The Glutathione Synthetase of *Schizosaccharomyces pombe* Is Synthesized as a Homodimer but Retains Full Activity When Present as a Heterotetramer*

Glutathione synthetase was overexpressed as a histidine-tagged protein in *Schizosaccharomyces pombe* and purified by two-step affinity chromatography. The recovered enzyme occurred in two different forms: a homodimeric protein consisting of two identical 56-kDa subunits and a heterotetrameric protein composed of two 32-kDa and two 24-kDa subfragments. Both forms are encoded by the GSH2 gene. The 56-Da protein corresponds to the complete GSH2 open reading frame, while the subfragments are produced following the cleavage of this larger protein by a metalloprotease. A stable homodimer was obtained by site-directed mutagenesis to remove the protease cleavage site, and this showed normal activity. A structural model of the fission yeast glutathione synthetase was produced, based on the x-ray coordinates of the human enzyme. According to this model the interacting domains of the proteolytic subfragments are strongly entangled. The subfragments were therefore coexpressed as independent proteins. These subfragments assembled correctly to yield functional heterotetramers with equivalent activity to the wild type enzyme. Furthermore, a permutated version of the protein was created. This also showed normal levels of glutathione synthetase activity. These data provide novel insight into the mechanisms of protein folding and the structure and evolution of the glutathione synthetase family.

Glutathione (GSH,\(^1\) γ-Glu-Cys-Gly) is a biologically important thiol found in almost all procaryotic and eucaryotic cells. It has been assigned several cellular functions, including protection against oxidative damage, maintenance of a reducing cellular thiol-disulfide balance, electron donation for a number of enzymes, protection of protein sulfhydryls from irreversible oxidation, and detoxification of foreign compounds (1–3). Glutathione is synthesized enzymatically from its constituent amino acids in two consecutive reactions. Glutathione synthetase (EC 6.3.2.3) catalyzes the second step, the addition of reduced GSH to L-glutamylcysteine.

In most eucaryotic organisms, including *Saccharomyces cerevisiae*, *Arabidopsis thaliana*, *Homo sapiens*, and *Rattus norvegicus*, glutathione synthetase is a 104–112-kDa homodimer composed of two identical 52–56-kDa subunits (4–10). In contrast, the structure of the *Schizosaccharomyces pombe* enzyme has not been completely elucidated. The enzyme was purified as a 120-kDa heterotetramer consisting of two kinds of subunits, called the “small” and “large” subunits, with apparent molecular masses of 26 and 33 kDa (11). While the gene encoding the 26-kDa subunit was unknown, the gene encoding the 33-kDa subunit, GSH2, had been cloned, and the coding region had been assigned to the 3′ end (12). When the GSH2 gene was re-sequenced at a later date, however, frameshift errors in the original sequence were identified (13, 14). These errors resulted in a shortened open reading frame, downstream from the correct start codon. A protein derived from the full-length GSH2 gene would have a molecular mass of 56 kDa, and BLAST searches showed that this protein is homologous to the glutathione synthetase proteins of other eucaryotic organisms. Wang and Oliver (13) proposed that the 56-kDa protein was the primary translation product, from which the subunits could be derived by proteolysis.

Here we report the homologous expression and purification of a histidine-tagged form of the fission yeast glutathione synthetase. We show that a 56-kDa protein corresponding to the whole open reading frame is indeed formed. Proteolytic cleavage then yields the small subunit, the 24-kDa N-terminal fragment, and the large subunit, the 32-kDa C-terminal fragment of this protein. Moreover, a model of the tertiary and quaternary structure of the fission yeast glutathione synthetase was generated, based on the structure of the human ortholog. On the basis of these results, in *vitro* mutagenesis experiments were performed to shed light on the subunit structure of the enzyme. The mutated recombinant proteins were expressed and affinity purified; in *vivo* as well as *in vitro* activities were determined. Our experiments provided novel data relating to folding and evolution of glutathione synthetase.

**EXPERIMENTAL PROCEDURES**

**Materials**—The chemicals used were of molecular biology grade or pro analysis grade.

**DNA Methods**—Standard molecular biology techniques were used for DNA isolation, analysis, and cloning (15, 16). The identity of all clones was tested by DNA sequencing, which was carried out by SequiServe (Vaterstetten, Germany) following the method of Sanger.

**Histidine Tagging of the Gene and Plasmid Construction**—Table I shows the oligonucleotides (part A) and the amplified fragments and
In part A the oligonucleotides, their sequences and restriction sites are indicated. Part B provides an overview of the cloning experiments performed, i.e. the amplified fragments of the GSH2 gene, the oligonucleotides used for PCR, the vectors in which the fragments were inserted and the restriction sites used. Part C shows the resulting vectors used for expression in the fission yeast. ORF, open reading frame.

### Table I

| A. Oligonucleotide | Sequence | Restriction site |
|--------------------|----------|-----------------|
| GSH2-Fwd           | 5\'–AAA GGA TCC ATT GAA ATT GAG AAT TAC ACA CAG GAG-3\' | BamHI, Smal |
| GSH2-Rev           | 5\'–AAA CCC GGG TTA ATG ATT ATG ATG TTG AAC AGA TTC ACT ACT AG-3\' | BamHI, Smal |
| GSH2M214F          | 5\'–AAA GGA TCC ATT GAA ATT GAG AAT TAC ACA CAG GAG-3\' | BamHI, Smal |
| CWGLU4A            | 5\'–AAA GAA TTC TCA AAT ACA TGT ATA ATT TT-3\' | EcoRI |
| GSH2Irev           | 5\'–AAA GGT ACC GTA GAC TAC AGC ATT-3\' | KpnI |
| GSH2IIFwd          | 5\'–AAA GGT ACC GAT AAC AGC AAA CCC ATT-3\' | KpnI |
| CW8DDA             | 5\'–AAA GGA TCC AAT ATC TTC TTT GAG GCA-3\' | BamHI |
| PermutGr           | 5\'–AAA GGT ACC TTC GGA AGG TAC ACT AG-3\' | KpnI |
| PermutKr           | 5\'–AAA GGT ACC GAT AAT GAG TAA ACA C-3\' | EcoRI, Smal |
| PNMTIF             | 5\'–AAA GAG CTG GCC ATA AAA GAC AGA ATG CAT C-3\' | |

| B. Oligonucleotides | Fragment amplified by PCR | Restriction site | Vector |
|--------------------|--------------------------|-----------------|--------|
| GSH2-Fwd           | 1533-bp fragment of S. pombe GSH2 ORF with C-terminal. | BamHI, Smal, Uc9, |
| GSH2-Rev           | HIS-tag                  | Smal, Uc9, PEP2 |
| GSH2M214F          | 884-bp fragment of large subunit of S. pombe GSH2 ORF | BamHI, Smal, PEP2 |
| GSH2-Rev           | With C-terminal HIS-tag | Smal, Uc9, PEP2 |
| 6H-GSH2F           | 678-bp fragment of small subunit of S. pombe GSH2 ORF | BamHI, Smal, Uc9 |
| GSH2K123R          | With N-terminal HIS-tag | Smal, Uc9, PEP2 |
| CWGLU4A            | 980-bp region upstream of the region to delete in S. pombe | EcoRI, Smal |
| GSH2II              | GSH2 ORF                 | KpnI, Smal, Uc9 |
| GSH2II Fwd         | 1142-bp region downstream of the region to delete in | KpnI, Smal, Uc9 |
| CW8DDA             | S. pombe GSH2 ORF        | BamHI, Smal, Uc9 |
| GSH2-Fwd           | Addition of C-terminal HIS-tag to fragment with deletion of | BamHI, Smal, Uc9 |
| GSH2-Rev           | 15 amino acids of S. pombe GSH2 ORF resulting in 1494-bp fragment | Smal, PEP2 |
| GSH2M214F          | 873-bp fragment of large subunit of S. pombe GSH2 ORF | BamHI, Smal, Uc9 |
| PermutGr           | Without STOP codon (for permutation experiment) | KpnI, Smal, Uc9 |
| PermutKr           | 678-bp fragment of small subunit of S. pombe GSH2 ORF | KpnI, Smal, Uc9 |
| PermutKr           | With N-terminal linker (amino acids PEG) and C-terminal HIS-tag (for permutation experiment) | EcoRI, Smal, Uc9 |
| GSH2M214F          | 1560-bp fragment of permuted S. pombe GSH2 ORF with C- | BamHI, PEP2, Smal |

| C. Plasmid | Vector | Expressed protein | HIS-tag |
|------------|--------|------------------|---------|
| pREP2-gsh2.hft | pREP2 | Full-length S. pombe glutathione synthetase (498 amino acids) | C-terminal |
| pREP2-gsh2-D15.hft | pREP2 | S. pombe glutathione synthetase with deletion of amino acid residues 204–218 | C-terminal |
| pREP1-KUgsh2 | pREP1 | Amino acid residues 1–213 of S. pombe glutathione synthetase ("small subunit") | N-terminal |
| pREP2-GUgsh2 | pREP2 | Amino acid residues 214–498 of S. pombe glutathione synthetase ("large subunit"); 24-kDa subfragment | C-terminal |
| pREP2-2g-SH2-perm | pREP2 | Permuted S. pombe glutathione synthetase consisting of amino acid residues 214–498 followed by a 5-amino acid linker (GTPSG0) and the amino acid residues 1–213 | C-terminal |

The fission yeast strain 48leu4/2 (ΔGSH2, geneticin', ura', leu') was generated as a segregant of the strains 46a14h (ΔGSH2, geneticin', ura') and leu1–232 (leu'). This strain was transformed with the plasmids pREP2-GUgsh2.hft (Table I, part C) and pREP1-KUgsh2 (Table I, part C) to express the regions of the putative large and small subunit of glutathione synthetase separately and with plasmid pREP2-2g-SH2-perm (Table I, part C) to express the permuted enzyme.

Cells were grown at 28°C in Edinburgh minimal medium (18). Microbiological Methods—Standard techniques were applied for yeast transformation, crosses, the plasmid loss test, and other microbiological methods (18, 19).

**Physiological Tests**—For heavy metal tolerance tests, the minimal inhibitory concentration of cadmium was determined using Edinburgh minimal medium containing different concentrations of CdCl. GSH and phytochelatin concentrations were determined by BP-HPLC as described previously (20). Phytochelatin standards were purchased from AppliChem (Darmstadt, Germany).

Protein Expression and Purification—Cells were grown in 3 × 500 ml minimal medium with gentle shaking at 28°C for 24 h, harvested by centrifugation (10 min, 1500 × g, SS34 rotor, 4°C), washed twice with distilled water, and lyophilized. Cell disruption was performed in a cell mill using glass beads (3 × 3 min dry; 6 × 3 min with 10 ml of immobilized metal ion affinity chromatography (IMAC) binding buffer of lyophilized cells). After centrifugation (30 min, 1500 × g, SS34 rotor, 4°C) the supernatant was used for the following purification.
procedure, with all steps carried out at 4 °C. For IMAC chelating Sepharose fast flow was loaded with nickel as indicated by the manufacturer (Amersham Biosciences). After applying the cell extract to the column and washing with IMAC binding buffer (20 mM Na2HPO4/NaH2PO4, 500 mM NaCl, 10 mM imidazole, pH 7.4), the bound proteins were eluted by increasing the imidazole concentration stepwise to 50 mM and then to 100 mM. Glutathione synthetase eluted at an imidazole concentration of 100 mM. For reversible dye affinity chromatography, the sample was adjusted to the appropriate buffer (10 mM Tris-HCl, 20 mM NaCl, pH 7.8) by Sephadex G-25 gel filtration chromatography and applied to the column packed with Cibacron blue 3GA-agarose. After a 10-mL wash, elution was carried out by increasing the NaCl concentration to 250 mM.

Assay for Glutathione Synthetase—Purified glutathione synthetase was equilibrated to 100 mM Tris-HCl buffer, pH 8.2, by Sephadex G-25 gel filtration chromatography. Glutathione synthetase activity was assayed according to Huang et al. (10) using γ-glutamyl cysteine trifluoroacetate salt (Sigma) as a substrate. One unit of enzyme is defined as the amount that catalyzes the formation of 1 μmol of product/min. Protein concentrations were determined according to Bradford (21) using bovine serum albumin as a standard.

Protease Inhibition Experiments—For protease inhibition experiments the strain 46a14h pREP2-gsh2:hft expressing the histidine-tagged wild type glutathione synthetase was used. Cell disruption was performed in the presence of different protease inhibitors at the following concentrations (aprotinin, 50 μg/mL; benzamidine HCl, 10 mM; EDTA-Na2, 10 mM; E-64, 5 μg/mL; leupeptin-hemisulfate, 5 μg/mL; pepstatin A, 7 μg/mL; PMSF, 0.5 μM). After cell disruption, the cell extracts were incubated at 4 °C. Samples were taken immediately and after 1, 5, and 21 h of incubation. Histidine-tagged glutathione synthetase was detected by immunoblot assay after SDS-PAGE and Western blot.

SDS-PAGE, Western Blot, and Immunodetection of the Histidine Tag—SDS-PAGE was performed following the method of Laemmli (22) using the M12 molecular mass standard (Novex, San Diego, CA). For Western blotting the proteins were electrotransferred, after SDS-PAGE, to a PVDF membrane in CAPS buffer (10 mM CAPS, pH 11.0, 20% methanol) at 870 mA for 2 h. The prestained marker 7B (Sigma) served as a molecular mass standard. Proteins containing a histidine tag were localized on the PVDF membrane by applying an (Sigma) laser was used. Pulses of 3 ns at 337 nm were used at a voltage of 20 kV.

Native PAGE analysis of different forms of the fission yeast glutathione synthetase after purification. The lanes show: the molecular mass standard M12 with bands of 116, 97, 66, 55, 37, and 22 kDa (lane 1); the wild type enzyme immediately after purification (lane 2); the wild type enzyme after complete cleavage of the 56-kDa protein, producing the 24- and 32-kDa subfragments (lane 3); the enzyme after deletion of residues 204–218 (lane 4); and the enzyme composed of separately encoded 24- and 32-kDa subfragments (lane 5).

FIG. 1. SDS-PAGE analysis of the different engineered forms of the fission yeast glutathione synthetase after purification.

The alignment of the amino acid sequences from S. pombe, S. cerevisiae, Fichia angusta, A. italiana, and H. sapiens was performed with the GUC package (Aceslrys Inc., San Diego, CA). As the crystal structure of the fission yeast glutathione synthetase has not yet been determined, homology modeling was used to create a structural model. The crystal structure of the human glutathione synthetase (24) was used as the template. The model was generated using the Modeller program under the homology module of Insight II. To simplify the procedure, the full-length 56-kDa form of the S. pombe monomer was analyzed. The template structure of the human protein was adopted for continuously aligned sequence stretches between the human and the S. pombe proteins. Sequence insertions and deletions were only allowed outside secondary structure elements. Insertions larger than three amino acids were modeled in an extended conformation.

RESULTS

Cloning and Expression of the Fission Yeast Glutathione Synthetase—An 18-bp sequence encoding six histidine residues was attached to the C terminus of glutathione synthetase by PCR. The protein was expressed in a ΔGSH2 fission yeast strain under control of the nmt1 promoter using the expression vector pREP2 (25). The presence of the plasmid was confirmed by PCR. Plasmid loss tests were performed as a control.

Purification of the Fission Yeast Glutathione Synthetase and Subunit Structure of the Enzyme—The histidine-tagged glutathione synthetase was purified to apparent homogeneity in a two-step affinity chromatography procedure. In the first step (IMAC), glutathione synthetase coeluted with other contami-
nating proteins. These were removed in the second step by reactive dye affinity chromatography (Fig. 1).

The purified enzyme produced a single band in a native PAGE gel (Fig. 2). The gel did not allow the accurate determination of the molecular mass. In gel filtration chromatography one protein peak was observed. The observed elution volume was slightly lower than that of *S. cerevisiae* alcohol dehydrogenase (141 kDa), the protein used for calibration (data not shown). These results are consistent with the value of 120 kDa reported for the molecular mass of the fission yeast glutathione synthetase by Nakagawa et al. (11). In SDS-PAGE a strong band of about 56 kDa and two faint bands of about 32 and 24 kDa were visible immediately after purification (Fig. 1). The 56- and 32-kDa proteins contained the C-terminal His6-tag, which was identified by Western blot and immunodetection using a monoclonal anti-His6 antibody (data not shown). Upon prolonged storage of the protein (10 mM Tris-HCl, pH 7.8, 20 mM NaCl, 4 °C); however, the 56-kDa band faded, while the other two bands became more intense. The 56-kDa protein was completely cleaved to the 32- and 24-kDa proteins when partially purified by IMAC and kept at 4 °C for 4 weeks (Fig. 1).

However, the 56-kDa protein was still present in fractions of the protein purified by IMAC and reactive dye affinity chromatography in which no contaminating proteins were detectable by SDS-PAGE after storage for more than 6 months under the conditions given above (data not shown).

The N-terminal sequence of the 32-kDa protein was identified as "SDNTKPIVLF" by Edman degradation. This truncated protein starts with the serine residue at position 218 and not with the methionine residue at position 214, as previously assumed (12). The 56-kDa protein and the 24-kDa protein were N-terminally blocked.

**Tryptic Digestion and Peptide Analysis of the 24-kDa Protein**—Because the N terminus of the 24-kDa protein was blocked, this protein was further investigated by analysis of the fragments obtained after tryptic digestion by MALDI-TOF-MS. Molecular masses between 400.0 and 2900.0 Da could be detected by the parameter file employed for MALDI-TOF-MS. Nineteen fragments theoretically arising from tryptic digestion of the N-terminal region (residues 1–218) of the 56-kDa protein were within this molecular mass range. Eleven of these fragments were recorded (Table II).

![FIG. 3. Schematic representation of the fission yeast GSH2 gene. The regions of the small and large subunit are colored gray and white, respectively, while the C-terminal His6-tag (H) added is shown in black. The area occurring only in the fission yeast enzyme is marked by a hatched box. Several important amino acids are marked by arrows (M 1, the N-terminal amino acid; Met284 (M 214), the previously assumed N terminus of the large subunit; Arg266 (A 204), and Ser216 (S 218), residues flanking the 15-amino acid sequence unique to the *S. pombe* enzyme; and Glu250 (E 498), the C-terminal amino acid).](image)

| Mass (experimental) | Position | Peptide sequence | Mass (calculated) | Deviation |
|---------------------|----------|------------------|-------------------|-----------|
| Da                  | %        |
| 939.6               | 85-92    | LHLQSIK         | 939.5             | 0.011     |
| 946.4               | 93-99    | YDEFMNK         | 946.3             | 0.011     |
| 964.5               | 100-106  | LWNLYQK         | 964.4             | 0.010     |
| 1140.6              | 76-84    | IANDYEFKL       | 1140.5            | 0.009     |
| 1306.7              | 6-16     | YTEPQIEELGK     | 1306.4            | 0.023     |
| 1534.8              | 116-128  | ENQPFPLSLGVFR   | 1534.5            | 0.020     |
| 1590.5              | 6-19     | YTEPQIEELGK-GAR | 1590.5            | -0.019    |
| 1696.9              | 144-159  | QEVTNISVPFGVSXK | 1696.5            | 0.024     |
| 1768.8              | 160-175  | AVSNLHAYCSSLGVR | 1768.6            | 0.011     |
| 1853.7              | 20-36    | DFAFAHGVFTELSVK | 1853.7            | 0.011     |
| 2196.8              | 20-39    | DFAFAHGVFTELSVK-EGR | 2196.1 | 0.002     |

* a Fragments detected as larger peptides (due to one miscleavage).

The purified enzyme produced a single band in a native PAGE gel (Fig. 2). The gel did not allow the accurate determination of the molecular mass. In gel filtration chromatography one protein peak was observed. The observed elution volume was slightly lower than that of *S. cerevisiae* alcohol dehydrogenase (141 kDa), the protein used for calibration (data not shown). These results are consistent with the value of 120 kDa reported for the molecular mass of the fission yeast glutathione synthetase by Nakagawa et al. (11). In SDS-PAGE a strong band of about 56 kDa and two faint bands of about 32 and 24 kDa were visible immediately after purification (Fig. 1). The 56- and 32-kDa proteins contained the C-terminal His6-tag, which was identified by Western blot and immunodetection using a monoclonal anti-His6 antibody (data not shown). Upon prolonged storage of the protein (10 mM Tris-HCl, pH 7.8, 20 mM NaCl, 4 °C); however, the 56-kDa band faded, while the other two bands became more intense. The 56-kDa protein was completely cleaved to the 32- and 24-kDa proteins when partially purified by IMAC and kept at 4 °C for 4 weeks (Fig. 1).

However, the 56-kDa protein was still present in fractions of the protein purified by IMAC and reactive dye affinity chromatography in which no contaminating proteins were detectable by SDS-PAGE after storage for more than 6 months under the conditions given above (data not shown).

The N-terminal sequence of the 32-kDa protein was identified as “SDNTKPIVLF” by Edman degradation. This truncated protein starts with the serine residue at position 218 and not with the methionine residue at position 214, as previously assumed (12). The 56-kDa protein and the 24-kDa protein were N-terminally blocked.

**Tryptic Digestion and Peptide Analysis of the 24-kDa Protein**—Because the N terminus of the 24-kDa protein was blocked, this protein was further investigated by analysis of the fragments obtained after tryptic digestion by MALDI-TOF-MS. Molecular masses between 400.0 and 2900.0 Da could be detected by the parameter file employed for MALDI-TOF-MS. Nineteen fragments theoretically arising from tryptic digestion of the N-terminal region (residues 1-218) of the 56-kDa protein were within this molecular mass range. Eleven of these fragments were recorded (Table II).

The 1140.5-Da fragment (residues 76-84) was sequenced and the expected sequence “IANDYEFKL” was found. Thus, MALDI-TOF-MS analysis of the tryptic peptides derived from the 24-kDa subfragment provided further evidence that this protein was derived from the 5‘ region of the GSH2 gene. (Fig. 3) shows a schematic diagram of the regions of the GSH2 gene.
Amino acid sequence alignment of the glutathione synthetase proteins from *H. sapiens* (*Hs*), *A. thaliana* (*At*), *S. cerevisiae* (*Sc*), *P. angusta* (*Pa*), and *S. pombe* (*Sp*). In accordance to Polekhina et al. (24) the following coloring was chosen for the domains of the ATP-grasp superfamily: red, N-terminal domain; orange, linker domain to the lid region; green, lid domain; blue, C-terminal domain. The secondary
amino acid residues 204–218, which do not appear in other eucaryotic glutathione synthetases, are marked in yellow. Regions of the enzyme without homology to the human protein are colored green. The N and C termini (Met¹ (M 1) and Glu¹⁹⁸ (E 498)) of the protein are marked (N-ter, C-ter).

responding to amino acid residues 1–203 and 219–498 are colored in red and blue, respectively. The 15 amino acids missing in the human enzyme (residues 204–218) are colored in yellow. The predicted small and large subunits correspond to amino acids residues 1–217 and 218–498, respectively. Four additional small insertions and a single amino acid deletion are located in the vicinity of the large 15-amino acid insertion.

With the exception of the allocations discussed above, the modeled structure of the fission yeast glutathione synthetase is similar to the crystallographic structure of the human enzyme. The amino acids essential for glutathione, cofactor, and Mg²⁺ binding in the human enzyme have been determined (24). In the modeled structure of the fission yeast enzyme a similar, or identical to the crystallographic structure of the human enzyme. The superimposition was based on the substrate molecules observed in both structures, namely the two magnesium ions, the two phosphates of the bound ADP, as well as the sulfur atom of the sulfate replacing one phosphate. The E. coli enzyme is colored green. The human enzyme is colored red and blue, with red representing the 24-kDa N-terminal subfragment, and blue representing the 32-kDa C-terminal subfragment of the fission yeast enzyme. The 15 amino acids (204–218) that appear only in the fission yeast glutathione synthetase are indicated by the large yellow loop. The N and C termini of both the human and E. coli enzymes are indicated by green and red/blue spheres, respectively.

amplifying the upstream and downstream regions of the GSH2 gene and adding a His₆-tag sequence to the 3' end of the downstream fragment. The two fragments were combined and subcloned in the expression vector pREP2. Two additional amino acid residues (G and T) were inserted due to the incorporation of the KpnI restriction site used for cloning. The mutated protein was expressed in a ΔGSH2 fission yeast strain, isolated, and purified to apparent homogeneity by IMAC and reactive dye affinity chromatography. In SDS-PAGE (Fig. 1) and Western blot experiments, only the 56-kDa protein was detected. In native PAGE experiments, one band approximately equivalent in size to the wild type enzyme was identified (Fig. 2), indicating that this enzyme is a homodimer composed of two 56-kDa subunits. The 56-kDa protein was very stable as no cleavage was observed upon storage for more than six months in a buffer containing 10 mM Tris-HCl, pH 7.8, 20 mM NaCl at 4 °C.

Molecular Masses of the Subunits and Localization of the Protease Cleavage Site—The increased stability of the protein following deletion of amino acid residues 204–218 suggested that this region contains a protease cleavage site. N-terminal sequencing revealed that the 32-kDa protein starts with the serine residue at position 218 (Fig. 3). MALDI-TOF-MS confirmed the molecular masses of the 56-, 32-, and 24-kDa proteins to be 56.981 kDa (His₆-tagged), 32.630 kDa (His₆-tagged), and 24.352 kDa, respectively (Table III). The difference between the calculated masses and the experimentally determined masses is consistent with the accuracy of MALDI-TOF mass spectrometry of proteins in this size range. Taking this into consideration, the MALDI-TOF data support the protein sequencing data, showing that the cleavage takes place between the amino acid residues Ala and Ser at positions 217 and 218.
Identification of the Protease That Cleaves the 56-kDa Protein—Cell disruption of strain 46a14h-pREP2-gsh2:hft was performed in the presence of different protease inhibitors, and glutathione synthetase was detected by SDS-PAGE, Western blot, and immunoassay for the histidine tag. Strain D18h− served as a negative control. This strain does not contain any proteins detectable by immunoassay using the anti-His6 antibody, and no signal was observed. In all the samples of strain 46a14h-pREP2-gsh2:hft, the intensity of the 56-kDa band decreased, while the intensity of the 32-kDa band increased in a time-dependent manner (Fig. 7). The 24-kDa band could not be detected using the anti-His6 antibody, because the tag was attached to the C terminus of the 56-kDa protein.

The addition of EDTA-Na2 strongly inhibited the cleavage of the 56-kDa protein (Fig. 7). The other protease inhibitors tested (aprotinin, benzamidine HCl, E-64, leupeptin-hemisulfate, pepstatin A, and PMSF) had no inhibitory effect on the cleavage of the 56-kDa protein at that position (data not shown). Therefore, we suggest that a metalloprotease is responsible for cleaving the 56-kDa protein yielding the 32-kDa and 24-kDa subfragments.

In addition to the 56- and 32-kDa bands, one more band of about 25 kDa was detected (Fig. 7). This represents a C-terminal fragment of glutathione synthetase, since the histidine tag is located at the C terminus of the protein. This additional product might arise through the digestion of glutathione synthetase by another protease present in the cell extract. The cleavage was inhibited by benzamidine HCl and PMSF, which suggests that a serine protease might be involved (data not shown). The protease is effectively separated from glutathione synthetase by the purification procedure as the additional degradation product is not observed when glutathione synthetase is purified by IMAC immediately after cell disruption.

Independent Expression of the 24- and 32-kDa Enzyme Subfragments—The coding regions corresponding to the 24- and 32-kDa subfragments of the fission yeast glutathione synthetase were cloned and expressed separately. Fragments of the subunits were amplified, and a His6-tag encoding sequence was attached to the N terminus of the 24-kDa subfragment (residues 1–213) and to the C terminus of the large subfragment (residues 214–498) by PCR. The two proteins were coexpressed in the ΔGSH2 fission yeast strain 46elu42 using the vectors shown in (Fig. 8). The protein was isolated and purified as stated above for the other forms of the enzyme. In SDS-PAGE experiments, the 24- and 32-kDa proteins were visible (Fig. 1); both proteins produced signals in Western blot and immuno-detection assays. Native PAGE showed one band equivalent in size to the wild type enzyme (Fig. 2). Therefore, the enzyme produced by coexpressing the small and large subfragments must be a heterotetramer of two 24-kDa and two 32-kDa proteins.

Permutation of S. pombe Glutathione Synthetase—According to the structural model generated for the S. pombe glutathione synthetase, the N and C termini of the protein are close to each other (Fig. 5). To analyze the calculated structure experimentally, these termini were linked. The protein was then cleaved at a different position, on the N-terminal side of amino acid residue 214, between the 24- and 32-kDa subfragments, to generate new termini. As a result, the fission yeast glutathione synthetase was artificially permuted by interchanging the positions of the subfragments within the protein. Five additional amino acid residues (GTPSG) were introduced between the two subfragments due to the oligonucleotides used for amplification and subcloning.

In Vivo Activities—S. pombe cannot grow without glutathione (27). To investigate whether the three different forms of glutathione synthetase are active in vivo, the proteins were expressed in a fission yeast ΔGSH2 strain. All the forms of the protein, i.e. the wild type glutathione synthetase, the recombinant protein with the 15-amino acid deletion, the protein comprising separately encoded and coexpressed 24- and 32-kDa subfragments, and the permuted protein, restored growth of the ΔGSH2 strain on minimal medium without glutathione (Fig. 10). As demonstrated by RP-HPLC measurements, all strains produced glutathione and, upon induction with cadmium, phytochelatins. The glutathione contents of all strains were similar to that of the wild type (Table IV).
strains expressing a functional glutathione synthetase (46a14h/H11002) growth of the with 10 mg/liter GSH was inhibited when only 10/H9262 glutathione.

The regions of the 32- and 24-kDa subfragments are colored white and gray, respectively, while the C-terminal His6-tag (H) is shown in black. They are separated by a five amino acid linker (GTPSG), also colored black. Some important amino acids are indicated by arrows (Met214 (M 214), the N-terminal amino acid of the 32-kDa subfragment, and the permuted protein; Glu498 (E 498), the last amino acid of the 32-kDa subfragment; and Lys213 (K 213), the C-terminal amino acid of both the 24-kDa subfragment and the full-length protein).

Moreover, all recombinant yeast strains expressing the different glutathione synthetase mutants showed heavy metal tolerances similar to the wild type. They all grew on minimal medium containing up to 750 μM CdCl2. On the contrary, growth of the ΔGSH2 strain on minimal medium supplemented with 10 mg/liter GSH was inhibited when only 10 μM CdCl2 was added.

In Vitro Glutathione Synthetase Activities—The wild type form and the three different recombinant forms of the fission yeast glutathione synthetase were purified and their specific activities determined. All proteins exhibited similar activities of about 17 to 22 units/mg of protein (Table V).

The presence of six extra histidines at the C terminus did not affect the catalytic rate, as the specific activity of 20.1 units/mg observed for the wild type enzyme purified in this manner was higher than the value of 14.1 units/mg given for the native enzyme (without a histidine tag) in the literature (11). The lower activity reported for the native enzyme is probably due to the extended purification protocol.

**DISCUSSION**

The fission yeast glutathione synthetase is, like the homologous enzymes of other eukaryotic organisms, a homodimer composed of two identical subunits encoded by the GSH2 gene. The subunit structure of two small and two large subunits observed by Nakagawa et al. (11) results from proteolytic cleavage of the 56-kDa protein, encoded by the complete GSH2 open reading frame. Proteolysis probably takes place after cell disruption. The cleavage is catalyzed by a S. pombe protease, which has not been characterized thus far. It is highly unlikely that the cleavage is an autocatalytic process, because the rate of cleavage is inversely proportional to the purity of the protein. The resulting fragments of 24 and 32 kDa are stable; further degradation is not observed. The protease responsible for the cleavage was assigned to the metalloprotease family by protease inhibition experiments. Residual activities have been reported for several metalloproteases after EDTA treatment (28–30). The metal ions cannot always be effectively removed because the catalytic sites of metalloenzymes can bind metal ions with a very high affinity. A very small amount of metalloprotease might be copurified with glutathione synthetase by Ni2+-IMAC because of the affinity of metalloproteases to metal ions, particularly since IMAC has been described as a method to purify metalloenzymes (31).

The cleavage site of the metalloprotease that generates the 24- and 32-kDa subfragments is localized on the N-terminal side of serine 218, as determined by N-terminal sequencing and MALDI-TOF mass spectrometry. Site-directed mutagenesis, to delete amino acid residues 204–218 situated around the cleavage site, yields a stable homodimer composed of two 56-kDa subunits. This region is not essential, since the recombinant enzyme shows full in vivo and in vitro activity.

Glutathione synthetase still functions in vitro upon complete cleavage of the 56-kDa protein yielding the 24- and 32-kDa subfragments. Therefore an enzyme composed of the subfragments encoded on different plasmids was expressed in a ΔGSH2 strain. The activity of this enzyme is very surprising, since the model of the structure of the fission yeast glutathione synthetase (Fig. 5) indicates that the domains of the small and large subunit form a classical quaternary structure. Instead of a classical double domain architecture, the two subunits are strongly entangled. This has several interesting implications when the two major concepts of protein folding are considered. According to the cotranslational theory, proteins fold while the polypeptide chain is synthesized at the ribosome. The posttranslational theory suggests that proteins start to fold after the polypeptide chain has emerged from the ribosome and that folding requires the assistance of chaperones (32). For the heterotetramer to function, the subfragments must interact and assemble in the correct manner even though they are synthesized at discrete sites on separate ribosomes. This cannot be explained by the separate folding of polypeptide chains and subsequent joining of the proteins. On the contrary, according to the structure modeled for the fission yeast enzyme, the subfragments must contact each other while they are in an at least partially unfolded state. The nascent polypeptide chains of the subfragments might be stabilized by ribosome-associated chaperones and transferred to further downstream chaperones where they interact and the folding of the full-length protein takes place. The assistance of molecular chaperones in protein folding is a commonly accepted principle occurring in procaryotic as well as eucaryotic cells (for review see Refs. 33–35). Moreover, a chaperone-mediated transport of
polypeptide chains between different chaperones (36) and a processivity of chaperone action have been described.

One should consider, however, that the structure of the fission yeast glutathione synthetase presented here is a model relying on the x-ray structure of the human enzyme. Although the two structures are very similar, there is no final proof that the fission yeast enzyme in fact folds in this way. However, major differences in the structures of the two enzymes are very unlikely. Essential and highly conserved residues of the catastrophic site. Two flexible loops allow entry of the substrates and the outer regions are deleted. As a result, N- and C-terminal parts of the protein become exchanged (41, 42). Naturally occurring permutations have been observed in many protein families (for review, see Ref. 43). There are also numerous examples of proteins that have been artificially permuted (44–47). Prerequisites that are necessary for protein permutation in most cases include close proximity between the N and C termini in the tertiary structure of the protein and surface loops that can be cleaved to generate new termini (40, 48). These conditions should apply to glutathione synthetase (Figs. 5 and 6). The bacterial protein probably represents the original form of glutathione synthetase. The permutation event occurred in the early eucaryotic lineage (24), after the separation of the eucaryotic branch for this enzyme. According to the structure-based alignment and the overlay of the human and E. coli enzymes (Fig. 6), the original N- and C-terminal domains of the ATP-grasp superfamily, and as the prototype of this family, the E. coli glutathione synthetase, are situated in some and degraded. This indicates that the cell can distinguish between functional and non-functional proteins.

Another important issue is the physiological relevance of the cleavage reaction. Although cleavage of glutathione synthetase appears to occur only during cell disruption, the specificity of the cleavage and the stability of the cleavage products suggests that the reaction may fulfill a specific functional or regulatory role. To resolve this issue further studies will be necessary. The protein composed of the separately encoded subunits as well as the permuted version of the protein illustrate the evolution of glutathione synthetase. While there is no detectable sequence similarity between the bacterial and eucaryotic enzymes, structure-based alignments between the E. coli and eucaryotic glutathione synthetases reveal common conserved structural motifs. This is due to a gene permutation, which led to a circular shift of the conserved secondary structure elements in the eucaryotic protein (24). Because of its crystal structure the human enzyme was assigned to the ATP-grasp superfamily, which is characterized by three typical domains, each centered around a four- to six-stranded β-sheet. The β-sheet of the central and the C-terminal domain form an ATP binding site. Two flexible loops allow entry of the substrates and ATP and also protect the reaction intermediates during catalysis (39).

Permutation events play an important role in molecular evolution (40). Circular permutation of a protein can occur by gene duplication, in-frame fusion and partial deletion or mutation of the resulting tandem protein. Point mutations can produce new start and stop codons at the appropriate positions and the outer regions are deleted. As a result, N- and C-terminal parts of the protein become exchanged (41, 42). Naturally occurring permutations have been observed in many protein families (for review, see Ref. 43). There are also numerous examples of proteins that have been artificially permuted (44–47). Prerequisites that are necessary for protein permutation in most cases include close proximity between the N and C termini in the tertiary structure of the protein and surface loops that can be cleaved to generate new termini (40, 48). These conditions should apply to glutathione synthetase (Figs. 5 and 6). The bacterial protein probably represents the original form of glutathione synthetase. The permutation event occurred in the early eucaryotic lineage (24), after the separation of the eucaryotic branch for this enzyme. According to the structure-based alignment and the overlay of the human and E. coli enzymes (Fig. 6), the original N- and C-terminal domains of the ATP-grasp superfamily, and as the prototype of this family, the E. coli glutathione synthetase, are situated in

### Table IV

**GSH and phytochelatin contents of different S. pombe strains determined by RP-HPLC**

Cells were grown in minimal medium; for the induction of phytochelatin formation cadmium was added in the logarithmic growth phase. The values are given in nmol/mg of protein S.D. was below 10%.

| Strain | Form of glutathione synthetase over-expressed | Without induction | After induction of phytochelatins |
|--------|-----------------------------------------------|-------------------|----------------------------------|
|        |                                               | Cys | GSH | Total thiols | Cys | GSH | PC2 | PC3 | Total thiols |
| D18h- pREP2 46a14h- | Wild type enzyme                          | 6.8 | 41.3 | 48.0        | 5.8 | 3.1 | 10.3 | 7.4 | 51.7        |
| pREP2-gsh2:hft/45 46a14h- pREP2-gab2-D18h/25 | Enzyme after deletion of residues 204-218 | 3.7 | 42.0 | 45.8        | 6.4 | 15.5 | 12.9 | 10.7 | 81.1        |
| 46leu/42 Enzyme composed of separately encoded 24- and 32-kDa subunits permuted enzyme | 4.4 | 42.0 | 46.3        | 4.6 | 5.7 | 6.9 | 7.3 | 46.6        |
| pREP2-GU/gsh2:hft + pREP1-hft:KUgsh2 46leu/42 pREP2-Sp-GSH2- perm | 1.7 | 48.2 | 49.9        | 6.2 | 58.3 | 37.4 | 26.3 | 218.3       |

### Table V

**In vitro activities of different forms of glutathione synthetase**

The specific activities of the different forms of the enzyme were determined photometrically in an assay coupling NADH oxidation to glutathione formation. The activity of 1 unit is defined as formation of 1 μmol of GSH/min. The average values of two independent experiments are shown.

| Protein | Specific activity |
|---------|------------------|
| Wild type glutathione synthetase | 20.1 ± 0.3 |
| Wild type glutathione synthetase after complete cleavage | 17.2 ± 0.6 |
| Glutathione synthetase after deletion of 15 amino acids | 19.3 ± 0.3 |
| Separately expressed 24- and 32-kDa subfragments | 17.3 ± 0.4 |
| Permuted glutathione synthetase | 21.9 ± 0.1 |
the middle region of the eucaryotic protein, between helix α 8 and strand β 6. This region also contains the 15-amino acid insertion unique to the fission yeast enzyme (residues 204–218). The N- and C-terminal domains of the ATP-grasp superfamily were linked in the eucaryotic enzymes by gene duplication. It is likely that the two genes were fused in-frame, but not directly connected, i.e. there was some distance between them. Over evolutionary time, the tandem gene accumulated further mutations, and the external and internal parts were probably deleted.

Amino acid alignments of the different eucaryotic glutathione synthetases (Fig. 4) demonstrate that, in addition to the S. pombe insert, a much shorter insertion is present in the enzymes of the yeasts S. cerevisiae and P. angusta, but not in the enzymes of A. thaliana and H. sapiens. The presence of an exon sequence in the human glutathione synthetase gene, C-terminal to the position of the 15-amino acid residue insertion in the S. pombe enzyme, might indicate the mechanism by which this insertion was eliminated during the evolution of metazoans. As shown in this study, the additional region of the fission yeast enzyme is not essential, so there was no selection pressure to maintain it. The large size of the insert in S. pombe, together with the other structural variations at this site, might explain the proteolytic degradation of glutathione synthetase specifically in S. pombe.

The permuted fission yeast enzyme is functional. This supports the permutation event described by Polekhina et al. (24) has taken place during the evolution of the protein and illustrates that the model calculated for the fission yeast protein (Fig. 5) is a good approximation of the natural structure. Our findings show that the fission yeast glutathione synthetase can serve as a model for protein folding. Experiments on the heterotetrameric enzyme consisting of the separately encoded subfragments might provide further insight into the mechanisms of protein folding within the cell. Moreover, calorimetric studies of the different versions of the recombinant protein could provide valuable data on protein stability.

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