	10
Glr1 sequence		M	L	S	A	T	K	Q	T	F	R
RNA sequence	UUU/ACC	AUG	CUU	UCU	GCA	ACC	AAA	CAA	ACA	UUU	AGA
		11	12	13	14	15	16	17	18	19	20
		S	L	Q	I	R	T	M	S	T	N
		AGU	CUA	CAG	AUA	AGA	ACU	AUG	UCC	ACG	AAC
		21	22	23	24	25	26	27	28	29	30
		T	K	H	Y	D	Y	L	V	I	G
		ACC	AAG	CAU	UAC	GAU	UAC	CUC	GUC	AUC	GGG
		31	32	33	34	35	36	37	38	39	40
		G	G	S	G	G	V	A	S	A	R
		GGU	GGC	UCA	GGG	GGU	GUU	GCU	UCC	GCA	AGA

Figure 1. Predicted mRNA and protein sequences of Glr1 from position −1 to position 40. Differences exist upstream of the first AUG codon. Wild-type yeast strain BY4742 which express Glr1 in its natural chromosomal environment has UUU in position −1. Y258-GLR1-tag yeast strain, a genetically modified strain, has ACC codon in position −1. These differences have been associated with weak and strong contexts around the first AUG start codon for wild-type and GLR1-overexpressing strain Y258 strains, respectively. The second AUG start codon (17) is in a good context in both strains.

no fixed modifications were considered, and additional variable modifications were included in the search such as acetylation of lysine and acetylation of serine. In addition, [$^2H_9$]leucine was included as a variable modification when appropriate. For the ion-trap instrument, the peptide tolerance and MS/MS tolerance were set to 0.4 and 0.8 Da, respectively. Data files containing CID only and ETD only were searched using the ESI-trap and ETD-trap parameters of the Mascot search window.

RESULTS

The N-Terminus of Commercial Glutathione Reductase from Yeast

The two Glr1 translation products are distinguished by the presence of 16 amino acids (MLSATKQTFRSLQIRT) which appear to make up the mitochondrial presequence. As expected for a mitochondrial presequence, basic and polar residues are predominant, and acidic residues are absent.[26] Figure 1 shows the predicted mRNA and protein sequence of Glr1 from position 1 to position 40.

This high proportion of basic residues in the presequence will generate small tryptic peptides which will not be observed during standard mass spectrometric analyses. If Glr1 synthesis starts at the second AUG codon, the N-terminal sequence is (M)STNTKHYDYLVIGGGSGGVASAR, and the detectability of the initial small tryptic peptide (M)STNTK is also in doubt. In fact, mass spectrometric analysis of Glr1 was previously conducted, and this small peptide was not detected.[27] However, after acetylation of lysine side chains, trypsin recognizes only arginine, and thus, appropriately sized peptides for mass spectrometric analysis can be generated. In addition, the availability of [$^2H_6$]acetic anhydride allows in vivo and in vitro acetylation to be distinguished.

Aliquots of commercial Glr1 from whole yeast were acetylated using [$^1H_6$]acetic anhydride or [$^2H_6$]acetic anhydride, digested with trypsin, and analyzed by MALDI-TOF MS and MS/MS. When [$^1H_6$]acetic anhydride was used for acetylation, no evidence of the long Glr1 isoform, with N-terminal MLSATKQTFR, was detected, but a signal at m/z 2394.2, corresponding to ([$^1H_3$]Ac)STNT([$^1H_3$]Ac)KHYDYLVIGGGSGGVASAR was observed. Similarly, when [$^2H_6$]-acetic anhydride was used, a peak at m/z 2397.2, corresponding to ([$^1H_3$]Ac)STNT([$^2H_3$]Ac)KHYDYLVIGGGSGGVASAR, was observed in the spectrum (the MS/MS spectra are shown in Figure R1 of SI). This represents the cytosolic glutathione reductase, which is synthesized from the second AUG codon and processed by removal of methionine and acetylation of the resulting N-terminal serine. N-terminal acetylation of serine is a common modification in vivo and has been extensively reported as a post-translational modification in the cytosol of yeast cells.[28−30]

Cytosolic and Mitochondrial Glr1 from *S. cerevisiae* BY4742 (Wild-Type) Strain

S. cerevisiae BY4742 strain was grown on lactate medium to encourage mitochondrial production, and the mitochondrial and cytosolic fractions were separated as described in the Materials and Methods section. The fractions were treated separately with adenosine 2′, 5′-diphosphate agarose, an affinity matrix which has been used to purify nicotinamide adenine dinucleotide phosphate (NADP)-dependent enzymes such as glutathione reductase.[31] SDS-PAGE of the cytosolic fraction before and after this purification strategy shows that at least 10 bands were present in the affinity-enriched cytosolic fraction (see SI, Figure R2). This is not surprising because this affinity material is not Glr1 specific; it can also bind other NADP-dependent dehydrogenases. The enriched fraction was acetylated with [$^1H_6$]acetic anhydride, digested with trypsin, and analyzed by LC−ESI−MS/MS, see Figure R3 in SI. Three Glr1 peptides were identified, including the N-terminal peptide, which was also seen when commercial glutathione reductase was used to perform the preliminary experiments. Two annotated MS/MS spectra of this peptide (using different mass spectrometers) are shown in Figures R4 and R5 of the SI and confirm the N-terminal sequence of the cytosolic Glr1.

The presence of glutathione reductase in the mitochondrial fraction was demonstrated by LC−ESI−MS/MS (table in SI, Figure R6) following tryptic digestion (without acetylation). Three peptides were clearly observed in this experiment, but they did not include the N-terminal peptide. Unfortunately, because the protein was present in only very small quantities, the acetylation−tryptic digestion procedure was unsuccessful, and the N-terminal peptide could not be identified.

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Cytosolic and Mitochondrial Glr1 from *S. cerevisiae* GLR1-Overexpressing Strain Y258

The low abundance of glutathione reductase in the mitochondria of the wild-type strain prompted numerous experiments, involving different strains of yeast in different growth media, which were ultimately unsuccessful in identifying the N-terminal peptide. We therefore turned our attention to GLR1-overexpressing strain Y258 in which Glr1 is expressed as a C-terminal fusion protein under *GAL1* control, which is suitable for tandem affinity purification with IgG.[21,32] This strain was grown on S-URA medium containing [$^2H_{10}$]leucine instead of [$^1H_{10}$]leucine, and the mitochondrial and cytosolic fractions were separated as described in the Materials and Methods section. Subsequently, cytosolic and mitochondrial Glr1 were obtained by purification on IgG Sepharose. Labeled leucine was used because it was required for turnover measurements which were made in the course of the same experiments. Although [$^2H_{10}$]leucine contains 10 deuterium atoms, the α-hydrogen is readily exchanged, and thus, only nine deuterium atoms are incorporated into proteins.

Glutathione Reductase Is Able To Migrate to Mitochondria. The first concern was whether glutathione reductase from GLR1-overexpressing strain Y258 migrated effectively into the mitochondria. Western-blot analysis was performed in order to confirm the existence of Glr1 in both the cytosol and the mitochondrial compartments. The results are shown in Figure 2.

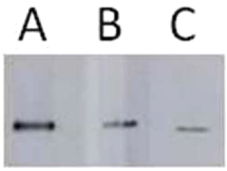

Figure 2. Western-blot analysis of Glr1 from the cytosolic and mitochondrial fraction of Y258-GLR1-tag *S. cerevisiae* strain purified from IgG Sepharose before incubation with 3C-protease. The cytosolic fraction (A) is 20 times more diluted than the mitochondrial fraction (B). The bands appeared at ~72 kDa which is in agreement with Glr1 (M_w 53 kDa) plus Protein A (M_w 19 kDa). As a positive control, 12.5 ng of prepurified yeast hybrid protein, adenylosuccinate synthase (Ade12), was loaded in the gel (C).

The Western-blot result shows that Glr1 exists in both the cytosol and mitochondria and the C-terminal tag is no impediment to migration of Glr1 from the cytosol into the mitochondria.

Both Cytosolic Glutathione Reductase and the Long Form of Mitochondrial Glutathione Reductase Are Found in the Cytosol. Cytosolic glutathione reductase from the GLR1-overexpressing strain Y258 was reduced, alkylated, and subjected to tryptic digestion. The resulting peptides were analyzed by HPLC–ESI–MS/MS. Glutathione reductase, as well as heat shock protein Ssa1/Ssa2, was identified with sequence coverage of 63% and 17% respectively. It appears that Ssa1/Ssa2 binds to Glr1, but we did not investigate whether the binding was specific or nonspecific.

Glr1-derived peptides confidently identified are shown in Figure R7 in the SI. Surprisingly, one of the peptides observed in this experiment corresponded to the N-terminal peptide of Glr1, long form, with one missed cleavage (([$^1H_3$]Ac)-MLSATKQTFR). Long (mitochondrial) Glr1 had never been observed in the cytosolic fractions derived from the wild-type strain. In order to determine whether the N-terminal peptide of the short (cytosolic) form was also present, another sample of the purified cytosolic protein was acetylated with [$^1H_6$]acetic anhydride and digested with trypsin. Three peptides of particular interest were identified in this sample, and the ESI–MS/MS spectra obtained under low-energy CID regime are shown in Figures R8–R10 of SI. These peptides are the N-terminal peptide of Glr1 long (mitochondrial) form ([$^1H_3$]-Ac)M[$^2H_9$]LSAT([$^1H_3$]Ac)KQTFR, the N-terminal peptide of Glr1 short (cytosolic) form ([$^1H_3$]Ac)STNT([$^1H_3$]Ac)-KHYDY[$^2H_9$]LVIGGGSGGVASAR, and the peptide TMSTNT([$^1H_3$]Ac)KHYDY[$^2H_9$]LVIGGGSGGVASAR derived from the long form after tryptic cleavage at arginine. In all the spectra presented in Figures R8–R10, b- and y-series of fragment ions confirm the sites of acetylation. These results (which were also confirmed by ESI–MS/MS on an Amazon ion trap mass spectrometer using CID and ETD, SI Figures R11 and R12) show that only midchain lysine residues and the N-terminal methionine and serine residues are acetylated.

When [$^2H_6$]acetic anhydride replaced the [$^1H_6$]acetic anhydride in this experiment, methionine and serine of the N-terminal peptides, ([$^1H_3$]Ac)M[$^2H_9$]LSAT([$^2H_3$]Ac)-KQTFR and ([$^1H_3$]Ac)STNT([$^2H_3$]Ac)KHYDY[$^2H_9$]LVIG-GGSGGVASAR respectively were unlabeled, demonstrating that both these acetylations take place in vivo (see ESI–MS/MS spectra shown in Figures R13 and R14 in the SI). Removal of methionine and acetylation of serine is a common post-translational modification in eukaryotic organisms.[28–30] How-

Table 1. Summary of the Mass Spectrometric Approaches Used To Identify the N-Terminal Glr1 Peptide(s) of Both Wild-Type and Overproducing Yeast Strains

			Mass Spectrometer		
			Q-TOF	Ion-Trap	
Yeast Strain	Compartment	Amino Acid Sequence (N-terminal Glr1 peptide)	CID[a]	CID[b]	ETD[b]
Wild-type	Cytosol	Ac-STNTKHYDYLVIGGGSGGVASAR	yes	–	–
	Mitochondria	Not identified	×	–	–
GLR1-overexpressing strain Y258	Cytosol	Ac-MLSATKQTFR	yes	yes	yes
		Ac-STNTKHYDYLVIGGGSGGVASAR	yes	yes	yes
		TMSTNTKHYDYLVIGGGSGGVASAR	yes	yes	yes
	Mitochondria	MSTNTKHYDYLVIGGGSGGVASAR	yes	–	–
		STNTKHYDYLVIGGGSGGVASAR	yes	–	–
		TKHYDYLVIGGGSGGVASAR	yes	–	–

[a]×: N-terminal peptide not identified. [b]–: Mass spectrometric technique not used.

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Table 2. Summary of the Cytosolic Glr1 SILAC Pairs Selected for Turnover Measurements Which Were Observed from MALDI-TOF Mass Spectra

Peptide	Sequence[a]	m/z light	m/z heavy	Δm (Da)	K (h^{-1})[b] (mean ± SD)
P1	LFGPE**K**FR	1035.56	1044.56	9	0.20 ± 0.02
P2	VELTPVAIAAGR	1196.81	1205.81	9	0.21 ± 0.01
P3	**K**LSNRLFGPE**K**FR	1675.93	1694.00	18	0.22 ± 0.03
P4	**S**TNT**K**HYDYLVIGGGSGGVASAR	2394.17	2403.17	9	0.20 ± 0.02
P5	TMSTNT**K**HYDYLVIGGGSGGVASAR	2585.28	2594.28	9	0.24 ± 0.02
P6	LNGIYQ**K**NLEKEKVDVVFGWAR	2733.44	2751.44	18	0.21 ± 0.02
P7	VSHANEYGLYQNLPLD**K**EHLTFNWPEF**K**Q**K**R	3927.94	3963.87	36	0.20 ± 0.02

[a]Acetylated amino acids (lysine and N-termini) are indicated in bold. [b]K values (determined using eq 3), in h^{-1}, mean values and standard deviation of three independent measurements are shown.

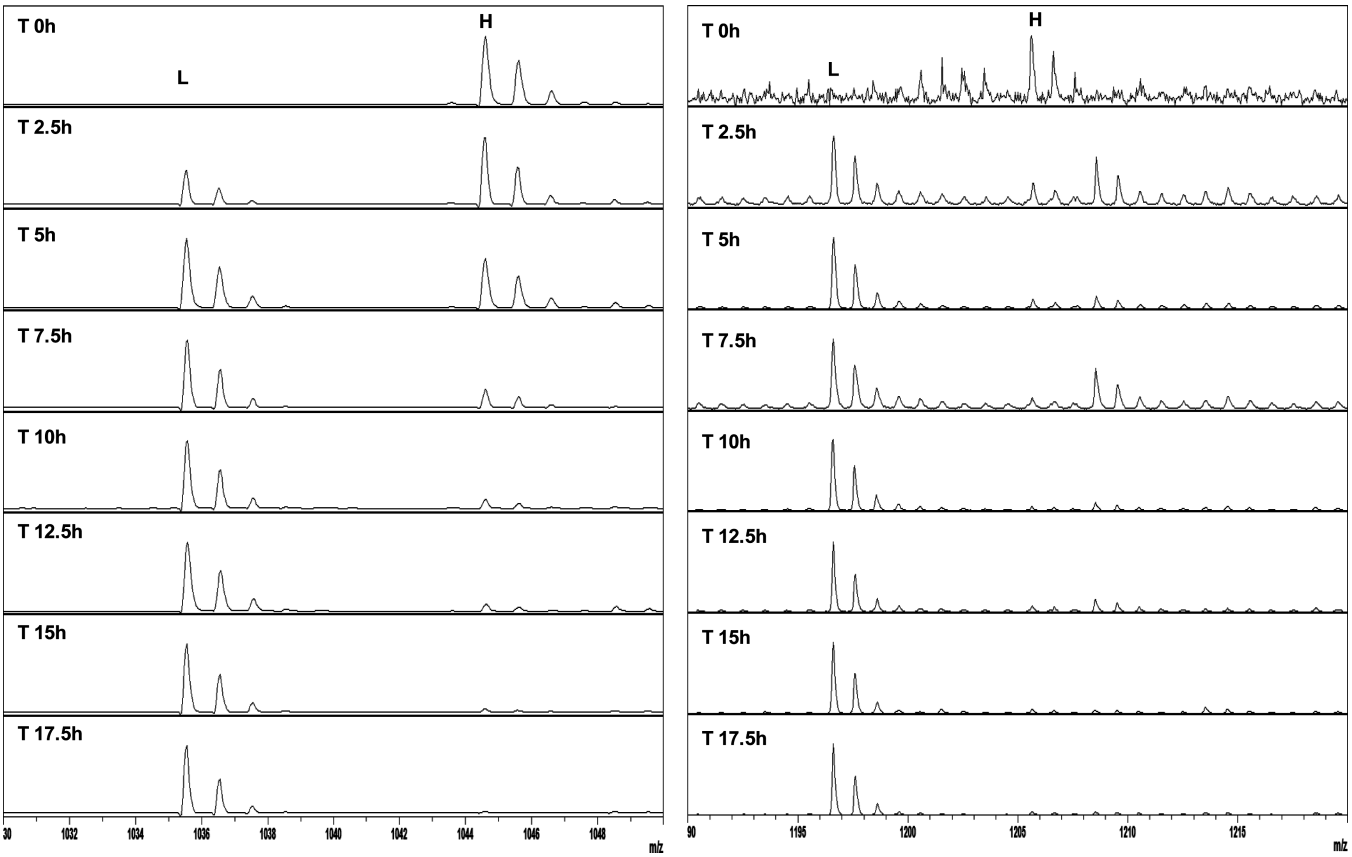

Figure 3. Changes in peptide profile during the unlabeled period obtained by MALDI-TOF MS for the cytosolic (on the left) and the mitochondrial (on the right) Glr1.The heavy and light versions of the peptide LFGPEKFR at m/z 1044.66 (H) and m/z 1035.66 (L) in the cytosol are shown on the left, and the peptide VELTPVAIAAGR, at m/z 1205.81(H) and m/z 1196.81 (L) are shown on the right. The H and L versions of SILAC pairs are shifted 9 Da.

ever, in the Glr1 long form, methionine is not lost but is itself acetylated in vivo. Acetylation of N-terminal methionine is a relatively uncommon modification, although it has been reported in the literature.[29]

Mitochondrial Glr1 Has Several N-Terminal Peptides. Estimated by Bradford assay, 6 μg of purified Glr1 was obtained from the mitochondrial fraction (compared with 300 μg from the cytosolic fraction) from 1.5 L of culture. Mitochondrial Glr1, extracted from GLR1-overexpressing strain Y258,was now digested with trypsin, with and without prior acetylation with [$^2H_6$]acetic anhydride, and both samples were subjected to HPLC−ESI−MS/MS as described above for the cytosolic fraction. Analysis of the non-acetylated sample indicated the presence of glutathione reductase with sequence coverage of 46%. The acetylated sample was also analyzed by HPLC−ESI−MS/MS, and the data were inspected manually and with the use of Mascot in order to decipher Glr1 N-terminal peptide in the mitochondria. The triply protonated peptide ions at: m/z 832.4 (MSTNT([$^2H_3$]Ac)KHYDY[$^2H_9$]LVIGGGSGGV-ASAR,), m/z 788.75 (STNT([$^2H_3$]Ac)KHYDY[$^2H_9$]LVIG-GGSGGVASAR) and m/z 688.05 (T([$^2H_3$]Ac)KHYDY[$^2H_9$]-LVIGGGSGGVASAR) with peptide scores of 68, 71, and 58, respectively were identified when "no-enzyme" was set during the Mascot search. The "no-enzyme" option was required to identify peptides that are produced by processes other than tryptic cleavage.) MS/MS spectra obtained using CID are shown in the SI, Figure R15. There is no evidence of acetylation of the N-terminal residues either in vivo (in which case the acetyl groups would be unlabeled) or in vitro (in which case the acetyl groups would be deuterated).

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Table 1 summarizes the N-terminal peptides obtained from cytosolic and mitochondrial Glr1 from wild-type and GLR1-overexpressing Y258 strains, using several different mass spectrometric approaches.

Glutathione Reductase Turnover in the Cytosol and in the Mitochondria of Yeast Cells

To monitor protein turnover, yeast cells (often auxotrophic for leucine) may be grown in the presence of [$^2H_{10}$]leucine and then transferred to a medium in which [$^1H_{10}$]leucine is the sole source of leucine. Samples are collected at intervals and the heavy:light isotope ratios analyzed. This strategy has two minor disadvantages. First, quantification by LC−MS may be compromised by the increased hydrophobicity of deuterated leucine, leading to labeled and unlabeled peptide not eluting exactly together in reverse-phase chromatography.[33] Second, not all peptides contain leucine, but since leucine is a very common amino acid, this is seldom a very serious concern. The alternative strategy in which $^{13}C/^{15}N$-labeled arginine and lysine form the source of the isotopic label overcomes both of these problems when trypsin is the proteolytic enzyme, but introduces two more: high cost and the biosynthetic conversion of arginine to proline, compromising the integrity of the label.[34] For this study, involving a low-abundance protein and requiring the separation of the mitochondria from the cytosol, large-scale cultures were required, and thus, [$^2H_{10}$]leucine was the preferred source of label.

A pseudocontinuous culture was used in order to maintain a constant growth rate during the experiment. Cells isolated at eight time points were lysed, the mitochondrial and cytosolic fractions were separated, and Glr1 was purified on IgG Sepharose beads. The total amount of glutathione reductase at each time point varied between 100 and 150 μg for the cytosolic fractions and 4−10 μg for the mitochondrial fractions.

Samples were acetylated, using unlabeled acetic anhydride, and digested with trypsin. MALDI-TOF mass spectra showed seven pairs of Glr1 peptides (labeled and unlabeled peptides, known as SILAC pairs[35]) derived from the cytosolic fraction, (see Table 2), and a single SILAC pair derived from the mitochondrial fraction, reflecting the much smaller amount of analyte. These eight peptides were selected to perform the turnover measurements.

Turnover in the cytosol is most clearly seen for the peptide LFGPEKFR and in the mitochondria for peptide VELTPVAIAAGR. Figure 3 shows the change in their isotopic compositions during the time course of the experiment.

Administration of [$^2H_{10}$]Leucine Results in Incorporation of [$^2H_9$]Leucine in Glutathione Reductase. Figure 3 (and Figure R16 of the SI) clearly shows a transition from the completely heavy to the completely light version of Glr1 peptides. The same behavior was seen with the other observable pairs of peptides (data not shown). Interestingly, the heavy peptide is not 10, but 9, mass units heavier than the light peptide. In all the SILAC pairs, the use of [$^2H_{10}$]leucine results in incorporation of [$^2H_9$]leucine into the protein. This is most striking for P7 which contains four leucine residues (see Table 2). This effect has also been observed by others.[36] These results show immediately that the α-deuterium of heavy leucine is completely exchanged. This is known to be catalyzed by cellular transaminases in a fast step, which need not be followed by transamination.[37]

Mitochondrial Glr1 Is in Fast Turnover Relative to Cytosolic Glr1. As shown above, when the GLR1-overexpressing Y258 strain is used, cytosolic Glr1 is a mixture of short (cytosolic) and long (unprocessed mitochondrial) forms. Of the seven SILAC pairs identified in the cytosolic fraction, one (P4) is unique to the cytosolic form of the protein, and one (P5) is unique to the unprocessed mitochondrial form. The other peptides are internal and common to both the mitochondrial and cytosolic forms of the protein.

To measure relative rates of Glr1 degradation in both cytosolic and mitochondrial compartments, we used a strategy previously reported by Pratt et al.[36]

At each time point, triplicate MALDI-TOF MS analyses were performed, and RIA (relative isotopic abundance) was calculated according to eq 1 where I_H is the intensity of the monoisotopic peak of the heavy peptide and I_L is the intensity of the monoisotopic peak of the light counterpart:

$$\mathrm{RIA}_t = \frac{I_H}{(I_L + I_H)} \qquad \text{(eq 1)}$$

Figure 4 shows the RIA values for the common peptide P2 (VELTPVAIAAGR) in the cytosolic and mitochondrial compartments plotted against time. On the basis of the data for this peptide, a clear difference between the rate of decay in the mitochondria and cytosol is observed, and a clear difference in the turnover rate of the cytosolic and the mitochondrial forms of Glr1 may be inferred. This inference is supported by the data for the other internal cytosolic peptides (P1, P3, P6, and P7) as shown in Figures R17 and R18 of the SI, which are superimposable on the data for cytosolic P2.

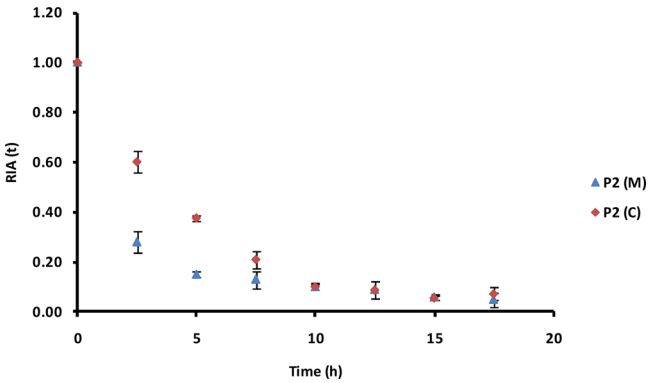

Figure 4. Graphical representation of RIA (relative isotopic abundance) values vs time for VELTPVAIAAGR peptide from the cytosol, P2 (C), and from the mitochondria, P2 (M).

This graphical representation is consistent with first-order kinetics where the rate constant reflects the combined rates of dilution and degradation. The first-order kinetics are expressed by eq 2, where the RIA values at time zero and infinity are 1 and zero, respectively, k is defined as the rate constant of loss of labeled peptide which is related to protein syntheses, protein degradation, and dilution.

$$\mathrm{RIA}_t = \mathrm{RIA}_\infty + (\mathrm{RIA}_o - \mathrm{RIA}_\infty) \times \exp^{(-kt)} \qquad \text{(eq 2)}$$

By simplifying eq 2, eq 3 can be obtained:

$$\ln(\mathrm{RIA}_t) = -kt \qquad \text{(eq 3)}$$

By applying eq 3, k was calculated for three sets of cytosolic Glr1 peptides, and values of 0.20, 0.24, and 0.21 h^{-1} for P4, P5, and internal peptides, respectively, were obtained (see Table 2). This value (k) reflects the rate of protein degradation and

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dilution. The dilution rate is approximately 0.06 h^{-1}, and thus, the rates of protein degradation for glutathione reductase can be estimated as 0.14, 0.18, and 0.15 h^{-1} for P4, P5 and internal peptides of cytosolic Glr1, respectively. The apparent k exhibited by the internal peptides represents a weighted average of the two forms, suggesting that the short form is more abundant by a factor of ~3. These results show that the degradation of the long form of Glr1 in the cytosol is faster than that of the true cytosolic protein. On the basis of the k values, the half-lives ($t_{1/2} = \ln 2/k$) of the true cytosolic and the long mitochondrial Glr1 in the cytosol were estimated as 5.0 and 3.9 h, respectively.

The rate constant of degradation was also calculated for the mitochondrial peptide, P2, and the mean value of k was 0.39 h^{-1} was obtained. Assuming the same dilution rate as before, the rate of degradation can be estimated as 0.33 h^{-1} and the half-life was calculated to be 2.1 h.

DISCUSSION

We have shown that in wild-type *S. cerevisiae*, cytosolic glutathione reductase is preferentially translated from the second AUG codon and processed by loss of methionine and acetylation of the resulting N-terminal serine. It has been reported that in *S. cerevisiae* mRNA, a strong context contains A in the position −3 and U in the position +4 relative to the AUG start codon.[20] The first AUG codon is in an unfavorable context for translation, since it contains U at −3 position. On the other hand, an A in the position −3 of the second in-frame AUG start codon confers a strong context.

In order to carry out measurements of turnover, it was necessary (because of the low abundance of the mitochondrial form) to overproduce glutathione reductase, and a GLR1-overexpressing strain Y258 (in which Protein A is fused at the C-terminus and expression is under control of a *GAL1* promoter) was used for further experiments. While the downstream context of the first AUG is, of course, unchanged from wild-type, the upstream context before the first AUG codon is now different, with an A in position −3 conferring a strong context at this position. Although the short form of the protein was still abundant, it was now also possible to detect the long form of glutathione reductase in the cytosol. The N-terminus was the anticipated methionine and was approximately 90% acetylated. We conclude that, when the GLR1-overexpressing strain Y258 is used, import of Glr1 to the mitochondria is rate limiting and the unprocessed protein is able to accumulate in the cytosol. It is not clear whether the methionine acetylation has biological significance (for example to mark the protein for rapid destruction—see turnover measurements below) or whether it is completely non-specific and without purpose

The use of the GLR1-overexpressing strain Y258 also allowed us to isolate and analyze Glr1 from the mitochondria. Although the entire mitochondrial Glr1 fraction was (as expected) truncated by removal of the signaling sequence, mitochondrial glutathione reductase had three different N-termini. The predicted sequence of mitochondrial glutathione reductase begins MSTNTK. We also found protein in which the N-terminal methionine was missing and protein which began TK (i.e., four residues had been removed from the N-terminus). None of these N-termini is acetylated. We have not quantified the three mitochondrial N-terminal peptides relative to one another, but the intensity of the signal due to the shortest peptide is about a third of the signals of each of the other two. The removal of a single residue from the N-terminus of a mitochondrial protein has previously been observed,[28,29] and glutathione reductase was one of a number of proteins shown to exhibit this behavior. However, we know of no example of the removal of four residues. We note that mass spectrometry has seldom been specifically employed to analyze the N-termini of mitochondrial proteins and that surprises are possible if such analysis is performed. An analysis of the mass spectra of cytosolic Glr1 in "enzyme-free" mode revealed no unexpected N-termini.

Others have reported[38] that acetic anhydride treatment of proteins results in acetylation at both lysine side chains and the N-terminus, if it is not modified in vivo. Under our experimental conditions, we observed no acetylation of the N-terminal protein residues, in agreement with Morten et al.[39]

Although the N-terminal presequence is necessary for Glr1 import into the mitochondria, it is well established that it is normally removed during or after import by specific mitochondrial proteases. Some presequence peptidases have been reported in the literature including the mitochondrial processing peptidase (MPP), mitochondrial intermediate peptidase (Oct1), and the inner membrane peptidase (Imp). The first of these is located in the mitochondrial matrix. However, its specificity is not clear yet, and it may be that it cleaves in a somewhat random way. Oct1 has been reported to have a role in processing several presequences by removal of characteristic octapeptides.[40] Hydrophobic regions of some proteins facing the inner membrane can be cleaved by the Imp peptidase which is, in fact, composed of two catalytic subunits, Imp1 and Imp2.[41] In addition, the m-AAA protease (ATPases associated with a number of cellular activities) and the rhomboid protease, Pcp1, of the inner membrane have been shown to cleave presequence from a few preproteins.[42] An intermediate cleavage peptidase (Icp55) has been recently characterized and is involved in removal of an amino acid from a characteristic set of MPP-generated N-termini.[43,44] We believe that the identification of three different N-termini for the same protein is a new finding that arises from the precision that mass spectrometry brings to this type of study. The explanation most immediately consistent with current understanding is generation of Glr1 with N-terminal MSTNTKHYDYLVIGGGSGGVASAR and TKHYDYLVIGGGSGGVASAR by direct action of MPP, with Icp55 removing N-terminal methionine from the longer protein to yield the protein with N-terminal STNTKHYDYLVIGGGSGGVASAR.

Glr1 turnover rates in both compartments were established using mass spectrometric techniques combined with a SILAC approach.[35] The mitochondrial forms of glutathione reductase (unfortunately the three forms could not be distinguished in these experiments) degrade faster than the two cytosolic forms. The two cytosolic Glr1 proteins also show different turnover rates, with the long form (produced from the first AUG) degrading faster than the short counterpart produced using the second AUG.

In both the cytosolic and mitochondrial compartments, Glr1 is involved in the reduction of GSSG. Mitochondria are the principal source of reactive oxygen species, and GSH is a scavenger, neutralizing their harmful effects. Higher activity of Glr1 is expected in the mitochondrial matrix relative to activity of the cytosol, as a consequence of the more oxidized environment and higher rate of ROS formation in this compartment.[45,46] Relatively rapid turnover of the mitochondrial Glr1 compared with that of its cytosolic counterpart is

consistent both with its function and its structure. The more oxidizing environment of the mitochondria leads us to expect that mitochondrial Glr1 will catalyze more events in a given time than the cytosolic form, increasing the risk of oxidative damage. In addition, it is reported that N-terminal acetylation of yeast proteins can have a stabilizing effect; therefore, mitochondrial Glr1 is likely to be more susceptible to degradation than the cytosol isoform.[28,29]

A comparison of the turnover rates of Glr1 in the two compartments gives surprising data about the synthesis rates from the two start codons. In the GLR1-overexpressing strain Y258, the upstream context of the first AUG has been rendered favorable; however, "leaky scanning" is still a very significant process leading to accumulation of the short, cytosolic glutathione reductase. The amounts of the two forms in the cytosol are not very different; the apparent turnover rate of the common peptides suggests that the short form is about 3 times more abundant. The turnover rates of the two forms are significantly different but by less than a factor of 1.5, suggesting that (in the overproducing strain) the two AUG start codons are used approximately equally (perhaps by a factor of 2 in favor of the second codon) and that the unfavorable downstream context of the first AUG is highly significant, leading to extensive "leaky scanning".

The turnover of mitochondrial Glr1 is (only) 2−3 times faster that of the cytosolic counterpart. Thus, extrapolating back to the wild-type strain (in which the long mitochondrial protein does not accumulate in the cytosol, so the cytosolic protein is 20 times more abundant than the mitochondrial protein), we may infer that the second AUG is responsible for the synthesis of around 90% of Glr1. Although "leaky scanning" has been well-known for many years, these experiments allow us to appreciate its potential extent. A poor context, as found for the first AUG of Glr1, can result in a second in-frame AUG being very strongly favored.

■ CONCLUSION

Protein turnover studies are scarce and have been made with highly abundant proteins which are relatively stable in the cell. Protein turnover measurements of low abundance proteins distributed in two different compartments of the cell have never previously been reported. This study demonstrates the potential of mass spectrometry as a sensitive and selective tool for understanding the structures of proteins in both spatial and temporal domains.

■ ASSOCIATED CONTENT

Supporting Information

Media preparations as well as the growth conditions, description of Bradford, SDS-PAGE, and Western-blot assays, LC−ESI−MS/MS, MS/MS, spectra, SDS-PAGE of the cytosolic fraction, identified Glr1-derived peptides, MS/MS spectra obtained using CID. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: jill.barber@manchester.ac.uk. Tel: 44-161-275-2369. Fax: 44-161-275-2396.

Present Addresses

[‡]ChELSI Institute, Chemical and Process Engineering, University of Sheffield, Mappin Street, Sheffield S1 3JD, United Kingdom.

[∥]School of Life Sciences, University of Warwick, Coventry CV4 7AL, United Kingdom.

[§]Queen Mary University of London, London E1 4NS, United Kingdom.

Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

Glr1, glutathione reductase; GLR1, glutathione reductase gene; GSH, reduced glutathione; GSSG, oxidized glutathione; ROS, reactive oxygen species; IgG, immunoglobulin; NADP, nicotinamide adenine dinucleotide phosphate; MPP, mitochondrial processing peptidase; Imp, inner membrane protease; Oct1, mitochondrial intermediate peptidase; Icp55, Intermediate cleaving peptidase 55; Pcp1, rhomboid protein 1; m-AAA, mitochondrial ATPases associated with a number of cellular activities; MALDI, matrix-assisted laser desorption/ionization; ESI, electrospray ionization; MS, mass spectrometry; MS/MS, tandem mass spectrometry; SILAC, stable isotope labeling with amino acids in cell culture; Ac, acetyl group

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