Biochemical Characterization and Mass Spectrometric Disulfide Bond Mapping of Periplasmic α-Amylase MalS of Escherichia coli*

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Periplasmic α-amylase of Escherichia coli, the malS gene product, hydrolyzes linear maltodextrins. The purified enzyme exhibited a $K_m$ of 49 $\mu$M and a $V_{max}$ of 0.36 $\mu$mol of p-nitrophenylhexaose hydrolyzed per min per mg of protein. Amylase activity was optimal at pH 8 and was dependent on divalent cations such as Ca$^{2+}$. MalS exhibited altered migration on SDS-polyacrylamide gel electrophoresis under nonreducing conditions. Analytical ultracentrifugation and electrospray mass spectrometry indicated that MalS is monomeric. The four cysteine residues are involved in intramolecular disulfide bonds. To map disulfide bonds, MalS was proteolytically digested. The resulting peptides were separated by reverse-phase high-performance liquid chromatography, and matrix-assisted laser desorption/ionization mass spectrometry analysis indicated the presence of two disulfide bonds, i.e. Cys$^{40-58}$ and Cys$^{104-209}$. The disulfide bond at Cys$^{40-58}$ is located in an N-terminal extension of about 160 amino acids which has no homology to other amyloses but to the proposed peptide binding domain of $\alpha$-amylase family that shares a characteristic ($\beta\alpha$)-barrel domain containing the active site (8). The four best conserved regions that are present in the active site of the α-amylase family are located toward the C terminus of MalS, i.e. between amino acids 304 and 566 (9). From amino acid sequence alignments it can be concluded that MalS has an N-terminal extension of about 160 amino acids of unknown function (8, 9). Four Cys residues are found at positions 40, 58, 104, and 520 of the mature MalS protein, three of which are located in the N-terminal extension of MalS, which is not homologous to other amylases.

In this study we describe an improved purification procedure, biochemical characterization and mass spectrometric identification of the disulfide bond structure of native MalS. Mass spectrometric molecular weight analyses using electrospray-ionization mass spectrometry (ESI-MS) and matrix-assisted laser-desorption/ionization mass spectrometry (MALDI-MS) peptide mapping analyses were applied for identification of the molecular structure of native MalS. Proper formation of disulfide bonds is crucial for attaining the correct three-dimensional structure of proteins (10–13). Therefore, it was important to determine the location of all disulfide bonds in MalS. Mass spectrometric methods have successfully been employed for the study of disulfide bridges of peptides and proteins (14–18), and several comprehensive reviews are available on this subject (19–21). In our studies we used proteolytic cleavage techniques, followed by high performance liquid chromatography (HPLC) separation and MALDI-MS for the rapid identification of the disulfide bridges.

MATERIALS AND METHODS

**Bacteria and Plasmids—E. coli strains are derivatives of MC4100 which is F$^{−}$ lacU169 araD1396 rbsR relA rpsL thi (22). CS10 is MC4100 malT$^{−}$ malP-lacZ malQ malZ dex7. CS4 is CS10, trxB::kan. CS5 is CS10, gsh::Tn10kan. CS4 is CS4, $\alpha$ara714 leu $\cdot$714 leu::Tn5. CS16 is CS5, $\alpha$ara714 leu::Tn5. CS68 is CS16, malE::Tn10. pUMA103 is a pbR232-derived plasmid that expresses malS under its own promoter (34). pCS7 is a pBAD18s derivative and expresses malS with a deletion of its signal sequence under control of the arabinose promoter (23). pBAD18s is a pbR232-derived high copy number plasmid that has the

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1 The abbreviations used are: ESI-MS, electrospray-ionization mass spectrometry; MALDI-MS, matrix-assisted laser-desorption/ionization mass spectrometry; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography; PN6, p-nitrophenylhexaose; HCCA, 4-hydroxy-α-cyanocinnamic acid; TCEP, Tris(2-carboxyethyl)phosphine; BisTris, 2-[bis(2-hydroxyethyl)-amino]-2-(hydroxymethyl)-propane-1,3-diol.
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3AID135 encodes alkaline phosphatase with a signal sequence (Δ22–25).

Purification of MalS—Strain C886 expressing malS from pUMA103 was grown in minimal medium 9 (M9) (26) supplemented with 0.2% casamino acids (27). Cells were harvested after centrifugation from a 2.5-liter overnight culture. Cells containing 100 μg/ml ampicillin were harvested by centrifugation (10 min, 5000 rpm, 21 °C, GS-3 rotor) and resuspended in 100 ml of 10 mM Tris-HCl, pH 7.5, prior to preparation of cold osmotic shock fractions. The cold osmotic shock procedure was carried out according to Neu and Heppel (27) with the exception of using CaCl2 instead of MgCl2. Remaining cells were removed by centrifugation (9000 rpm, 30 min, 4 °C, SS34 rotor). Subsequently, the periplasmic extract was lyophilized, and proteins were solubilized in buffer A (20 mM Tris-HCl, pH 7.5) at a concentration of 10 mg/ml. After dialysis overnight in buffer A, insoluble proteins were removed by centrifugation (9000 rpm, 15 min, 4 °C, SS34 rotor).

The resulting supernatant fraction was loaded on a MonoQ column (from Pharmacia Biotech Inc.), which had been equilibrated with buffer A. Non-interacting proteins were washed off with 10 ml of buffer A, and the remaining proteins were eluted with a linear gradient from 0 to 200 mM NaCl in 40 ml of buffer A at a flow rate of 0.5 ml/min. MalS eluted at about 80 mM NaCl.

SDS-PAGE was done as described by Laemmli (28). SDS gels were stained with Coomassie Blue (29). To detect MalS on Western blots, an amylase activity assay was used (30). To detect MalS by Western dot blot analysis, a polyclonal antiserum against MalS was used.

SDS-PAGE—Analytical Sedimentation Equilibrium Ultracentrifugation—

Analytical ultracentrifugation experiments were carried out in a Beckman Instruments Optima XL-A analytical ultracentrifuge. A solution of 0.2 μM in buffer containing 20 mM Tris-Cl, pH 7.5, and 75 mM NaCl was measured at 10,000 rpm at 4 °C. For the determination of the molecular weight the data were fit to Equation 1.

\[
\Lambda = \Lambda_0 \exp \left[ \frac{M \cdot (1 - \nu \cdot v) - \omega^2 \cdot (r^2 - r_0^2)}{2 \cdot RT} \right] + E
\]

(Eq. 1)

In Equation 1, \(\Lambda_0\) and \(\Lambda\) are the absorbance at a radial position \(r\) and at the meniscus \(r_0\) (a reference position), respectively. The parameter \(M\) is the molecular weight of the macromolecule that is derived from the fit; \(\nu\) is the partial specific volume that was calculated from the amino acid composition of the protein to be 0.720 ml g⁻¹ (35, 36); \(\rho\) is the density of the solvent that was measured to be 1.01 g ml⁻¹; \(\omega\) is the angular velocity; \(R\) is the absolute temperature; \(T\) is the absolute temperature, and \(E\) is the base line that was determined at 10,000 rpm after sedimentation of the protein sample at 48,000 rpm for 6 h.

Tryptic Digestion of Unmodified, Reduced, and Carboxamidomethylation of MalS—MalS (50 μl, 2 mg/ml) in 50 mM ammonium bicarbonate, pH 7.5, was mixed with a solution (2.7 μl) of Tris-(2-carboxyethyl)phosphine (TCEP) in 10 mM ammonium bicarbonate, pH 7.5 (35, 36). Final concentration of TCEP was 5.4 mM, resulting in a molar ratio of MalS to TCEP of 1:200 (4 Cys per MalS). The solution was incubated at 37 °C for 1 h. A 100 mM iodoacetamide solution in H₂O (0.7 μl) was added, and the mixture was incubated at 23 °C for 30 min in the dark, pH 7.5. The molar ratio of MalS to iodoacetamide was 1.50. The reaction was terminated by ultrafiltration using a microconcentrator device (Amicon, microcon, cutoff 10 kDa). Retentates were washed three times with 200 μl of a solution consisting of 30% (v/v) methanol in 35 mM ammonium bicarbonate, pH 8. The final protein concentration was adjusted to 1 μg/ml. Tryptic digestion was carried out with MalS and carboxamidomethylated MalS (1 mg/ml each) in 100 μl 35 mM ammonium bicarbonate solution containing 30% (v/v) methanol, pH 8. A trypsin solution (10 μl, 1 mg/ml; 1 mM HCl) was added to yield a final pH of 7.5 (E/S = 1:10). Samples were incubated at 37 °C for 2 h. Aliquots (0.5 μl) were used for MALDI-MS analysis without further purification. For subsequent HPLC separation the peptide mixture (50 μg) was lyophilized and redissolved in 100 μl of HPLC solvent A.

HPLC Separation of MalS and Tryptic Peptides from MalS—A Waters Millipore solvent delivery system, consisting of two HPLC pumps (Waters M510 and Waters M45), was used. Purification of MalS was done using a 250 × 8.5 mm Grom-Sil 200 Butyl-1 ST reversed phase C-4 column (300 Å, 11 μm) equipped with a Grom precolumn. Separations of the tryptic peptide mixture were carried out using a 250 × 4.6-mm Vydac reversed phase C-18 column (300 Å, 10 μm) equipped with a Vydac precolumn. For each run, solvent A was 0.1% trifluoroacetic acid in H₂O and solvent B was 0.07% trifluoroacetic acid in acetonitrile. For MalS purification the flow rate was adjusted to 2.4 ml/min, and after sample injection, the solvent mixture was kept constant at 10% B for 5 min and was raised to 90% B over a period of 55 min. MalS-containing samples were collected, lyophilized, and redissolved for ESI-MS analysis in 10% acetic acid/2,2,2-trifluoroethanol (7:3), pH 2, to a final concentration of 50 μm. After centrifugation, a solution of 25 mM TCEP in 50 mM ammonium bicarbonate (1 μl; pH 7.5) and acetonitrile (1 μl) was added to yield a final pH of 7.5. The peptide mixture-containing solution was injected. The flow rate was adjusted to 1 ml/min and the solvent mixture was kept constant at 10% B for 5 min and was raised to 55% B over a period of 45 min. The lyophilized HPLC fractions were dissolved in 5 μl of acetonitrile, 0.1% trifluoroacetic acid (2:1), pH 2, and were used for subsequent MALDI-MS analysis.

Reduction of the Disulfide Bond-containing Tryptic Peptides of MalS—For reduction of the disulfide bond-containing peptides in solution, 1 μl of each HPLC fraction was mixed with 5 μl of 50 mM ammonium bicarbonate solution, pH 8, and a solution of 1 μl of 100 mM 2-mercaptoethanol in H₂O was added. The mixture was incubated at 37 °C for 15 min. Aliquots (0.5 μl) were used for MALDI-MS analysis without further purification. For on-target reduction, a solution of 25 mM TCEP in 50 mM ammonium bicarbonate (1 μl; pH 7.5) and acetonitrile (1 μl) was added to the solid matrix/peptide mixture and mixed gently until all solid material was completely redissolved. The reaction mixture was adjusted to pH 4. After 15 min (23 °C) the solvent was evaporated. The matrix/peptide mixture was washed once with 2 μl of 0.1% trifluoroacetic acid, pH 2, and was recrystallized once from 100 μl of 50 mM ammonium bicarbonate solution containing 30% (v/v) methanol, pH 8. A trypsin solution (10 μl, 1 mg/ml; 1 mM HCl) and acetonitrile (1 μl) were used for MALDI-MS analysis.

Mass Spectrometric Molecular Weight Determination and Peptide Mapping—Matrix-assisted laser desorption/ionization-time-of-flight—mass spectrometric (MALDI-TOF-MS) analyses were carried out using a Bruker Biflex time-of-flight mass spectrometer (Bruker Franzen, Bremen, Germany), equipped with a UV nitrogen laser (337 nm) and a dual microchannel plate detector. For the molecular weight determination...
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Purification and characterization of MalS. A, lane 1, crude extract from CS66 grown in M9 containing casamino acids (0.2% final concentration); lane 2, crude extract from CS66 harboring pUMA103 grown in the same medium; lane 3, cold osmotic shock fraction; lane 4, pooled fractions after MonoQ ion-exchange chromatography; lane 5, molecular mass standards. 10 μg of protein was applied to each slot. The position of MalS is indicated. B, activity of MalS was assayed in 50 mM Tris-HCl in the range of pH 6 to 9.5 and in 50 mM BisTris-HCl between pH 5.0 and 8.0.

RESULTS

MalS was purified using a simple procedure involving cold osmotic shock followed by ion exchange chromatography (Fig. 1A). Purified MalS migrated on a 10% SDS-PAGE gel under reducing conditions according to its predicted molecular mass of 74 kDa, and the sample showed homogeneity (>95%) as no further protein bands were observed.

To determine optimal conditions for assay of the amylase, several parameters were investigated. The pH optimum of MalS was found to be between pH 8.0 and 8.5 (Fig. 1B). Therefore, we used Tris-HCl buffer, pH 8.0, in all assays. The kinetic parameters of pure MalS were determined with PNP6 as substrate by varying substrate concentrations between 0.5 and 500 μM. The Kₘ value was 49 μM, and the maximum velocity (Vₘₐₓ) was 0.36 μmol min⁻¹ mg⁻¹ protein.

Since the activity of many amylases is dependent on Ca²⁺, we tested whether MalS activity was inhibited by EDTA and whether the inhibitory effect could be reversed by addition of divalent cations. The addition of 1 mM EDTA to the reaction buffer abolished MalS activity. After adding back Ca²⁺ and Ba²⁺, enzymatic activity was restored. We detected that Ba²⁺ even stimulated amylase activity slightly. Although Mg²⁺ and Mn²⁺ restored MalS activity to 77 and 74%, respectively, the addition of Zn²⁺ did not abolish the inhibition by EDTA (Table I).

We determined whether conditions of high or low concentrations of salt would affect MalS activity. MalS activity was highest (350 nmol min⁻¹ mg⁻¹) when no NaCl was added to the standard assay buffer and decreased with increasing concentrations of salt. It was about 2-fold lower in the presence of 1 M NaCl.

Migration of MalS on SDS-PAGE under Reducing and Nonreducing Conditions—MalS migrated at its predicted molecular mass under reducing conditions, i.e. 74 kDa. Under nonreducing conditions, MalS migrated near 90 kDa. Furthermore, the same shift from 74 to 90 kDa was detected after reoxidation of reduced MalS using oxidized glutathione, indicating the presence of disulfide bonds (Fig. 2A). However, since proteins containing disulfide bonds normally migrate at lower apparent molecular weight than under reducing conditions, we investigated the possibility that the unusual behavior of MalS may be due to other explanations, e.g. other post-translational modifications.

Table I

| Cation | MalS activity (nmol/min mg protein) |
|--------|------------------------------------|
| Ca²⁺   | 44                                 |
| Ba²⁺   | 400                                |
| Mg²⁺   | 520                                |
| Mn²⁺   | 309                                |
| Zn²⁺   | 296                                |

Peptide Mapping Using Reduced MalS—To further investigate the molecular structure of MalS, the reduced and iodoacetamide alkylated protein was cleaved proteolytically with trypsin, and the fragment mixture was subsequently analyzed by MALDI-MS (mass spectrometric peptide mapping). The results (Table II) confirmed the amino acid sequence (Fig. 3) as very good sequence coverage was obtained. Only in the cases of...
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**Figure 2.** Reduction and reoxidation of MalS and ESI-MS mass spectrum of HPLC purified MalS. A, 1.2 μg of purified MalS was subjected to SDS-PAGE. Prior to electrophoresis, samples were incubated without DTT (lane 1), with DTT (lane 2), and DTT followed by reoxidation with oxidized glutathione (lane 3). The positions of molecular mass standards of 67 and 94 kDa are indicated. B, ESI-MS molecular weight analysis of nonreduced MalS showed a series of multiply charged molecular ions centering around the [M + 43H]⁺ molecular ion. Analysis was performed by injection of 10 μl of nonreduced MalS (1.4 μμ) in 10% acetic acid/2,2,2-trifluoroethanol (7:3), pH 2. The molecular mass of MalS was determined to 73,978 Da. * indicates the 8+ to 10+ molecular ions of hen egg white lysozyme used for internal calibration.

Partial sequences with closely spaced lysine or arginine residues were not able to identify the resulting short peptides directly from peptide mapping data due to superimposition with matrix ions. However, the correct N terminus (amino acids 1–27; fragment T1) was ascertained by an ion signal at m/z 2837, and the C-terminal trypptic peptide (amino acids 103–106)-S-S-(517–534) 2543.9 2544 of the protein was identified as an ion signal at m/z 2544 for carboxamidomethylated T2 and at m/z 2404 for carboxamidomethylated T3 (Table II).

**Table II**

| Peptide | Partial sequence (calc.) | Peptide | Partial sequence (calc.) |
|---------|--------------------------|---------|--------------------------|
| T2-S-S-T3 | (28–48)-S-S-(49–66) 4515.1 | T7-S-S-T42 | (103–106)-S-S-(517–534) 2543.9 |
| T1 | 1–27 2837.1 | T4 | 49–66 2172.9 |
| T2 | 28–48 2460.7 | T5 | 57–80 1311.4 |
| T3 | 68–80 2100.5 | T6 | 81–102 2256.6 |
| T4-T5 | 67–80 1272.9 | T7 | 107–127 2205.2 |
| T5 | 70–80 1586.6 | T8 | 128–138 1328.5 |
| T6 | 81–102 1586.6 | T9 | 139–158 2140.5 |
| T7 | 159–181 2657.8 | T10 | 182–195 1586.6 |
| T8 | 182–195 1586.6 | T11 | 196–212 1584.0 |
| T9 | 213–247 3813.3 | T12 | 213–247 3813.3 |
| T10 | 213–247 3813.3 | T13-T14 | 218–247 3299.7 |
| T11 | 218–247 3299.7 | T15-T16 | 248–277 3536.7 |
| T12 | 248–277 3536.7 | T16 | 278–286 1027.1 |
| T13-T14 | 278–286 1027.1 | T17 | 290–323 3841.3 |
| T14 | 290–323 3841.3 | T18 | 320–353 2901.2 |
| T15-T16 | 320–353 2901.2 | T19 | 354–358 606.6 |
| T16 | 354–358 606.6 | T20 | 354–362 1164.3 |
| T17 | 354–362 1164.3 | T21 | 363–366 588.8 |
| T18 | 363–366 588.8 | T22 | 367–391 2775.0 |
| T19 | 367–391 2775.0 | T23 | 392–405 1501.7 |
| T20 | 392–405 1501.7 | T24 | 408–411 1000.2 |
| T21 | 408–411 1000.2 | T25 | 408–414 4086.6 |
| T22 | 408–414 4086.6 | T26 | 414–421 893.0 |
| T23 | 414–421 893.0 | T27 | 422–433 1604.8 |
| T24 | 422–433 1604.8 | T28 | 434–441 943.0 |
| T25 | 434–441 943.0 | T29 | 442–446 1457.6 |
| T26 | 442–446 1457.6 | T30 | 447–457 1349.6 |
| T27 | 447–457 1349.6 | T31 | 458–465 819.9 |
| T28 | 458–465 819.9 | T32 | 462–476 1027.1 |
| T29 | 462–476 1027.1 | T33 | 475–499 2962.3 |
| T30 | 475–499 2962.3 | T34 | 499–516 1988.1 |
| T31 | 499–516 1988.1 | T35 | 515–525 1835.0 |
| T32 | 515–525 1835.0 | T36 | 535–550 2256.6 |
| T33 | 535–550 2256.6 | T37 | 554–594 4220.7 |
| T34 | 554–594 4220.7 | T38 | 555–559 3733.2 |
| T35 | 555–559 3733.2 | T39 | 596–605 1267.2 |
| T36 | 596–605 1267.2 | T40 | 608–615 1082.5 |
| T37 | 608–615 1082.5 | T41 | 616–620 650.8 |
| T38 | 616–620 650.8 | T42 | 621–630 978.1 |
| T39 | 621–630 978.1 | T43 | 631–637 1586.6 |
| T40 | 631–637 1586.6 | T44 | 638–644 826.9 |
| T41 | 638–644 826.9 | T45 | 645–659 1681.8 |
| T42 | 645–659 1681.8 | T46 | 651–659 1000.2 |

* Numbers denote amino acid positions as found in the sequence of the mature protein.
* Reduced and carboxamidomethylated peptide.

2586 coeluted in one HPLC fraction and could only be separated by 2-fold rechromatography (cf. Fig. 4C). The HPLC fractionated peptide with m/z 4515 (Fig. 4A) was reduced separately in ammonium bicarbonate solution, and MALDI-MS analysis was repeated (Fig. 4B). In addition to the complete disappearance of the dipeptide ion signal at m/z 4515, two strong ion signals were observed upon reduction at m/z 2404 which corresponds to T2 and m/z 2114 corresponding to T3 proving the presence of the disulfide bond Cys⁴⁰–Cys⁶⁰. By contrast, the disulfide-linked dipeptide T7–S–S–T42 (m/z 2544) was reduced with TCEP on the MALDI target in the presence of the HCCA matrix at pH 4. TCEP was chosen for these experiments as this reducing agent is applicable even at acidic pH (39). The ion signal at m/z 2544 disappeared, although not completely, and two new strong ion signals at m/z 2070 (T42) and 2259 (T42-HCCA adduct) were observed. An ion signal for T7 (m/z 2577) was not observed due to suppression of the ion. However, the mass difference of 474 (3 mass units lower than calculated due to three additional protons added by reduction and protonation) between the ion signal at m/z 2544 for the
FIG. 3. Peptide mapping and amino acid sequence of MalS. Trypsin cleavage sites (T) and disulfide bonds of MalS are indicated. Tryptic peptide cleavages given in parentheses refer to peptides to the left.

FIG. 4. MALDI mass spectra of disulfide bond containing peptides after tryptic digestion and HPLC separation. A, before and B, after reduction of peptide T2–S–S–T3 with 2-mercaptoethanol in solution, indicating the disulfide bond Cys40–Cys58. C, before and D, after reduction of peptide T7–S–S–T42 with TCEP on-target, demonstrating the presence of the disulfide bond Cys104–Cys520. HCCA was used as matrix.
**Table III**

Enzymatic activity of signal sequenceless MalS and of signal sequenceless AP in trxB::kan and gsh::kan mutants

| Relevant genotype | MalS activity | AP activity |
|-------------------|---------------|-------------|
|                   | units/mg      | units/mg    |
| Wild type         | 0.2           | 60.0        |
| trxB::kan         | <0.1          | 454.0       |
| gsh::kan          | <0.1          | 137.9       |

**Figure 5.** Expression of signal sequenceless MalS in the cytoplasm. Whole cell extracts of strains CS10 (wt), CS14 (trxB::kan), and CS16 (gsh::Tnl0), expressing ΔmalS from pCS7 were subjected to SDS-PAGE followed by Western blotting using a polyclonal antiserum to MalS. Electrophoresis was carried out under oxidizing conditions. Free thiol groups were blocked with iodoacetamide. The position of reduced and of oxidized forms of MalS is indicated.

The absence of MalS activity could be correlated to its inability to form disulfide bonds since only reduced MalS migrating at 74 kDa after the addition of DTT indicating that disulfide bonds were reduced completely by using 10 mM DTT. Only at SDS concentrations above results, the activity of native MalS under reducing conditions, stability of MalS toward thermal inactivation was investigated under reducing and nonreducing conditions, i.e. in the presence and absence of DTT. As determined by MalS assays, stability of reduced MalS was only slightly less than that of oxidized MalS. A complete loss of MalS activity was observed at 61 °C under nonreducing conditions as well as under reducing conditions. Similar results were obtained when thermal denaturation of MalS was assayed in whole cells. In this case, MalS activity was abolished at 55 °C under nonreducing conditions as well as under reducing conditions (data not shown).

**Table IV**

Thermal stability of oxidized and reduced MalS

| Temperature/°C | -DTT MalS activity | +DTT MalS activity |
|----------------|---------------------|-------------------|
| 22             | 283                 | 342               |
| 37             | 261                 | 256               |
| 42             | 264                 | 262               |
| 47             | 237                 | 192               |
| 50             | 222                 | 209               |
| 56             | 205                 | 156               |
| 61             | 7                   | 21                |

**Table V**

Effect of SDS on the activity of oxidized and reduced MalS

| SDS | MalS activity |
|-----|---------------|
|     | % nmol/min × mg protein | % nmol/min × mg protein |
| 0   | 270           | 236               |
| 0.001 | 253           | 208               |
| 0.010 | 367           | 321               |
| 0.100 | 240           | 187               |
| 0.250 | 119           | 68                |
| 0.500 | 55            | 25                |
| 0.750 | 29            | 8                 |
| 1.000 | 24            | 7                 |
| 2.000 | 1             | 0                 |

were not heat-treated prior to electrophoresis. Also, mass spectrometric peptide mapping analyses of reduced MalS confirmed that both disulfide bonds were reduced completely by using 200-fold molar access of DTT and could be alkylated quantitatively with iodoacetamide (see above).

**MalS Activity under Reducing and Denaturing Conditions**—Disulfide bonds enhance the thermal stability of many proteins (42–45). Since we detected no loss of MalS activity under reducing conditions, stability of MalS toward thermal inactivation was investigated under reducing and nonreducing conditions, i.e. in the presence and absence of DTT. As determined by MalS assays, stability of reduced MalS was only slightly less than that of oxidized MalS (Table IV). A complete loss of MalS activity was observed at 61 °C under nonreducing conditions as well as under reducing conditions. Similar results were obtained when thermal denaturation of MalS was assayed in whole cells. In this case, MalS activity was abolished at 55 °C under nonreducing conditions as well as under reducing conditions (data not shown).

SDS resistance has been reported for a fungal α-amylase (46). However, MalS was shown to be sensitive to SDS. Half-maximal activity was detected near 0.2% SDS (Table V). Subsequently, we tested whether the presence of disulfide bonds would enhance the tolerance of MalS toward SDS. Only at SDS concentrations above 0.25% was the difference in enzymatic activity between reduced and oxidized MalS 50% or greater.

**Analytical Sedimentation Equilibrium Ultracentrifugation**—The analysis of the MalS protein by sedimentation equilibrium ultracentrifugation yielded a molecular mass of 70 ± 4 kDa under oxidizing conditions. This was in agreement with the calculated molecular weight within the error of the measurement. The fit of the data to Equation 1 is very good and shows no signs of the presence of additional species, as can be deduced from the residuals of the fit which show a random scattering around the fit curve. Thus, the results demonstrate unambiguously that the protein is present as a monomer at concentra-
Electron Microscopy—Electron microscopy of purified MalS was used to obtain indications about the structure of MalS under nonreducing and reducing conditions. MalS is a globular protein with the shape of a horseshoe (Fig. 7). Using negatively stained samples, the height could be determined to 80.5 Å \(\pm 0.63\) and the inner diameter of the hollow space was 23.0 Å \(\pm 0.43\).

After incubation with DTT, MalS aggregated to form large clumps (Fig. 7C). Thus, reduction of disulfide bonds lead to at least partial denaturation of MalS. It could be speculated that hydrophobic segments may be exposed at the surface causing intermolecular interaction. Since no loss of enzymatic activity was detected under these conditions, the observed partial denaturation did not affect the active site.

**DISCUSSION**

MalS differs from the numerous amylases identified and characterized to date. MalS has an N-terminal extension of about 160 amino acids that is not homologous to other amylases but rather shows homology to the proposed peptide binding domain of GroEL (Fig. 8). The function of the MalS N terminus, which is linked via a disulfide bond formed between Cys\(^{104}\) and Cys\(^{520}\) to the C-terminal amylase domain, could be to assist in folding of the amylase domain. Reduction of disulfide bonds, which most likely leads to detachment of the N and C termini of MalS, caused aggregation. Since one of the main functions of molecular chaperones is to prevent aggregation, it is tempting to speculate on an autochaperone activity of the MalS N terminus.

MalS contains a second disulfide bond formed between Cys residues 40 and 58, located in its N-terminal domain. Of the Cys residues involved in disulfide bond formation, only Cys\(^{520}\) is conserved in a number of amylases, e.g., of *Alteromonas haloplanktis* (47), *Aspergillus shirousamii* (48), *Schwannomyces occidentalis* (49), and *Saccharomycopsis fibuligera* (50), where this residue, together with other conserved Cys residues, is involved in disulfide bond formation within the amylase domains. However, Cys\(^{40}\), Cys\(^{58}\), and Cys\(^{104}\) seem to be uniquely present in MalS. This may explain that MalS was active even after reduction of disulfide bonds.

Initial evidence for the importance of disulfide bonds for folding of MalS was obtained by expression of MalS under reducing conditions in the cytoplasm, which yielded inactive protein. The inability of MalS to become active in the cytoplasm, even when the trxB and gsh mutations were present, was surprising since several periplasmic proteins have been shown to be actively expressed in the cytoplasm; examples include maltose binding protein (51), alkaline phosphatase (41), β-lactamase (52), and trehalase TreA.\(^2\) It could be argued that the inability of signal sequenceless MalS to fold into its active conformation in the cytoplasm may be an artifact caused by the genetic manipulation of signal sequence removal. This explanation can be excluded since it was shown earlier that this MalS construct could be functionally expressed in the cytoplasm.

\(2\) K. Uhland and M. Ehrmann, unpublished results.
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periplasm when exported by an altered secretion machinery, i.e. in prfA mutants (23).

Locating cystine bridges in proteins generally involves cleavage of the protein by enzymatic or chemical means under which disulfide scrambling is avoided. However, with non-denatured and disulfide bond containing proteins, nonspecific or incomplete proteolytic cleavages are frequently observed (e.g. Refs. 15 and 37). Therefore, cleavage by trypsin was first carried out with irreversibly denatured, i.e. reduced and carboxamidomethylated, MalS to study specific cleavage products. Disulfide bonds were destroyed in this sample, but sequence verification was possible as almost all predicted peptide ions were observed (cf. Table II). Using this information, reversibly denatured MalS (by addition of 30% methanol (37)) was digested with trypsin for 2 h at pH 7.5, a rather short cleavage period, but these conditions were chosen to minimize disulfide bond scrambling (15). Subsequently, disulfide bonds in MalS were identified by mass spectrometric analyses.

To determine whether or not an observed peak in the recorded mass spectrum is due to disulfide linkages, the corresponding ion signal should completely, or at least mostly, be eliminated by reduction. This is important as the recently discovered MALDI-induced cleavage of disulfide bonds (53–55), which was also observed in our analyses (cf. Fig. 4, A and C), may obscure the results, particularly when peptide mixtures are analyzed. Thus, chemical reduction either in solution or on-target, as demonstrated here, is necessary to address disulfide-linked peptides unambiguously. Whereas reduction in solution (at pH 8) led to complete reduction of the disulfide-linked peptides, reduction on the MALDI target in the presence of the matrix (at pH 4) was incomplete and additionally formed strong peptide-matrix adduct ions (Fig. 4D). The presence of TCEP on the target did not interfere with peptide ion detection. Thus, this strategy proved successful for identification of disulfide bonds even with very little material, e.g. after several chromatographic separation steps.

Further information on the structure of MalS was obtained by electron microscopy and analytical ultracentrifugation. The EM data indicated that MalS is present as a U-shaped structure which looked similar to amylopullulanase (56) and a glycoamylase of Clostridium thermosaccharolyticum (57). The latter is also a monomer as has been determined by analytical ultracentrifugation (58). A topological model of the MalS protein was derived from these structural data (Fig. 9). We propose that MalS is composed of at least two domains, an N-terminal extension carrying a disulfide bond and a C-terminal amylose domain that is connected to the N-terminal segment via the second disulfide bond. The presence of extensions was reported for other amyloses; however, these extensions are not N-terminal, are not connected via a disulfide bond to the amylose domain, and do not exhibit homology to the relevant domains of MalS or GroEL (59–61).

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