Role of an Intrusubunit Disulfide in the Association State of the Cytosolic Homo-oligomer Methionine Adenosyltransferase*

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Recombinant rat liver methionine adenosyltransferase has been refolded into fully active tetramers (MAT I) and dimers (MAT III), using as a source chotrope-solubilized aggregates resulting from specific washes of inclusion bodies. The conditions of refolding, dialysis in the presence of 10 mM dithiothreitol or 10 mM GSH with 1 mM GSSG, allowed the production of both isoforms, the nature of the redox agent determining the capacity of the final product (MAT I/III) to interconvert. Refolding in the presence of 10 mM dithiothreitol yielded mainly MAT III in a concentration-dependent equilibrium with the homotetramer MAT I. However, refolding in the presence of the redox pair GSH/GSSG resulted in a stable MAT I and III mixture. Blockage of dimer-tetramer interconversion has been found related to the production of a single intramolecular disulfide in methionine adenosyltransferase during the GSH/GSSG folding process. The residues involved in this disulfide have been identified by mass spectrometry and using a set of single cysteine mutants as cysteines 35 and 61. In addition, a kinetic intermediate in the MAT I dissociation to MAT III has been detected. The physiological importance of these results is discussed in light of the structural and regulatory data available.

Cysteines are one of the least abundant but highly reactive amino acids present in polypeptide chains. Their appearance is, in many cases, related to a key role of the thiol/disulfide group on the structural and functional features of the protein. These side chains have the capacity to actively participate in catalysis, can be posttranslationally modified, or can lead to the apparition of redox sensitive sites that could have effects either on modulation of catalysis or because of the generation of conformational restrictions (1–5). Posttranslational modifications, such as prenylation and acylation, take place in the sulfhydryl group of cysteine residues located at or near the C terminus of the protein, leading to translocation from the cytosol to membranes (4, 5). Nitrosylation occurs in residues related to activity control, either activating (ryanodine receptor) or inactivating key proteins (MAT)1 (6). Zinc chelation determines the acquisition of polypeptide geometries compatible with the recognition of nucleic acids (zinc fingers) (7). Moreover, the presence of cysteine thiol/disulfide groups may also play an essential role in folding and association, because of disulfide bond formation (8, 9). To form this covalent bond, the residues must come to a distance such that the C atoms of the cysteines are within 3.8–4.5 Å of each other (9). These disulfides can be established within the same subunit, linking different areas of the polypeptide chain that may be quite distant in the sequence, or between subunits, binding each monomer on the right position to the others (10, 11).

The production and stability of disulfides have been related to the existence of an oxidative environment. Thus, the number of extracellular or secreted proteins that contain disulfide bridges in their structure is large, because of their passage through a highly oxidative environment, such as that of the endoplasmic reticulum, where the presence of thiol/disulfide oxidoreductases favors their production (12). However, in vitro experiments have demonstrated that it is possible to obtain disulfide bonds even under strong reducing conditions, provided the local concentration of sulfhydryls is high enough (13). Therefore, the presence of disulfides in cytosolic proteins is possible, and in fact, there have been some reports describing their existence (14–15). The role for these covalent bonds is not clear and may be related to the control of protein activity.

One of the few cases where the presence of a cystosolic disulfide has been described is the liver-specific methionine adenosyltransferase (MAT), a homo-oligomeric protein that is isolated as stable tetramers (MAT I) and dimers (MAT III), differing in the affinity for methionine (16). MAT I is 10-fold more active than MAT III under physiological concentrations of the amino acid (60 μM) (17), and hence the ratio of isoforms determine the activity level displayed in the cell. This is of special relevance if we take into account that the product of MAT reaction is S-adenosylmethionine, the main methyl donor for the transmethylation reactions (18). This enzyme presents 10 cysteine residues/subunit (19, 20), and under several in vitro and in vivo conditions it has been possible to demonstrate their role on the activity and oligomeric state: (a) inactivation and dissociation of MAT I is produced by N-ethylmaleimide (NEM) modification of two –SH groups (21, 22); (b) site-directed mutagenesis of Cys60 renders the enzyme mainly as dimers (23); (c) all of the mutants on the cysteines comprised between

1 The abbreviations used are: MAT, ATP: methionine adenosyltransferase; DTT, dithiothreitol; NEM, N-ethylmaleimide; ANS, 8-anilino-naphthalene-1-sulfonic acid; EP, ethylpyridylated; c-MAT, E.coli methionine adenosyltransferase; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; CM, carboxamidomethylated.
residues 35 and 105 have some effect on the MAT I/MAT III interaction fluid was obtained from E&G Wallac (Milton Keynes, UK). Cation exchanger AG-50W-X4, goat anti-rabbit IgG-horseradish peroxidase, 2 ml samples of the column fractions (maximum volume, 1 cm

\( Y = Ae^{-i \theta} + Y \)  
\( Y = Ae^{-i \phi} + Ae^{-2i \phi} + Y \).

where \( Y \) is the fluorescence intensity at 470 nm; \( A \) is the amplitude of the signal; \( i \) is the time; \( k \), \( X \), and \( Z \), the rate constants; and \( Y \), is the fluorescence intensity at an infinite time.

**Experimental Procedures**

**Materials**

Methionine, ATP, phenylmethylsulfonyl fluoride, pepstatin A, aprotinin, leupeptin, antipain, DTT, ampicillin, GSH, GSSG, cyanogen bromide, 4-vinylpyridine, iodacetamide, and the molecular mass standards for gel filtration chromatography were products from Sigma. NEM and trypsin sequencing grade were purchased from Serva (Heidel-}

The total free energy of the process, from MAT I to the unfolded protein, can be calculated using the following expression.

\( \Delta G = \Delta G(H_2O) - mx = -RT \ln(K) \)  
\( \Delta G_{\text{unfolding}} = \Delta G + 2AG \)  

where \( f_D \) is the fraction of protein as dimer; \( c \) is the protein concentration; \( R \) is the gas constant; \( T \) is the absolute temperature; \( \Delta G \) is the free energy of the process; \( \Delta G_{\text{dimerization}} \) is the free energy of the tetramer-dimer conversion; \( \Delta G_{\text{unfolding}} \) is the free energy calculated for the dimer unfolded; \( m \) in this particular case is the slope of the dependence of the thermodynamical parameter upon protein concentration; \( \Delta G_{\text{unfolding}} \) is the free energy of the total process, tetramer to monomer unfolding; and \( K \) is the equilibrium constant.

**Dot Blot**—The samples of the column fractions (maximum volume, 30 ml) were spotted on nitrocellulose membranes. After denaturation using 6 M guanidinium chloride (50 \( \mu \)l), the membrane was washed twice with TTBS (20 ml Tris/HC1, pH 7.5, 500 ml NaCl, 0.05% (v/v) Tween 20), before the blocking step using low fat dry milk (3% w/v).

\[ K f_D + 4c f_D^2 - K = 0 \]

\( \Delta G = \Delta G(H_2O) - mx = -RT \ln(K) \)

\( \Delta G_{\text{unfolding}} = \Delta G + 2AG \)
The membrane was washed again with TTBS and incubated with a 1:20000 (v/v) solution of an anti-MAT polyclonal antibody raised in our laboratory using DTT-refolded MAT. Under these conditions the only band detected corresponds to MAT in SDS-PAGE gels. The membranes were revealed using Renaissance, the exposed films were subjected to densitometric scanning, and the data were used for the corresponding calculations.

**Determination of the Free Sulfhydryl Content and Location of the Disulfide Bond**—The number of free –SH groups for wild type and mutant MATs was determined as described previously using NEM labeling (15, 22). In addition, for quantitation of free cysteine residues and disulfide bonds in wild type and mutant MAT proteins, samples of the purified proteins were dialyzed against ammonium acetate extensively and lyophilized, and the content of reduced and oxidized cysteine residues was determined by mass spectrometry. To this end, ~1 mg/ml of protein in 150 mM Tris/HCl, pH 8.6, 1 mM EDTA, 6 mM guanidinium chloride was heated for 5 min at 80 °C, cooled down to room temperature, incubated either with 10 mM iodoacetamide or 1 h at room temperature or with 1% 2-mercaptoethanol for 2 min at 100 °C, followed by the addition of a 5-fold molar excess of 4-vinylpyridine or iodoacetamide over reducing agent, and incubated for 1 h at room temperature. The samples were dialyzed against deionized MilliQ water and lyophilized. Aliquots of these samples were subjected to matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry using a PE Biosciences Voyager DE-Pro instrument and sinapinic acid (saturated in 0.1% trifluoroacetic acid in 50% acetonitrile) as matrix.

To locate free cysteines and disulfide bonds within the primary structure of MAT (Swiss-Prot S06114), native and ethylpyridylated proteins (10 mg/ml in 70% formic acid) were degraded with cyanogen bromide (100 mg/ml) overnight in the dark under a nitrogen atmosphere. In addition, native and carboxamidomethylated proteins (1 mg/ml in 100 mM ammonium bicarbonate, pH 8.3, 10 mM iodoacetamide) were degraded with sequencing grade trypsin at an enzyme/substrate ratio of 1:50 (w/w) overnight at 37 °C. Mass fingerprinting of the digests was done by MALDI-TOF mass spectrometry using α-cyano-4-hydroxy-cinnamic acid (saturated in 0.1% trifluoroacetic acid in 50% acetonitrile) as matrix.

**N-terminal Sequencing**—N-terminal sequence analyses of the purified wild type and mutant MAT proteins were done using an Applied Biosystems 473A sequencer following the manufacturer’s instructions. The results indicated that 70% of the protein had a complete N-terminal sequence (MNGPVDGL), whereas the rest had lost the initial methionine. These data were taken into account in the MALDI-TOF mass spectrometry calculations.

**Sedimentation Velocity Experiments**—The samples (0.5–5 mg/ml) were loaded on the An50Ti rotor of a Beckman Optima XL-A analytical ultracentrifuge (Beckman Instruments Inc.) equipped with absorbance optics, and the experiments were performed at 50,000 rpm and 18 °C. Absorbance scans (step size, 0.005 cm) were taken at 280 nm. The sedimentation velocity data were analyzed with the program Svedberg (35), and the sedimentation velocity coefficients were corrected for solvent composition and temperature to obtain s_{20,w} (36).

**Determination of the Protein Concentration**—Protein concentration of the samples after ultrafiltration was measured using the Bio-Rad kit I and using bovine serum albumin as a standard. However, protein concentration on the fractions collected from analytical phenyl Sepharose columns was determined after trichloroacetic acid precipitation using the BCA system, because of the presence of MeSO.

**MAT Activity Measurements**—MAT activity was measured as described by Gil et al. (37) using 160-µl samples of the column fractions. Kinetics for methionine and ATP were performed in the concentration range comprised from 1 μM to 10 mM or for one of the substrates while keeping the other constant at 5 mM (2). Oxidation constants and GSSG inhibition were measured as previously described (2, 38).

**RESULTS**

**Association/Dissociation Processes in Liver-specific MAT**—Rat liver MAT has been shown to appear in a concentration-dependent equilibrium upon its overexpression in E. coli cytosol. However, there are no indications that such equilibrium takes place in the rat liver-purified enzyme forms. In an attempt to clarify the difference among these proteins and get insight into the structure-function facts that regulate MAT oligomerization, we have used two refolding systems described previously in our laboratory. The use of DTT rendered only dimers at the protein concentrations used for refolding, but concentration by ultrafiltration caused the apparition of tetramers (Fig. 1, A and B). On the other hand, replacement of DTT by a GSH/GSSG redox buffer allowed the production of a mixture of tetramers and dimers, which do not associate upon enhancement of the protein concentration (Fig. 1, C and D). Reloading of both GSH/GSSG-refolded MAT I and III on gel filtration chromatography showed no variation in the elution position for both association states (Fig. 1E). These results were confirmed on analytical phenyl Sepharose chromatography, a procedure that allows the quantitative separation of MAT I and III forms by means of differences in hydrophobicity (39). Association was also studied by sedimentation velocity, but the use of this technique was precluded because complete dissociation of tetramers to dimers and monomers was already observed at the highest protein concentrations tested (2 mg/ml).

Dissociation of MAT I was then studied by analytical phenyl Sepharose chromatography. As can be observed, isolated DTT-refolded MAT I render tetramers and dimers upon reloading on

**FIG. 1.** Gel filtration chromatography analysis of wild type DTT- and GSH/GSSG-refolded samples. Wild type MAT was refolded using the DTT or GSH/GSSG refolding methods. Samples of the refolded mixtures (100 µl) were injected on a Superose 12 HR column at two protein concentrations and detected by measuring MAT activity (○) and by densitometric scanning of the dot blots corresponding to each collected fraction (○). A and B show the profiles for DTT-refolded MAT at 0.2 mg/ml (A) and 2 mg/ml (B); C and D depict the profiles for the GSH/GSSG-refolded MAT at 0.2 mg/ml (C) and 2 mg/ml (D). E shows the profiles for the rechromatography of MAT I (●) and III (□) and III (●) and □) from D (the data have been scaled for graphical purposes). Elution positions for rat liver purified MAT I (point a) and III (point b) are indicated in A. The figure shows the results of a typical experiment.
the hydrophobic columns, whereas GSH/GSSG-refolded MAT I appears as a stable tetramer (Fig. 2, A and F). More information about the dissociation process was then obtained by fluorescence spectroscopy of ANS-bound DTT-refolded MAT I samples (Fig. 3A). Dilution followed by immediate recording of the fluorescence emission at 470 nm revealed the presence of an exponential decay, until the emission levels corresponding to an ANS-bound dimer were reached (Fig. 3B). This decay was preceded by a short lag phase, suggesting the presence of at least one intermediate in the process (Fig. 3A, inset). The fluorescence intensity of MAT I corresponded to the double of that for MAT III, thus indicating that the dye binds in an area not related to association. This change cannot be followed by intrinsic fluorescence, because no significant changes in the Trp and Tyr emissions were detectable (data not shown). The same is true for other spectroscopic techniques, such as circular dichroism, because MAT I and III show identical spectra. The data were then analyzed using either one- or two-phase exponential decays (Equations 1 and 2), with the best fit being obtained by a single exponential. The calculated rate constant \((k_{\text{off}})\) for dissociation to MAT III is 0.022 s\(^{-1}\), and the calculated half-life for MAT I upon dilution is 14.69 ± 0.5 s at 25°C. Large-zone gel filtration chromatography was then used for the calculation of the dissociation constants (Fig. 4). Using large zones in the 0.05–5 mg/ml range, the centroid volumes for each protein concentration were calculated and used to determine the weight-average partition coefficients (Equation 3) and the dimer fraction (Equation 4). These data were then used to obtain the \(K_d\) value for the DTT-refolded MAT, which was found to be in the 10\(^{-6}\) M \(^{-1}\) range (Table I). Moreover, the free energy of association was calculated using this dissociation constant in Equation 6, as well as using the dimer fractions in Equations 5 and 6 (Table I).

**Role of the Sulfhydryl Groups in MAT Association**—MAT contains 10 cysteine residues/subunit that could be present either in a reduced or partially oxidized pattern. In the present study different oxid/reduction conditions were used for refolding of wild type MAT, and thus several parameters related to the redox status of the protein could differ among the forms obtained. Analysis of the free –SH group content of the different MAT forms was carried out by NEM or vinlypyridine labeling followed by mass spectrometry (Table II). The molecular mass of DTT-refolded MAT was 43,518 Da, which changed to 44,549 Da after treatment with vinlypyridine under denaturing but nonreducing conditions (Fig. 5, A and B). This value was not altered by reduction of the protein followed by ethylenediamine. The difference of 1031 Da corresponded to the addition of 10 (1031/106 = 9.7) EP groups, thus indicating the presence of 10 –SH groups of the DTT-refolded MAT subunit. On the other hand, the molecular mass of the GSH/GSSG-refolded MAT I and III increased by 823 Da upon treatment with vinlypyridine under denaturing but nonreducing conditions, and by 1061 Da when the proteins were fully reduced and ethylenediaminated (Fig. 5C). These results showed that the GSH/GSSG-refolded proteins contained eight titrable cysteine residues and a disulfide bond.

The redox behavior of these proteins was further characterized. Wild type MAT refolded using both procedures is inhibited by GSSG, but the \(K_i\) values calculated are lower for the GSH/GSSG-refolded forms, indicating a higher susceptibility to this agent (Table II). In addition, modulation of the activity by redox buffers is also observed, and the \(K_{i,x}\) values were calculated on the basis of the previous knowledge for this enzyme (Table III). These values are close to the R[GSH] data determined in vivo under mild to severe oxidative stress (300–3 mM) (40).

**C35S and C61S Mutations Abrogate the Oligomer Interconversion Blockage**—The results presented above prompted us to explore the possibility that the presence of a disulfide bond could be responsible for the differences observed among DTT- and GSH/GSSG-refolded MATs. For this purpose, individual mutants on cysteine residues located at the dimer-dimer contact area, according to the crystal structure of the protein, were selected. Of the 10 cysteine residues of the MAT polypeptide chain, four are located at the \(\beta\)-sheet (B2) of contact between dimers (Cys\(^{35}\), Cys\(^{57}\), Cys\(^{61}\), and Cys\(^{69}\)) (Fig. 6). Among those, Cys\(^{61}\) and Cys\(^{69}\) are specific for the liver enzyme and hence may be directly linked to the special behavior shown by this MAT. These four residues were mutated to serine, and the recombinant proteins were refolded using either the DTT or GSH/GSSG system described above and purified. Characterization by CD and fluorescence spectroscopy showed no differ-
The capacity of association of the DTT- and GSH/GSSG-refolded mutants was then explored. As can be observed, all of them are in tetramer-dimer equilibrium upon DTT refolding, a behavior that is conserved in C35S and C61S after GSH/GSSG refolding (Fig. 2). The molecular masses of these MAT mutants, determined after vinylpyridine labeling by MALDI-TOF mass spectrometry were 44,455 Da (Fig. 5). The data were fitted to one- or two-phase exponential decays as described under "Experimental Procedures." The capacity of association of the DTT- and GSH/GSSG-refolded mutants was then explored. As can be observed, all of them are in tetramer-dimer equilibrium upon DTT refolding, a behavior that is conserved in C35S and C61S after GSH/GSSG refolding (Fig. 2). The molecular masses of these MAT mutants, determined after vinylpyridine labeling by MALDI-TOF mass spectrometry were 44,455 Da (Fig. 5), indicating the presence of nine free —SH groups in C35S and C61S under both refolding conditions (Table III). Dissociation constants were also calculated by large-zone gel filtration chromatography, the values being larger than that for the wild type MAT (Table I). On the other hand, stable MAT I/III forms were obtained when using the redox buffer system to refold C57S and C69S (Fig. 2). The fact that the association process is still observed in the absence of Cys35 and Cys61 is consistent with a role for these residues in the oligomerization process.

Identification of the Disulfide Bond in GSH/GSSG-refolded MAT—To ensure that a disulfide bridge between Cys35 and Cys61, such as that detected in liver-purified MAT, is responsible for the blockage of association, free sulphydrols of GSH/GSSG-refolded wild type MAT were ethylisopropylidated (EP) or carboxamidomethylated (CM), and the native and the EP- and CM-GSH/GSSG-refolded wild type MAT proteins were degraded with CNBr and trypsin. The proteins and their diges-

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**Table I**

| Protein       | $K_d$ (μM) | $\Delta G^{\circ}$ (H₂O) (kcal/mol) | $\Delta G^{\circ}$ (H₂O) (kcal/mol) |
|---------------|------------|-------------------------------------|-------------------------------------|
| Wild type     | 3.66 ± 0.64| -7.58 ± 1.3                         | -6.99 ± 0.1                         |
| C57S          | 10.79 ± 0.68| -8.22 ± 0.54                        | -7.79 ± 0.88                        |
| C61S          | 14.83 ± 0.78| -8.41 ± 0.45                        | -9.34 ± 0.14                        |
| DTT-refolding |            |                                     |                                     |
| Wild type     | 10.01 ± 0.47| -8.18 ± 0.39                        | -8.71 ± 0.97                        |
| C57S          | 7.54 ± 0.29 | -8.01 ± 0.3                         | -7.92 ± 0.74                        |

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**Fig. 4.** Determination of the $K_d$ for wild type MAT by large-zone gel filtration chromatography. Large zones for several protein concentrations (0.05–5 mg/ml) were loaded on a gel filtration column. $A_{280}$ was recorded, and fractions of the eluent were collected for its use in dot blot. The centrifoid volume for each zone was determined and used for the calculation of the weight-average partition coefficient and the $f_D$. The $K_d$ value was calculated from the plot of $f_D$ versus log of the dimer concentration (μM). The figure shows a plot of a typical experiment for DTT-refolded wild type MAT.
Wild type MAT samples were refolded with DTT or GSH/GSSG, and the oligomeric forms obtained were characterized and analyzed for their SH content and their behavior against GSSG and GSH/GSSG buffers. The data on the table are the means ± S.D. of the results obtained in experiments carried out in triplicate for the refolded MAT I/III.

**TABLE II**

|                      | DTT-refolded MAT III | GSH/GSSG-refolded MAT III | GSH/GSSG-refolded MAT I |
|----------------------|----------------------|---------------------------|-------------------------|
| $M_r$ on gel filtration chromatography (kDa) | 92,700               | 89,125                    | 194,900                 |
| Specific activity (nmol/min/mg)               | 110 ± 11             | 95.8 ± 4.4                | 100.3 ± 13.1            |
| Free –SH groups (nmol/mg)                     | 10.35 ± 0.4          | 8.04 ± 0.4               | 8.02 ± 0.3              |
| $K_{GSH}$ (nm)                                 | 2.15 ± 0.48          | 0.42 ± 0.07              | 1.02 ± 0.41             |
| $R_{0.5}$ GSH/GSSG                            | 4.01 ± 1.32          | 3.88 ± 0.41              | 6.27 ± 0.96             |
| $K_m$ (mM)                                      | 40.1                 | 38.8                      | 62.7                    |

**DISCUSSION**

MAT isoforms, as purified from the liver, appear as stable dimers and tetramers, whereas the recombinant protein, as isolated from *E. coli*, shows a concentration-dependent equilibrium between MAT I and III (29). These behaviors are paralleled by the MAT forms generated under two different refolding systems, DTT- and GSH/GSSG-based (31), thus providing a useful tool to explore the mechanisms that control association. Analysis of the association process by analytical ultracentrifugation techniques evidenced a dramatic sensitivity of the oligomeric assemblies to centrifugal forces. This phenomenon, experimentally manifested as dissociation, has been described in other oligomeric proteins, such as NAD-dependent dehydrogenases and tryptophan synthase (41). This instability can be experimentally manifested as dissociation, has been described in other oligomeric proteins, such as NAD-dependent dehydrogenases and tryptophan synthase (41). This instability can be explained by the low number of interactions between dimers shown in the MAT I crystal structure (28) (Fig. 7). In fact, only five polar interactions have been described to take place between dimers in contrast to what happens in the *E. coli* MAT (c-MAT) (42). In this last case the number of interactions between dimers in the tetramer structure is far larger than in MAT I, and no dimer structures have been obtained to date. Moreover, the particular arrangement of interactions observed is completely different to that shown in the crystal structure of c-MAT (Fig. 7). Rat liver presents a squared contact area formed by residues of its four subunits that is in direct contact with the solvent. On the other hand, c-MAT has this squared contact area exposed to the solvent, thus protecting those interactions and allowing a higher stability for the complex. Based on these considerations the techniques of choice for our experi-
Wild type MAT and mutants C35S and C61S were refolded in the presence of either DTT- or GSH/GSSG-containing buffers. The dimeric forms were purified in each case and characterized kinetically. In addition, samples were used for free –SH determination by MALDI-TOF. The data shown are the means ± S.D. of three independent experiments carried out in triplicate.

### Table III
Characterization of the refolded MAT III forms

| V\text{max} | S\text{cys Met} | S\text{cys ATP} | Free –SH groups |
|-------------|-----------------|-----------------|----------------|
| DTT-refolded |                 |                 |                |
| Wild type   | 110 ± 11        | 246 ± 76        | 588 ± 108      |
| C35S        | 94.47 ± 6.3     | 222 ± 61        | 493 ± 47.5     |
| C61S        | 69.07 ± 16.5    | 766 ± 31.9      | 3250 ± 220     |
| GSH/GSSG-refolded |         |                 |                |
| Wild type   | 95.8 ± 4.4      | 1120 ± 150      | 530 ± 48       |
| C35S        | 32.7 ± 0.56     | 449.6 ± 17.7    | 777.6 ± 47.8   |
| C61S        | 31.5 ± 0.21     | 793.8 ± 129.2   | 2540 ± 139     |

### Table IV
Results of the tryptic digestion analyzed by MALDI-TOF mass spectrometry

| Fragment | Sequence modifications |
|----------|-----------------------|
| Dn       |                       |
| 2551.9   | 34–48                 |
| 6588.3   | 55–62                 |
| 6727     | 49–54                 |
| 2306.6   | 63–82                 |
| 2264.8   | 63–82                 |
| 2233.1   | 63–82                 |
| 1006.0   | 80–98                 |
| 3261.1   | 99–126                |
| 2553.9   | 104–126               |
| 3775.2   | 127–160               |
| 503.6    | 161–184               |
| 605.1    | 165–189               |
| 4969.5   | 127–170               |
| 1510.2   | 170–182               |
| 1354.8   | 171–182               |
| 3321.8   | 171–200               |
| 1898.2   | 183–200               |
| 2306.3   | 201–220               |
| 1622.8   | 221–235               |
| 1182.6   | 225–235               |
| 584.7    | 235–250               |
| 584.7    | 236–259               |
| 1430.8   | 253–285               |
| 1080.4   | 291–300               |
| 1621.9   | 294–308               |
| 446.2    | 305–308               |
| 576.7    | 309–313               |
| 942.5    | 345–352               |
| 1286.5   | 353–363               |
| 1025.2   | 355–363               |
| 603.7    | 364–368               |
| 1098.1   | 375–383               |
| 1118.6   | 384–392               |

### Fig. 6
Localization of Cys\textsuperscript{35}, Cys\textsuperscript{57}, Cys\textsuperscript{61}, and Cys\textsuperscript{69} in rat liver MAT I crystal structure. The figure shows the two strands of \(\beta\)-sheet B2, where Cys\textsuperscript{57}, Cys\textsuperscript{61}, and Cys\textsuperscript{69} are located (Protein Data Bank code 1QM4). In addition, the position of Cys\textsuperscript{35} on \alpha\text{-}helix 1 is also included.

Sulfhydryl groups are shown in green, and the dashed lines indicate the hydrogen bonds stabilizing the \(\beta\)-sheet. In gray are the positions that Cys\textsuperscript{35} and Cys\textsuperscript{61} should have to form a disulfide bond.

Differences among the refolding systems used are based in their redox potential; thus it could be possible that the opposite association behavior shown by DTT- and GSH/GSSG-refolded MAT may be due to an effect related to the redox state of the cysteine sulfhydryls. MAT polypeptide contains 10 such residues/subunit that under the redox buffer could originate reduced or partially oxidized proteins (19, 20). Our results indicate that two of the 10 –SH groups are not accessible to alkylation, even after guanidinium chloride denaturation, thus suggesting their involvement in a disulfide. These residues have been identified using single cysteine mutants as Cys\textsuperscript{35} and Cys\textsuperscript{61}, the same residues involved in titration, thus suggesting their involvement in a disulfide.
association. The presence of these cysteines in the β-sheet of contact between dimers and the fact that Cys51 is a liver-specific amino acid may be related to the special features shown by the liver-specific MAT, such as its capacity to generate two oligomeric assemblies (16). In fact, the sulfhydryls of these two cysteines appear perfectly oriented and at bonding distance in the crystal structure of MAT I (obtained in the presence of DTT) (28). As for Cys57 and Cys69 (another liver-specific cysteine), both are also located in the β-sheet B2 and close enough as to form a disulfide bond, but their sulfhydryl groups are facing opposite directions (Fig. 6). Thus, it could be possible that under certain circumstances this second disulfide is formed, but for this purpose a slight torsion of the main chain of the protein would be needed. Therefore, it can be deduced that alterations in this β-sheet of contact could be responsible for the changes observed in the oligomerization.

Intramolecular disulfides are known to have a role in protein oligomerization (46) but are not commonly observed in such a reducing environment as cellular cytosol (10, 14). However, during folding it is possible that a large increase in the local sulfhydryl concentration occurs, allowing the production of disulfides. Moreover, in vitro experiments have shown the production of optimal oxidation rates during folding, even in the presence of significant concentrations of reductants (13). In vivo, using alcohol liver cirrhotic samples, an increase in the oxidation conditions is also observed that was caused by a 30% reduction in the GSH levels. Under these conditions a decrease in MAT activity correlated with its apparatus mainly as dimers has been described (27), but no explanation for this incapacity to associate has been obtained for the moment. Based on our data, we suggest that under cirrhotic oxidative conditions the production of dimers may be favored by allowing the disulfide bond (Cys55-Cys61) to form, before association to MAT I takes place. Another possibility that cannot yet be excluded is the production of another disulfide (Cys57-Cys69) or of both disulfide bridges (Cys55-Cys61 and Cys57-Cys69) in cirrhotic MAT dimers. In these cases, association may not be precluded exclusively by the presence of the disulfide bonds but also by the incapacity of establishing the correct polar interactions on the interface, because of the main chain torsion. Moreover, a third possibility exists: the presence of two types of dimers. Our results on the kinetics of MAT I dissociation suggest the presence of an intermediate in the pathway to MAT III. Even when it is not possible to infer the oligomeric state of such an intermediate, it is attractive to suggest that it may be a dimer just separated from MAT I, before undergoing the small changes that lead to MAT III. These changes should occur close to the ANS-binding site, located between Pro508-Gly509 and the sequence Val-Gly-Ala that limits the active-site loop as suggested by Sánchez del Pino et al. (47), because a kinetic intermediate is detectable by this modification. All of these data together showing the effects exerted by dissociation on areas related to the active site, such as the flexible loop and the central domain may explain the 10-fold decrease in methionine affinity shown by MAT III as compared with MAT I or its activation by Me₂SO (2, 17).

Using the previous knowledge on MAT III urea unfolding (47, 48) and the results described herein, we propose the following model for MAT I folding and association.

**Model 1**

The unfolded MAT polypeptide (U) associates to the dimer (MAT III) through a monomeric equilibrium-intermediate (I), which evolves to a monomeric kinetic intermediate (I²) and the dimer (MAT III) could then undergo oxidation of Cys35 and Cys61 to make a disulfide bond that precludes further association or evolve to a kinetic intermediate (I³) that leads to the tetramer (MAT I). The production of the Cys35-Cys61 disulfide in MAT I must then take place in this last kinetic intermediate before definitive association in the tetramer. The total free energy of the process can be calculated using Equation 7, and in our case this renders a ΔG(H₂O) of 24.41 kcal/mol.

Finally, all of these results indicate that the presence of a disulfide bond between Cys35 and Cys61 is responsible for the stabilization of tetramer and dimer forms, blocking the association process. The production of this disulfide takes place at the dimer level (MAT III and I²).

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