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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 μM and a V_{max} of 0.36 μmol of *p*-nitrophenylhexaoside 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 $Cys^{104–520}$. 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 amylases but to the proposed peptide binding domain of GroEL, the Hsp60 of *E. coli*. The N-terminal extension is linked to the C-terminal amylase domain via disulfide bond $Cys^{104–520}$. Reduction of disulfide bonds by dithiothreitol treatment led to aggregation suggesting that the N terminus of MalS may represent an internal chaperone domain.

In *Escherichia coli*, maltodextrins enter the periplasm preferentially via the outer membrane porin LamB (1). In the periplasm, maltodextrins are either cleaved by a periplasmic amylase, MalS, or are transported to the cytoplasm via the binding protein-dependent transport complex $MalEFGK_2$ (2). This transport system can only transport maltodextrins up to maltoheptaose. Longer dextrins have to be cleaved prior to transport. In the cytoplasm, linear maltodextrins are processed by maltodextrin phosphorylase, amylomaltase, or maltodextrin glucosidase (3–5).

The *malS* gene, located at 80.54 min on the *E. coli* chromosome, is part of the complex maltose regulon (6), which is involved in the uptake and degradation of maltodextrins. Thus, *malS* is controlled by MalT, the positive regulator of the system (7).

MalS hydrolyzes α-(1,4) glycosidic linkages in long maltodextrins but not in maltose. MalS is an enzyme of 659 amino acids with a molecular mass of 74 kDa (8). It belongs to the α-amylase family that shares a characteristic $(\beta/\alpha)_8$-barrel domain containing the active site (9). 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)[1] 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^- Δ*lacU169 araD136 rbsR relA rpsL thi* (22). CS10 is MC4100 *malT*^{con} Δ*malQ malP-lacZ* Δ*malS* Δ*malZ dex7*. CS4 is CS10, *trxB::kan*. CS5 is CS10, *gsh*::Tn*10kan*. CS14 is CS4, Δ*ara714 leu*::Tn*10*. CS16 is CS5, Δ*ara714 leu*::Tn*10*. CS66 is CS10, *malE*::Tn*10*. pUMA103 is a pBR322-derived plasmid that expresses *malS* under its own promoter (8). 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 pBR322-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; PNP6, *p*-nitrophenylhexaoside; HCCA, 4-hydroxy-α-cyanocinnamic acid; TCEP, Tris(2-carboxyethyl)phosphine; BisTris, 2-[bis(2-hydroxyethyl)-amino]-2-(hydroxymethyl)-propane-1,3-diol.

arabinose promoter followed by a linker containing multiple restriction sites (24). pAID135 encodes alkaline phosphatase with a signal sequence deletion (Δ2–22) (25).

Purification of MalS—Strain CS66 expressing *malS* from pUMA103 was grown in minimal medium 9 (M9) (26) supplemented with 0.2% casamino acids as carbon source. Cells from a 2.5-liter overnight culture 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 $CaCl_2$ instead of $MgCl_2$. 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, polyclonal antiserum against MalS was used.

For mass spectrometric structure determination, MalS solutions were concentrated by ultrafiltration using microconcentrator devices (Amicon, microcon, cutoff 10 kDa). Retentates were washed with 500 μl of 50 mM ammonium bicarbonate, pH 8, and collected to result in a final protein concentration of 6.9 mg/ml. Aliquots were used for further investigations.

Assay of MalS Activity—Amylase activity was determined using *p*-nitrophenylhexaoside (PNP6) as a substrate. The release of *p*-nitrophenol from PNP6 by MalS was assayed at room temperature.

Amylase assays of whole cells were performed as described with the exception that cells were grown overnight in M9 medium supplemented with 0.2% casamino acids and 100 μg/ml ampicillin (23).

Activity of purified MalS was determined in an assay buffer of 50 mM Tris-HCl, pH 8.0, 5 mM $CaCl_2$. Assays were carried out in a total volume of 125 μl at room temperature. MalS was added to a final concentration of 1.16 μg/ml (15.7 nM). The reaction was started by adding PNP6 to a final concentration of 1 mM. To determine the effect of various effectors, preincubation of MalS with the corresponding substance was performed for 5 min at room temperature prior to starting the reaction. After appearance of a pale yellow color the absorbance at 405 nm was determined with an Anthos HTII microplate reader. For determination of kinetic parameters, MalS was used at a concentration of 1.45 μg/ml, and PNP6 concentrations between 0.5 and 500 μM were assayed. All assays were performed at least in duplicate. Results varied by $<10\%$.

Assay of Alkaline Phosphatase Activity—Alkaline phosphatase activity in whole cells was assayed by measuring the rate of *p*-nitrophenyl phosphate hydrolysis by permeabilized cells as described (30). 1 unit of alkaline phosphatase activity corresponds to 1 μmol of *p*-nitrophenyl phosphate hydrolysis per min at room temperature. Specific activity is given as units per mg of cellular protein.

Reoxidation of Reduced MalS—1.7 μM MalS was reduced by incubation for 15 min in 30 mM DTT (final concentration). Reoxidation was obtained by adding oxidized glutathione to 130 mM. After incubating 40 min at room temperature, iodoacetamide was added to a final concentration of 150 mM, and after further incubation for 5 min at room temperature samples were precipitated with methanol/$CHCl_3$ (31). The resultant pellet was dissolved in sample buffer and subjected to SDS-PAGE.

Electron Microscopy—The protein was diluted in 20 mM triethanolamine buffer, pH 7.5, to concentrations from 12.5–50 μg/ml and was adsorbed on hydrophilized carbon films, briefly fixed in 0.1% glutaraldehyde in phosphate-buffered saline, washed in water, and negatively stained by 2% uranyl acetate, pH 4.5, or 1% phosphotungstic acid, pH 7.2. Reduced MalS was obtained by incubation with 30 mM DTT prior to adsorption on carbon films. In addition, specimens were prepared by the glycerol spray technique and shadow casted by platinum/carbon as described (32). Specimens were examined in a EM10 C2 (Zeiss, Oberkochen, Germany) under 80 kV at primary magnification of 36,000 ×.

Analytical Sedimentation Equilibrium Ultracentrifugation—Experiments were carried out in a Beckman Instruments Optima XL-A analytical ultracentrifuge. A solution of $A_{280} = 0.1–0.2$ (corresponding to a concentration of 0.6–1.1 μM in terms of protein monomer concentration) in a buffer containing 20 mM Tris-HCl, 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.

$$\Lambda_r = A_0 \cdot \exp\left(\frac{M \cdot (1 - \nu \cdot \rho) \cdot \omega^2 \cdot (r^2 - r_0^2)}{2 \cdot RT}\right) + E \qquad \text{(Eq. 1)}$$

In Equation 1 A_r and A_0 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; ν is the partial specific volume that was calculated from the amino acid composition of the protein to be 0.720 ml g^{-1} (33, 34); ρ is the density of the solvent that was measured to be 1.01 g ml^{-1}; ω is the angular velocity; R is the universal gas constant; 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 Carboxamidomethylated MalS—A MalS solution (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)phosphin (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 H_2O (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/μl. 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 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. In all cases, solvent A was 0.1% trifluoroacetic acid in H_2O 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 1–2 μM. For determination of the disulfide bond-containing peptides, 100 μl of the tryptic peptide-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 H_2O 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 adopted 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 1 μl of acetonitrile, 0.1% trifluoroacetic acid (2:1), pH 2, prior to 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 determina-

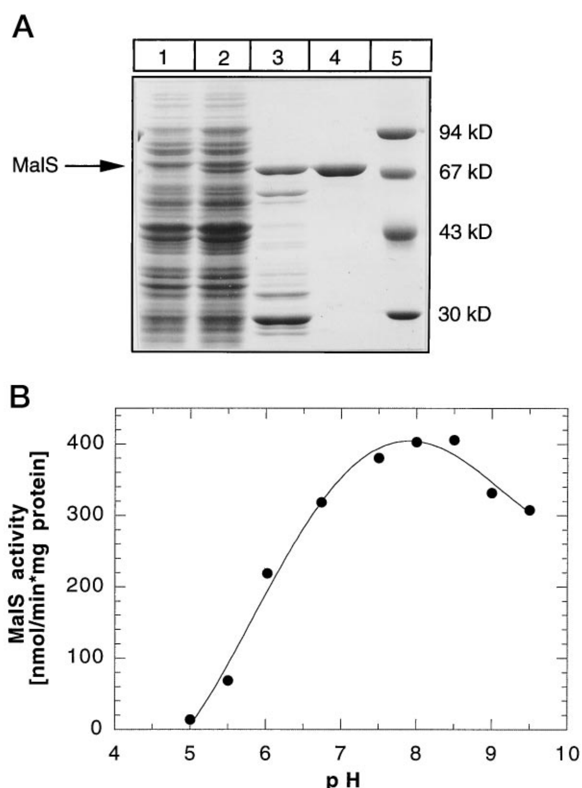

FIG. 1. **Purification and characterization of MalS.** *A, lane 1*, crude extract from CS66 grown in M9 containing casamino acids (0.2% final concentration); *lane 2*, crude extract from CS66 harboring pUMA103 grown in the same medium; *lane 3*, cold osmotic shock fraction; *lane 4*, pooled fractions 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.

tions, acceleration voltage was set to 25 kV, and spectra were calibrated with recombinant human macrophage-colony-stimulating factor (49,030 Da) (37). For peptide mapping experiments acceleration voltage was set to 20 kV and insulin was used for calibration. MalS- and tryptic peptide-containing solutions (0.5 μl) were mixed with 0.5 μl of matrix solution (10 μg/μl 4-hydroxy-α-cyanocinnamic acid, HCCA) dissolved in acetonitrile, 0.1% trifluoroacetic acid (2:1), pH 2, directly on the target. Spectra were recorded after evaporation of the solvent and processed using the X-MASS data system.

Electrospray ionization-mass spectrometry (ESI-MS) was performed with a Vestec-201A quadrupole mass spectrometer (Vestec, Houston, TX). The ion-spray interface temperature was approximately 45–55 °C for all measurements. The mass analyzer with a nominal m/z range of 2000 was operated at 1/8 unit resolution. An electrospray voltage at the tip of the stainless steel capillary needle of 1.4–1.6 kV and a nozzle-repeller voltage of 20–40 V were employed, respectively. Mass calibration was performed with the 8+ to 12+ charged ions of hen egg white lysozyme (14306.5 Da) and with the 13+ to 17+ charged ions of bovine trypsinogen (23980.9 Da). Raw data were analyzed using a Tecnivent Vector-2 data system. MalS samples were delivered into the mass spectrometer by infusion through a 50-μm (inner diameter) fused silica capillary at a flow rate of 1 μl/min using a Harvard-44 infusion pump.

RESULTS

MalS was purified using a simple procedure involving cold osmotic shock followed by ion exchange chromatography (Fig. 1*A*). 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. 1*B*). 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 K_m value was 49 μM, and the maximum velocity (V_{max}) was 0.36 μmol min^{-1} mg^{-1} protein.

TABLE I
Activation of Ca^{2+}-depleted MalS by various bivalent cations

Ca^{2+}-depleted MalS was generated by preincubation with 1 mM EDTA for 5 min. Reactivation assays were carried out by adding chloride solutions of the respective cations at concentrations of 10 mM. After incubation for additional 5 min, the amylase assay was carried out. Prior to EDTA treatment, MalS activity was 403 units.

Cation	MalS activity
	nmol/min × mg protein
	14
Ca^{2+}	400
Ba^{2+}	520
Mg^{2+}	309
Mn^{2+}	296
Zn^{2+}	5

Since the activity of many amylases is dependent on Ca^{2+}, 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 Ca^{2+} and Ba^{2+}, enzymatic activity was restored. We detected that Ba^{2+} even stimulated amylase activity slightly. Although Mg^{2+} and Mn^{2+} restored MalS activity to 77 and 74%, respectively, the addition of Zn^{2+} 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. 2*A*). 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.

ESI-MS molecular weight analysis of nonreduced MalS showed a series of multiply charged molecular ions centering around the $[M + 43H]^{43+}$ molecular ion (Fig. 2*B*), indicating the homogeneity of the sample. The experimentally determined molecular mass of 73,978 Da (±20) was in excellent agreement with the calculated molecular mass (73961 Da) from the translated amino acid sequence; thus, the existence of a covalent multimeric form of MalS or other post-translational modifications besides possible disulfide bond formation could be ruled out. In addition, iodoacetamide alkylation of nonreduced MalS did not yield carbamidomethylated product, indicating the absence of free cysteine residues (data not shown).

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

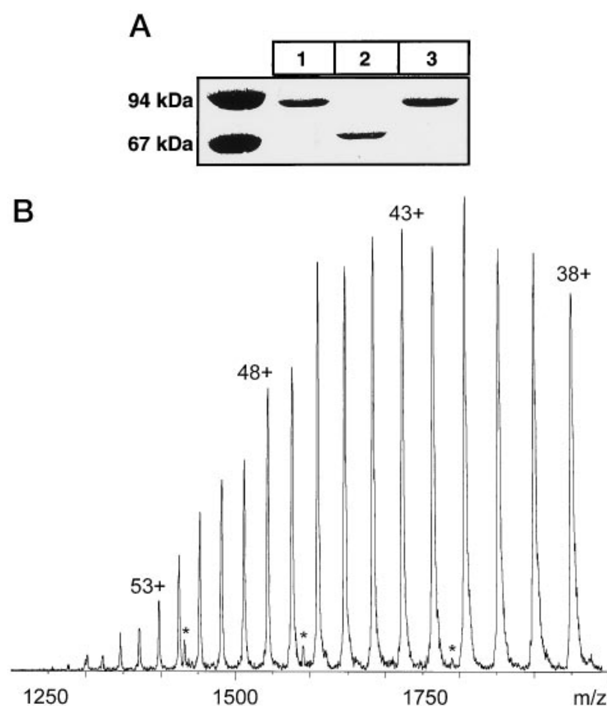

FIG. 2. **Reduction and reoxidation of MalS and ESI-MS mass spectrum of HPLC purified MalS.** *A*, 1.2 μg of purified MalS was subjected to SDS-PAGE. Prior to electrophoresis, samples were incubated without DTT (*lane 1*), with DTT (*lane 2*), and DTT followed by reoxidation with oxidized glutathione (*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 $[M + 43H]^{43+}$ molecular ion. Analysis was performed by injection of 10 μl of nonreduced MalS (1.4 μM) in 10% acetic acid/2,2,2-trifluoroethanol (7:3), pH 2. The molecular mass of MalS was determined to 73978 ± 20 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 we 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 tryptic peptide (amino acids 651–659) of the protein was identified as an ion signal at *m/z* 1000. Cysteine-containing peptide ions were observed, *e.g.* at *m/z* 2461 for carboxamidomethylated T2 and at *m/z* 2172 for carboxamidomethylated T3 (Table II).

Disulfide Bond Mapping—To map disulfide bonds, MalS was digested with trypsin prior to reduction. Digestion was carried out at pH 7.5 at 37 °C but was limited to 2 h to minimize possible disulfide bond scrambling (14, 15, 38). MALDI-MS peptide mapping showed comparable spectra as with reduced MalS, but additional ion signals were observed that could not be addressed as tryptic peptides but as disulfide-bonded dipeptides at *m/z* 4515, assignable as T2–S-S–T3; at *m/z* 2544, assignable as dipeptide T7–S-S–T42; and at *m/z* 2586, assignable as dipeptide T3–S-S–T7, respectively. As more than two possibly disulfide-linked peptide ion signals were detected in the peptide mapping experiments, and to reliably distinguish between the two possibilities T7–S-S–T42 and T3–S-S–T7, which gave only weak ion signals, the tryptic peptide mixture was fractionated by HPLC. MALDI-MS analyses were carried out on all fractions to identify the peptides (Table II, Fig. 4) and showed that separation of the complex peptide mixture was incomplete, and in nearly all fractions two or more peptides coeluted. Interestingly, the peptides with *m/z* 2544 and *m/z* 2586 coeluted in one HPLC fraction and could only be separated by 2-fold rechromatography (*cf.* Fig. 4*C*). The HPLC fractionated peptide with *m/z* 4515 (Fig. 4*A*) was reduced separately in ammonium bicarbonate solution, and MALDI-MS analysis was repeated (Fig. 4*B*). In addition to the complete disappearance of the dipeptide ion signal at *m/z* 4515, two new 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 Cys^{40}–Cys^{58}. 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 used 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* 477) 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

TABLE II
MALDI-MS peptide mapping analysis of tryptic peptides, thiol-alkylated peptides, and disulfide-linked peptides from MalS

Peptide	Partial sequence[a]	$[M + H]^+_{(calc.)}$	$[M + H]^+_{(obed.)}$
T2–S-S–T3	(28–48)-S-S-(49–66)	4515.1	4515
T7–S-S–T42	(103–106)-S-S-(517–534)	2543.9	2544
T1	1–27	2837.1	2837
T2[b]	28–48	2460.7	2461
T3[b]	49–66	2171.4	2172
T4–T5	67–80	1727.9	1729
T5	70–80	1311.4	1312
T6	81–102	2256.6	2259
T8	107–127	2202.5	2002
T9	128–138	1328.5	1329
T10	139–158	2140.5	2141
T11	159–181	2657.8	2658
T12	182–195	1586.6	1586
T13–T14	196–212	1842.0	1841
T15–T16	213–247	3813.3	3814
T16	218–247	3299.7	3302
T17	248–277	3536.7	3537
T18	278–286	1027.1	1028
T20	290–323	3841.3	3842
T23	330–353	2902.1	2901
T24	354–358	606.6	605
T24–T25	354–362	1164.3	1164
T26	363–366	588.8	589
T27	367–391	2775.0	2777
T28	392–405	1501.7	1502
T30–T33	408–441	4086.6	4085
T31	414–421	893.0	893
T32	422–433	1604.8	1605
T33	434–441	943.0	942
T33–T34	434–446	1457.6	1457
T35	447–457	1349.6	1349
T36	458–465	818.9	820
T34–T38	442–469	3235.8	3237
T40	475–499	2962.3	2962
T41	500–516	1986.1	1986
T43	535–550	1838.0	1835
T43–T44	535–553	2255.6	2255
T45–T46	554–594	4220.7	4220
T46	559–594	3733.2	3735
T47	595–605	1267.3	1268
T48	606–615	1085.2	1085
T49	616–620	650.8	651
T50–T51	621–630	978.1	978
T51	623–630	750.9	752
T52	631–637	817.0	817
T53	638–644	826.9	827
T54–T55	645–659	1681.8	1682
T55	651–659	1000.2	1000

[a] Numbers denote amino acid positions as found in the sequence of the mature protein.
[b] Reduced and carboxamidomethylated peptide.

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*.

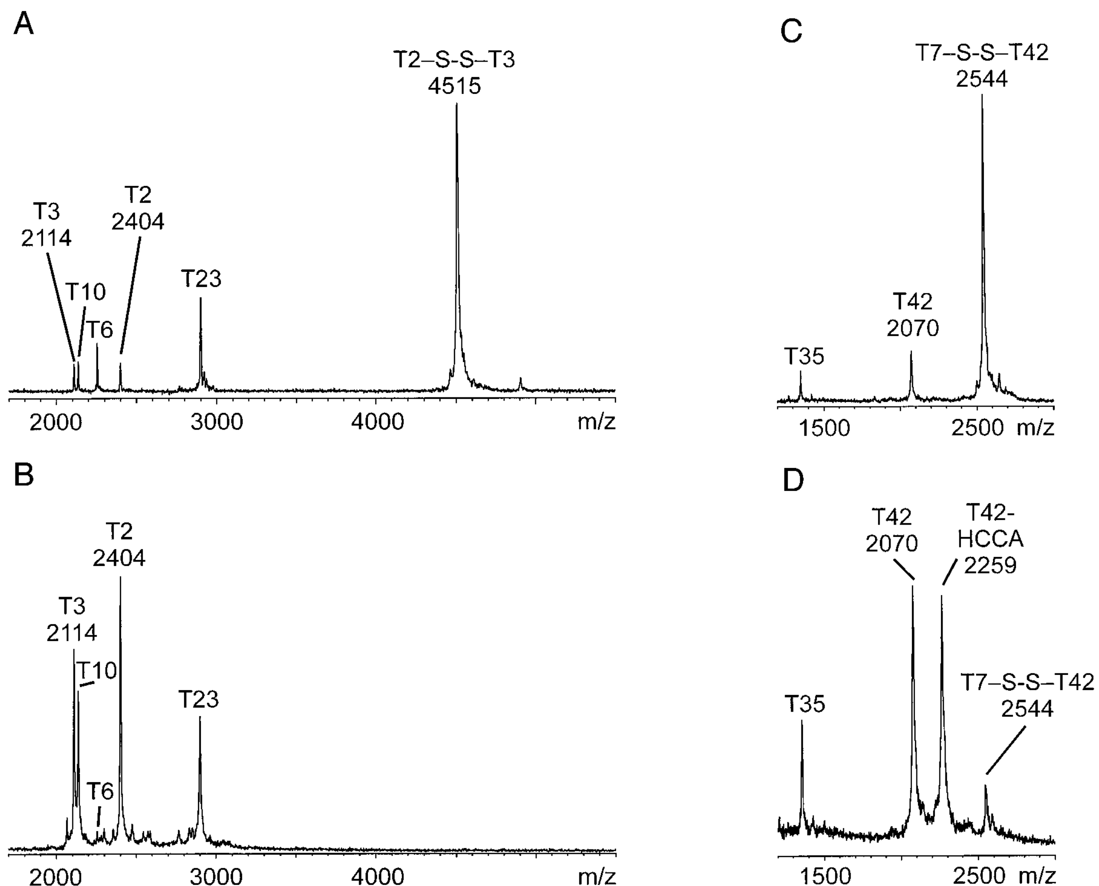

FIG. 4. **MALDI mass spectra of disulfide bond containing peptides after tryptic digestion and HPLC separation.** *A*, before and *B*, after reduction of peptide T2–S-S–T3 with 2-mercaptoethanol in solution, indicating the disulfide bond Cys^{40}–Cys^{58}. *C*, before and *D*, after reduction of peptide T7–S-S–T42 with TCEP on-target, demonstrating the presence of the disulfide bond Cys^{104}–Cys^{520}. 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

malS was expressed from pCS7 in strains CS10 (wild type), CS14 (*trxB::kan*), and CS16 (*gsh::kan*). *phoA* was expressed from pAD135 in strains CS10 (wild type), CS4 (*trxB::kan*), and CS5 (*gsh::kan*). *MalS* and alkaline phosphatase activity was determined in whole cells.

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

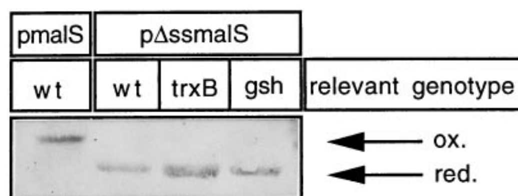

FIG. 5. **Expression of signal sequenceless MalS in the cytoplasm.** Whole cell extracts of strains CS10 (*wt*), CS14 (*trxB::kan*), and CS16 (*gsh::*Tn*10*), expressing Δss*malS* from pCS7 were subjected to SDS-PAGE followed by Western blotting using a polyclonal antiserum to MalS. Electrophoresis was carried out under oxidizing conditions. Free thiols were blocked with iodoacetamide. The position of reduced and of oxidized forms of MalS are indicated.

disulfide-linked dipeptide and the ion signal with *m/z* 2070 for T42 indicated the existence of T7 in this fraction. Thus, it can be concluded that mature MalS contained two disulfide bonds linking Cys^{104}–Cys^{520} and Cys^{40}–Cys^{58} (*cf.* Fig. 3 and Fig. 4). The ion signal at *m/z* 2586 resisted reduction and, thus, did not represent a disulfide-linked peptide. As this ion signal does not correlate to a tryptic peptide, it remains unassigned.

Expression of MalS in the Cytoplasm—To obtain initial evidence for the importance of disulfide bonds for proper folding of MalS, we asked whether MalS can fold into its active conformation when expressed in the reducing environment of the cytoplasm. When we expressed signal sequenceless MalS from pCS7 in wild-type cells, it was enzymatically inactive (Table III). Since alkaline phosphatase, which is known to require disulfide bonds for enzymatic activity (40), does not fold properly in the cytoplasm of wild-type cells, but can be actively expressed in the cytoplasm of *trxB* and *gsh* mutants (41), we tested whether the same result could be obtained for MalS. *gsh* encodes glutathione synthase which, like thioredoxin reductase (TrxB), is thought to be involved in maintaining a reducing environment in the cytoplasm. However, in all strains tested, MalS was inactive when expressed in the cytoplasm. In contrast to alkaline phosphatase, MalS could not fold into an active conformation even in *trxB* or *gsh* mutants (Table III). The absence of MalS activity could be correlated to its inability to form disulfide bonds since only reduced MalS migrating at 74 kDa was detected under nonreducing conditions on SDS-PAGE (Fig. 5).

MalS Activity under Reducing Conditions—In contrast to the above results, the activity of native MalS under reducing conditions indicates that disulfide bonds are not required for MalS function. At room temperature, properly folded MalS was active in standard assay buffer containing either 0.01, 0.1, 1, 10, or 30 mM DTT. In the absence of DTT amylase activity was 292 nmol min^{-1} mg^{-1}. In the presence of the given DTT concentrations it was 330 ± 30 nmol min^{-1} mg^{-1}. To confirm that disulfide bonds were reduced by the addition of DTT, we determined the migration of MalS on SDS-PAGE. MalS migrated at 74 kDa after the addition of DTT indicating that disulfide bonds were reduced (Fig. 2*A*). It should be noted that samples

TABLE IV
Thermal stability of oxidized and reduced MalS

MalS in a 15.7 nM concentration was reduced by incubation for 5 min with 10 mM DTT prior to heat treatment. Incubation at the indicated temperature was done for 5 min. Samples were cooled down to room temperature before starting the assay.

Temperature	−DTT MalS activity	+DTT MalS activity
°C	*nmol/min × mg protein*	*nmol/min × mg protein*
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

Reduction of MalS (15.7 nM) was achieved by preincubation with 10 mM DTT 5 min before addition of SDS.

SDS	MalS activity	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-

tions up to the μM range (Fig. 6).

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 ± 0.88. The complete diameter was 57.5 Å ± 0.63, and the inner diameter of the hollow space was 23.0 Å ± 0.43.

After incubation with DTT, MalS aggregated to form large clumps (Fig. 7*C*). 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.

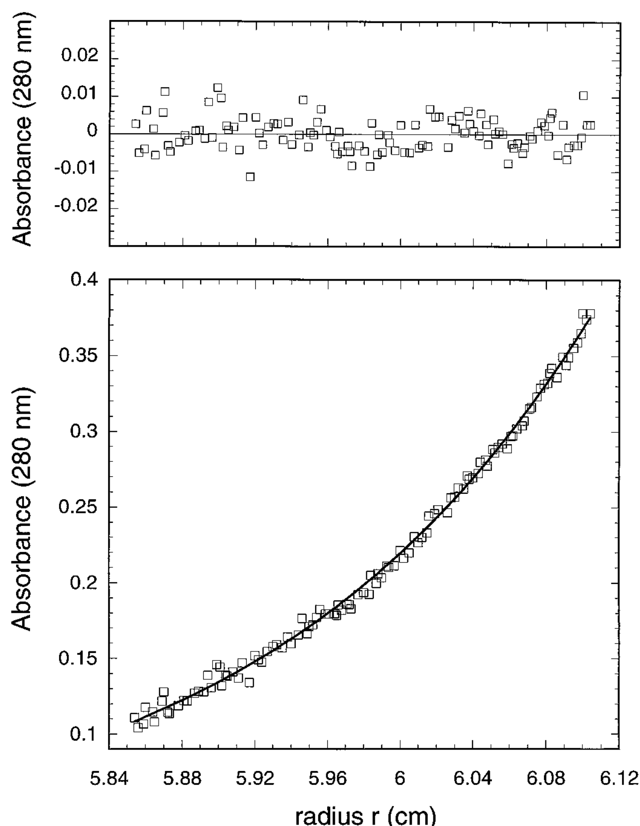

FIG. 6. **Analytical sedimentation equilibrium ultracentrifugation.** Centrifugation was carried out at 10,000 rpm and at 4 °C for 24 h as described under "Materials and Methods." The initial protein concentration was 1.1 μM MalS (A_{280} = 0.2). In the *bottom* part of the figure the absorbance measured at 280 nm (*open squares*) *versus* the radial position (distance to the center of the rotor) is shown. The *solid line* represents the result from the fit of the data points to Equation 1. A molecular mass of 70 kDa was determined from the analysis of this scan. The *top* part of the figure shows the residuals to the fit expressed as the difference between experimental and fitted values.

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 haloplanctis* (47), *Aspergillus shirousamii* (48), *Schwanniomyces 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

[2] K. Uhland and M. Ehrmann, unpublished results.

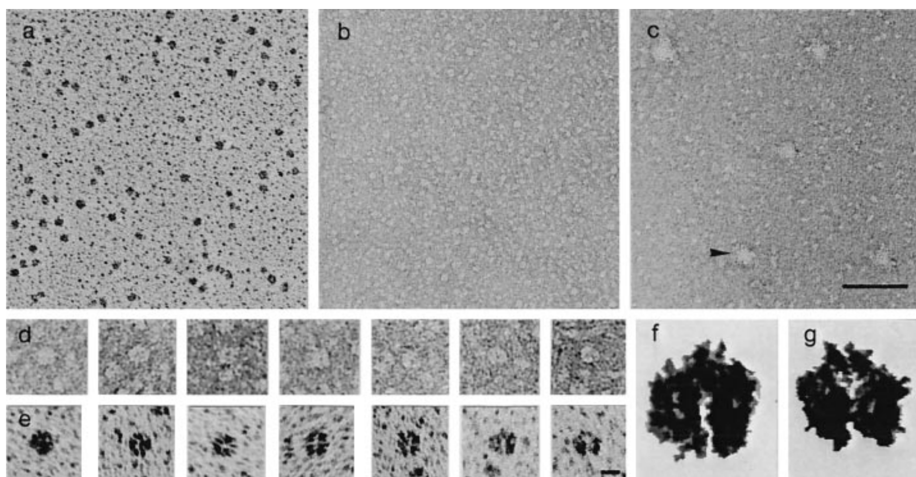

FIG. 7. **Electron microscopy of nonreduced and reduced MalS.** Electron micrographs of isolated MalS. The material was prepared by glycerol spray-Pt/C shadow casting (*a*) and uranyl acetate negative staining (*b* and *c*) techniques. *a* and *b* show the purity and homogeneity of the preparation. After incubation with DTT, MalS aggregated (*c, arrowhead*). Images in lines *d* and *e* show a selection of individual aspects found in specimens prepared by negative staining and shadow casting, respectively. The last two images in line *e* were further processed by background elimination, contrast enhancement, and over-magnification (*f* and *g*). *Bars* in *c* and *e* represent 100 and 10 nm, respectively.

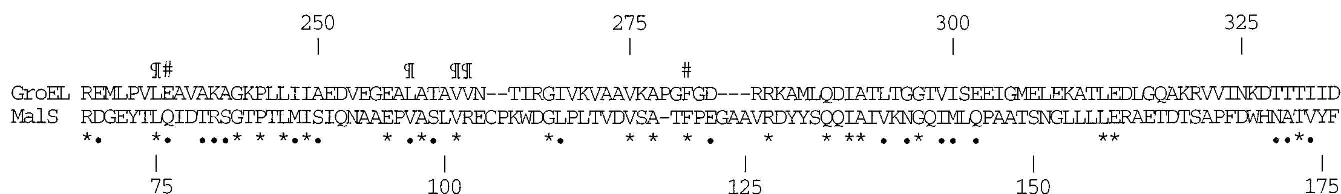

FIG. 8. **Amino acid sequence alignment of the N terminus of MalS and the apical domain of GroEL.** The amino acid sequence of GroEL (residues 230–333) was aligned to residues 69–176 of mature MalS using CLUSTALW 6.1. Point mutants in GroEL, leading to loss of peptide binding (¶) or aggregation of peptide (#) (62); *, identical; ●, conserved amino acid residues.

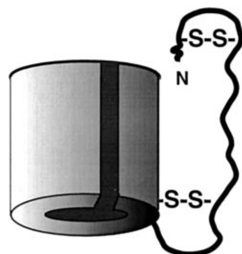

FIG. 9. **Topological model of MalS.** The amylase domain is shown as a *cylinder*. The N terminus is linked via a disulfide bond formed between Cys^{104} and Cys^{520} to the C-terminal amylase domain.

periplasm when exported by an altered secretion machinery, *i.e.* in *prlA* 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 nondenatured 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 dipeptides 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. 4*D*). 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 amylase domain that is connected to the N-terminal segment via the second disulfide bond. The presence of extensions was reported for other amylases; however, these extensions are not N-terminal, are not connected via a disulfide bond to the amylase domain, and do not exhibit homology to the relevant domains of MalS or GroEL (59–61).

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