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Assignment of a single disulfide bridge in rat liver methionine adenosyltransferase

María L. Martínez-Chantar and María A. Pajares

Instituto de Investigaciones Biomédicas 'Alberto Sols' (CSIC-UAM), Madrid, Spain

Rat liver methionine adenosyltransferase incorporated 8 mol of *N*-ethylmaleimide per mol of subunit upon denaturation in the presence of 8 M urea, whereas 10 such groups were labelled when dithiothreitol was also included. This observation prompted a re-examination of the state of the thiol groups, which was carried out using peptide mapping, amino acid analysis and N-terminal sequencing. The results obtained revealed a disulfide bridge between Cys35 and Cys61. This disulfide did not appear to be conserved because cysteines homologous to residue 61 do not exist in methionine adenosyltransferases of other origins, therefore suggesting its importance for the differential aspects of the liver-specific enzyme.

Keywords: disulfide bond; methionine adenosyltransferase; *N*-ethylmaleimide; sulfhydryl groups.

Methionine adenosyltransferases (MATs) are a family of cytosolic proteins that have been exceptionally well conserved during evolution [1]. The information encoding MAT is present in the genome of all organisms studied to date, except for some metanogenic archaebacteria [2,3]. The importance of this family of enzymes is due to the key role of their product, *S*-adenosylmethionine (AdoMet), as the main methyl donor for transmethylation reactions and the propylamine supplier for polyamine synthesis following decarboxylation [4]. It is synthesized from ATP and methionine, and pyrophosphate and inorganic phosphate are also formed as side products [5]. MAT enzymes show several common features: they appear as oligomeric proteins in all tissues and organisms studied, Mg^{2+} ions are needed for activity, monovalent cations (K^{+}) are activators of these enzymes, most of the enzyme forms show sigmoidal kinetics for their substrates, and they exhibit tripolyphosphatase activity [4]. In mammals, two isoenzymes have been detected, one ubiquitous (MAT II) and another liver specific [6]. This latter protein appears in two oligomeric forms, a tetramer (MAT I) and a dimer (MAT III), composed of the same type of subunit [7,8]. The dimer form of MAT differs from MAT I in its lower affinity for methionine. Decreases in MAT activity have been observed in several liver diseases, and in these cases changes in the MAT I/III ratio have been detected, the dimer being the prevalent form [9]. These data have led to speculation about the role of MAT I and MAT III in the liver, and the mechanisms that control the ratio of the forms [4].

The rat liver MAT gene has been cloned and its product found to be composed of 396 amino acid residues, including 10 cysteines [10,11]. Several experiments demonstrating the importance of such residues in the activity and oligomeric state of rat liver MAT have been reported previously. Thiopropyl–Sepharose chromatography of rat liver MAT I and elution in the presence of reduced glutathione (GSH) led to changes in its methionine kinetics, as well as in its electrophoretical mobility [12]. Rat liver MAT is inhibited by oxidized glutathione (GSSG) inactivation, which produces dissociation [12]. Inhibition by GSSG is modulated by GSH [12]. Substitution of Cys residues by Ser using site-directed mutagenesis produces changes in the MAT I/III ratio compared with the wild-type enzyme. This shift is specially important in the Cys69Ser mutant, which appears only as dimers [13]. Chemical modification of two sulfhydryl groups with *N*-ethylmaleimide produces inactivation of either MAT I or MAT III, and dissociation of the former to a dimer [14]. Modification by nitric oxide donors also leads to enzyme inactivation, which is not observed in the Cys121Ser mutant [15,16]. Finally, neither *N*-ethylmaleimide nor nitric oxide modifications, even using denatured MAT, lead to the labelling of more than eight Cys groups [17,18]. These results indicate the presence of two nonaccessible sulfhydryl groups, which may be involved in a protein disulfide.

Homology among the several MAT proteins cloned to date is high [1], and hence the conservation of residues important for its function and structure, such as Cys, is expected. However, neither the number of Cys residues (5–10 per monomer) nor their localization in the sequence is constant [13]. In fact, only Cys35, Cys57 and Cys105 (numbered according to the rat liver sequence) are present in most of the sequences from *Escherichia coli* to mammals, while Cys9, Cys61, Cys69, Cys121 and Cys377 seem to be liver specific [13]. These differences between liver and nonliver MATs, the importance that Cys69 seems to have in the oligomerization, and the possible presence of a disulfide bond may be related to the fact that only liver forms appear as stable tetramers and dimers. Therefore, this study focused on the identification of the nonaccessible Cys residues of rat liver MAT I/III and clearly establishing its possible involvement in a disulfide bond.

Correspondence to M. A. Parjares, Instituto de Investigaciones Biomédicas 'Alberto Sols' (CSIC-UAM), Arturo Duperier 4, 28029 Madrid, Spain. Fax: + 34 91 585 4587, Tel.: + 34 91 585 4601, E-mail: mapajares@iib.uam.es

Abbreviations: AdoMet, *S*-adenosylmethionine; GSSG, oxidized glutathione; GSH, reduced glutathione; MAT, methionine adenosyltransferase; PMSF, phenylmethanesufonyl fluoride; STI, soybean trypsin inhibitor; TFA, trifluoroacetic acid.

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MATERIALS AND METHODS

Materials

Methionine, ATP, benzamidine, phenylmethanesufonyl fluoride (PMSF), soybean trypsin inhibitor (STI), trypsin and dithiothreitol were from Sigma Chemical Co. *N*-Ethylmaleimide was purchased from Serva (Germany). [2-^{3}H]ATP (20 Ci·mmol^{-1}) and [1,4^{14}C] *N*-ethylmaleimide (2–10 mCi·mmol^{-1}) were supplied by Amersham International (Little Chalfont, UK) and ARC Inc. (St Louis, MO, USA), respectively. DEAE–Sephacel, phenyl Sepharose CL-4B and blue Sepharose CL-6B were purchased from Pharmacia LKB. Optiphase HiSafe 3 scintillation fluid was obtained from E & G Wallac (Milton Keynes, UK). Endoproteinase–Glu-C was from Boehringer Mannheim. Cation exchanger AG-50W-X4, Bio-Gel A 1.5-m, Biogel HTP, Bio-Rad protein assay and the electrophoresis reagents were from Bio-Rad. The Ultrasphere ODS C_{18} column was obtained from Beckman (San Ramón, CA, USA). Glass fibre filters and YM-30 ultrafiltration membranes were products of Whatman Ltd (Maidstone, UK) and AMICON Corp. (Beverly, MA, USA), respectively. Urea, 2-mercaptoethanol and dimethylsulfoxide were purchased from Merck (Darmstadt, Germany). All other buffers and reagents were of the best quality available commercially.

Purification of rat liver MAT

Rat liver MAT I and MAT III were purified as described by Pajares *et al*. [12], excluding the thiopropyl Sepharose chromatography.

Determination of MAT activity

MAT activity was measured essentially as described by Cabrero *et al*. [7]. The final reaction volume was 250 µL and contained saturating concentrations of the substrates [5 mM methionine and 5 mM [2-^{3}H] ATP (1 Ci·mol^{-1})]. Incubation was carried out for 30 min at 37 °C, and was stopped by the addition of 3 mL of distilled water. After separation of the product, radioactivity was determined in the presence of 1 mL acetic acid and 10 mL scintillation fluid.

Sulfhydryl group labelling

Purified proteins, MAT I and MAT III (1 mg), were lyophilized and resuspended in 100 µL of 85 mM Hepes/Na pH 8 containing 85 mM KCl, 58 mM $MgCl_2$ and 8 M urea, in the presence or absence of a 10-fold molar excess of dithiothreitol over the expected sulfhydryl groups, and the solution was incubated for 1 h at 37 °C. [^{14}C] *N*-Ethylmaleimide (5 µCi) was then added with cold *N*-ethylmaleimide in a fivefold molar excess over the total content of -SH groups (-SH groups of dithiothreitol plus -SH groups of protein) and the alkylating reaction was carried out at room temperature for 1 h. To stop the reaction a 10-fold molar excess of 2-mercaptoethanol was added and the protein was precipitated by the addition of 1 mL 10% (v/v) trichloroacetic acid. A portion of the sample (100 µg) was then loaded onto glass fibre filters, washed five times with 10 mL trichloroacetic acid 10%, dried and counted in the presence of 10 mL scintillation fluid in order to calculate the number of free -SH groups in each case. The remaining protein (0.9 mg) was washed three times with trichloroacetic acid 10%, and the final pellet was resuspended in 100 µL of buffer and subjected to proteolysis.

Proteolysis of the samples

Trypsinization was carried out as described by Deigner *et al*. [19]. Endoproteinase–Glu-C treatment was performed at 37 °C for 5 h, and initiated by the addition of 1 : 50 (w/w) of the protease to the labelled protein sample in 100 µL of 20 mM ammonium bicarbonate pH 7. The reaction was stopped by freezing and the samples were lyophilized.

Peptide mapping

Proteolysed samples were resuspended in 100 µL of 0.1% trifluoroacetic acid (TFA) in water (solvent A) and injected on a C_{18} HPLC column (250 × 4 mm; 10 µm particle diameter) equilibrated in this same buffer. Chromatography was carried out at 1 mL·min^{-1}, and after a 5-min washing period a three-step gradient was developed. The first step included a 13 min gradient from 100% solvent A to 21% 0.1% TFA/50% propanol-2 in water (solvent B). The second step was developed for 60 min to 79% solvent B and the third step for 10 min until 100% solvent B. Fractions were collected each 0.5 min and divided, 50 µL were counted and the rest lyophilized. Repurification of the isolated peptides was carried out using the same HPLC column and a gradient described previously using 0.1% TFA in water (solvent A) and 0.1% TFA in 50% acetonitrile (solvent B) [17].

Amino acid analysis and N-terminal sequencing of the *N*-ethylmaleimide-labelled peptides

Purified peptides were hydrolysed in the presence of 6 M HCl for 2 h at 110 °C, and amino acid analysis of the samples was carried out on a Beckman System 6300 Autoanalyzer as described previously by Deigner *et al*. [19]. As for peptide sequencing, samples were analysed on an Applied Biosystems Procise Sequencer.

SDS/PAGE

Denaturing gel electrophoresis was performed in order to establish the purity of the protein samples. For this purpose, samples containing 10–20 µg of protein were loaded onto 10% polyacrylamide/SDS gels under reducing conditions using the buffer system described by Laemmli [20]. Detection was performed using either Coomasie Brilliant Blue or silver staining.

Protein concentration determination

The protein concentration of the samples was measured using the method described by Bradford [21], with the Bio-Rad protein assay kit and BSA as the standard.

RESULTS AND DISCUSSION

Purified rat liver MAT can be labelled in its sulfhydryl groups using either *N*-ethylmaleimide or nitric oxide donors [14,15]. Maximum labelling was achieved using urea-denatured MAT I/III, and in that case only 8 of the 10 Cys sulfhydryls present in the sequence were modified [17,18]. However, the 10 residues were chemically modified when purified MAT I/III were incubated with urea and a 10-fold molar excess of

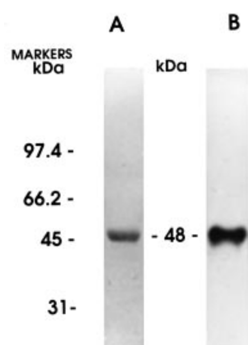

Fig. 1. SDS/PAGE of rat-liver-purified MAT I and MAT III. Rat liver MAT I and MAT III were purified as described in Materials and methods. Five micrograms of MAT I (A) and 8 µg of MAT III (B) were loaded onto a 10% SDS/PAGE gel and detected using Coomasie Brilliant Blue. The molecular mass of the markers is given on the left-hand side of the figure, and the M_r of the MAT band appears in the centre.

dithiothreitol. These results indicated the presence of two nonreacting thiols in each subunit, and suggested the possible presence of a disulfide bridge. Intersubunit disulfides have not been detected by SDS/PAGE under nonreducing conditions, but changes in electrophoretic mobility were obtained upon thiopropyl–Sepharose chromatography [12], thus suggesting an intrasubunit disulfide. Moreover, inhibition and oxidation constants for rat liver MAT in glutathione redox buffers have been calculated either in the presence or absence of thioltransferases, and the mechanism of the reaction appeared to involve the formation of intramolecular disulfides [12,22]. In order to study this possibility rat liver MAT I and MAT III were isolated to homogeneity (Fig. 1), and urea denatured either in the presence or absence of dithiothreitol. The samples were then labelled with ^{14}C-*N*-ethylmaleimide and proteolysed using trypsin. Table 1 shows the peptides expected to be labelled by this procedure. Peptides were separated on reverse-phase HPLC and the peptide maps obtained were compared (Fig. 2). Urea-denatured samples (U) of either MAT I or MAT III showed less labelling than those treated with urea and dithiothreitol (UDTT; Fig. 2A,C). In the latter case, most of the radioactivity appeared early in the chromatogram as a broad peak (fraction 29-UDTT), that coincided with the elution position for excess dithiothreitol. Moreover, both samples presented very similar profiles except for the disappearance of a shoulder on fraction 31-UDTT of the dithiothreitol/urea-denatured protein (Fig. 2A, arrow) and the apparition of a broader peak on fraction 42/43-U of the urea-treated MAT (Fig. 2C, arrow), either I and III. Changes in this area of the chromatograms were also detected when the absorbance profiles at 214 nm were compared (Fig. 2B,D). The absorbance for fraction 29-UDTT/31-UDTT was reduced on the urea-treated protein and a new peak corresponding to fraction 43-U was detectable (Fig. 2D, arrow). These changes were studied in more detail, and for this purpose the labelled peaks were collected and HPLC analysed again using a second set of conditions.

Peaks derived from dithiothreitol/urea-treated samples after the second HPLC analysis showed the presence of only one *N*-ethylmaleimide peptide, except for that corresponding to fraction 41-UDTT (Fig. 3A). In this later case two labelled peptides were separated. Repurified-labelled peaks were divided into three aliquots that were used for endoproteinase Glu-C treatment, amino acid analysis and N-terminal sequencing. Proteolysis with endoproteinase Glu-C was carried out in order to distinguish between those peptides containing several labelled residues, because the presence of glutamic acid residues in their sequences between Cys residues allowed this protease to cut tryptic peptides to obtain others containing a single Cys (Table 1). Fractions 46-UDTT, 51-UDTT and 91-UDTT contained protease susceptible peptides (Fig. 3D–F). Two labelled peaks were obtained upon endoproteinase Glu-C proteolysis of peaks 46-UDTT and 91-UDTT (Fig. 3D,F), and three from 51-UDTT (Fig. 3E). Amino acid analysis and N-terminal sequencing allowed the identification of these peptides as those corresponding to residues 55–62, 104–160 and 104–126, respectively (Table 2).

Urea-denatured MAT I and MAT III peptides were also analysed using a second HPLC gradient and the isolated peptides were divided into four aliquots and used for endoproteinase Glu-C treatment, dithiothreitol incubation, amino acid analysis and N-terminal sequencing. The results obtained were similar to those observed in the dithiothreitol/urea-treated samples, except for fraction 43-U. Upon incubation with dithiothreitol this new peak was reanalysed on HPLC using the first gradient. One peak of radioactivity was obtained (fraction 46, Fig. 4A), in addition to two peaks of absorbance

Table 1. Cysteine-containing tryptic peptides derived from rat liver methionine adenosyltransferase. The sequences of the tryptic peptides containing the 10 cysteine residues of MAT are shown below. Cysteine residues are underlined.

Peptide sequence	Cysteine-contained residues	Peptide
MNGPVDGLCDHSLSEEGAFMFTSESVGEGHPDK	Cys9	1–33
ICDQISDAVLDAHLK	Cys35	34–48
VACETVCK	Cys57 and Cys61	55–62
TGMVLLCGEITSMAMIDYQR	Cys69	63–85
TGMVLLCGEITSMAMIDYQRVVR	Cys69	63–88
TGMVLLCGEITSMAMIDYQRVVRDTIK	Cys69	63–92
TCNVLVALEQQSPDIAQCVHLDR	Cys105 and Cys121	104–126
TCNVLVALEQQSPDIAQCVHLDRNEEDVGAGDQGLMFGYATDETEECMPLTIVLAHK	Cys105, Cys121 and Cys150	104–160
NEEDVGAGDQGLMFGYATDETEECMPLTIVLAHK	Cys150	127–160
AGLCR	Cys312	309–313
AGLCRR	Cys312	309–314
AGLCRRVLVQVSYAIGVAEPLSISIFTYGTSK	Cys312	309–340
TACYGHFGR	Cys377	375–383
TACYGHFGRSEFPWEVPK	Cys377	375–392

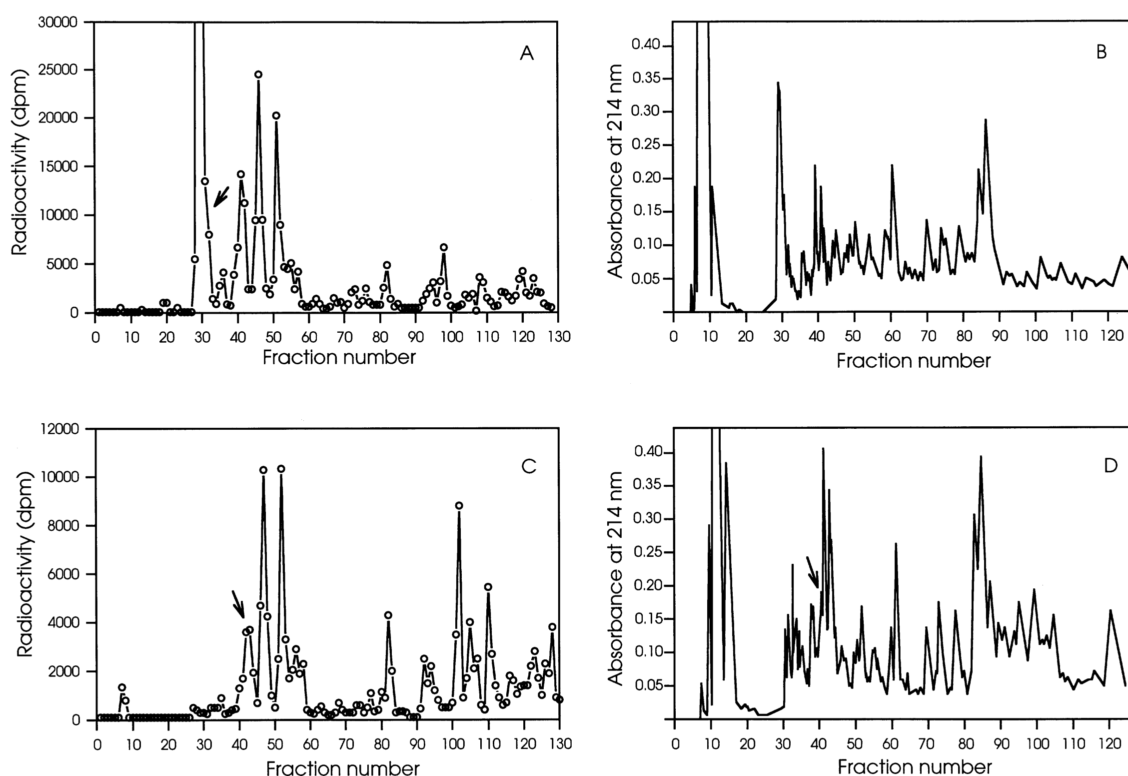

Fig. 2. Tryptic peptide maps of ^{14}C-*N*-ethylmaleimide-labelled MAT III. Purified rat liver MAT III was urea-denatured in either the presence (sample UDTT, A and B) or absence (sample U, C and D) of dithiothreitol. Protein samples were then chemically modified in their free -SH groups using ^{14}C-*N*-ethylmaleimide and proteolysed with trypsin. Peptides were separated on HPLC as described in Materials and methods at a flow rate of 1 mL·min^{-1}, and 500 μL fractions were collected. In order to detect the labelled peptides containing free -SH groups, a small sample (50 μL) of the fractions was counted in the presence of scintillation fluid (A and C). In addition, the absorbance at 214 nm was also recorded (B and D). The peaks containing the labelled peptides were collected separately and kept for further purification. The figure shows the part of the HPLC chromatogram where radioactivity was detected. Arrows indicate those areas of the chromatogram where changes were detected. Identical results were obtained using rat liver purified MAT I.

corresponding to fractions 31 and 46 (Fig. 4B). This behaviour is compatible with the presence of a disulfide bridge linking one peptide containing one cysteine to another containing two. Amino acid analysis and N-terminal sequencing of these fractions revealed that they correspond to peptides 34–48 and 55–62, respectively (Table 2). Therefore, a disulfide bond between Cys35 and Cys57 or Cys61 was present in the protein samples. To finally clarify this point a portion of fraction 43-U was treated with endoproteinase Glu-C, the labelled peak purified and used for N-terminal sequencing. The residues identified corresponded to VA-, results that were compatible with labelling on Cys57, and hence, clearly established the presence of a disulfide between Cys35 and Cys61 of the MAT sequence. The same results were obtained using either MAT I or MAT III.

Cysteines have been demonstrated to be important for the activity and oligomerization of rat liver MAT [13,14]. The importance of some of these residues suggested that their conservation should be fundamental for this family of proteins. Surprisingly, neither Cys69 nor Cys121 appeared in all the known sequences, but only in the liver-specific enzymes [13]. The same was true for Cys61, while Cys35 appeared in almost all the cloned MATs. This fact revealed some peculiarities of the hepatic forms probably related to its appearance as a tetramer and dimers, which may represent an advantage when responding to changes in substrate concentration. The availability of X-ray diffraction data for *E. coli* MAT [23] made possible the construction of a model for the rat liver enzyme structure [16]. In this model Cys35, Cys57, Cys61 and Cys69

Table 2. N-terminal sequencing and amino acid analysis of the labelled peptides. Peptides isolated after proteolysis of the urea/dithiothreitol-treated rat liver MAT III were repurified and used for the determination of their amino acid content and N-terminal sequencing. The results obtained are shown below and the cysteine residues are indicated on the side column. Identical results were obtained using urea/dithiothreitol-treated rat liver MAT I.

N-ethylmaleimide-labelled tryptic fraction	Amino acid analysis	Sequence	Cysteine-containing residues
31	34–48		Cys35
35	127–160	NEE-	Cys150
41	309–313 309–314	AGL-	Cys312
46	55–62	VA-E	Cys57 and Cys61
51	104–160	T-	Cys105, Cys121 and Cys150
55	375–383	TA-Y	Cys377
57	375–392		Cys377
82	1–33	MNGP-	Cys9
91	104–126		Cys105 and Cys121
93	309–340		Cys312
101	63–85		Cys69
104	63–88		Cys69
106	63–92		Cys69

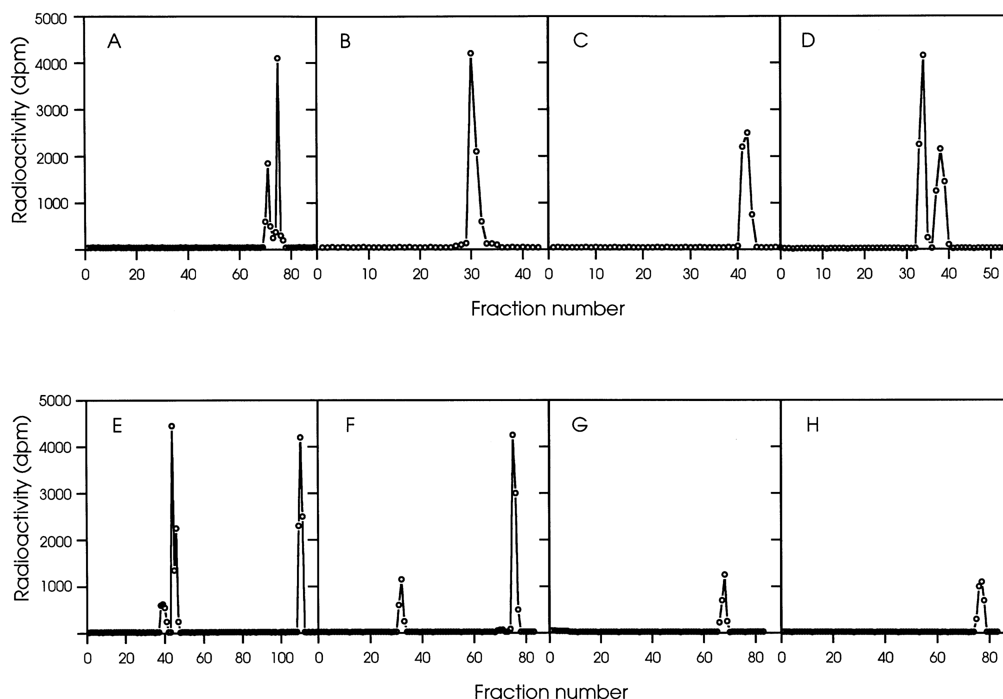

Fig. 3. Analysis of the ^{14}C-*N*-ethylmaleimide labelled MAT III tryptic peptides. Tryptic peptides derived from MAT III were collected and repurified using a second HPLC gradient as described in Materials and methods. A flow rate of 1 mL·min^{-1} was used and 500 μL fractions were collected. All the peaks analysed showed the presence of a unique labelled peptide, except for fraction 41 dithiothreitol (A). For further characterization of these peptides an aliquot was treated with endoproteinase Glu-C in order to distinguish between those containing two cysteines. The results obtained for the tryptic peptides are shown as follows: (B) 31-UDTT, (C) 41-UDTT, (D) 46-UDTT, (E) 51-UDTT, (F) 91-UDTT, (G) 93-UDTT and (H) 104-UDTT. The figure shows that part of the HPLC chromatograms where radioactivity was detected. Identical results were obtained when MAT I tryptic peptides were treated using the same procedures.

appeared very close in the structure, with the last three residues located in a β sheet that seemed to be the area of contact for two dimers. In fact, the Cys69Ser mutant of the protein led only to the production of dimer forms, a result that suggested its direct relationship with MAT oligomerization [13]. Moreover, only two of these cysteines seemed to be oriented properly and within the bonding distance as judged from the rat liver MAT crystal structure (J. Sanz-Aparicio and M. A. Pajares, unpublished results), Cys35 and Cys61. Our experimental data therefore coincide with the structural data in that the residues involved in the disulfide bond are those expected.

Disulfide bond formation between cysteine residues is one of the post-translational modifications most dependent on the protein conformation. Even when the cytosol is a strongly reducing environment and disulfide linked proteins are scarce [24–26], some of these bonds have been detected in proteins such as the cytosolic branched-chain amino transferase and the human glucocorticoid receptor [27,28]. The possibility exists that during protein folding the local concentration of sulfhydryls becomes high enough to allow disulfide bridge production in such a reducing environment. In fact, optimal rates of oxidative folding are obtained at significant reductant concentrations [29]. Comparison of MAT sequences showed that the liver enzymes have evolved with increasing numbers of cysteines in a particular location of the protein. The presence of a disulfide bridge at Cys35–Cys61, close to the area of contact between the dimers, could give more stability to the β sheet, and hence to the whole oligomer. Disappearance of this bond may destabilize this part of the structure allowing easier aggregation or dissociation as observed with *E. coli* overexpression of rat liver MAT [30]. The implication of intramolecular disulfides in protein oligomerization is not

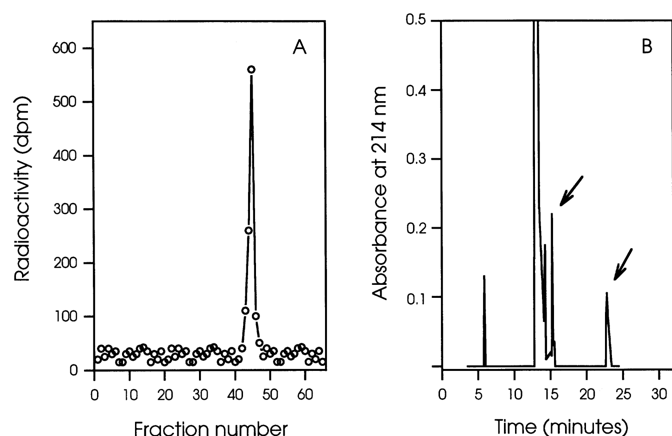

Fig. 4. Dithiothreitol treatment of fraction 43-U. Fraction 43-U obtained from the urea-denatured MAT III sample, after purification using the two HPLC methods described in Materials and methods, was incubated with dithiothreitol. The effect of this treatment was then analysed using the first HPLC purification gradient. Again 500 μL fractions were collected and 50 μL samples of those counted in the presence of scintillation fluid. (A) The radioactivity profile detected upon fraction collection, (B) the absorbance recorded at 214 nm. Peaks of interest are indicated by an arrow. Identical results were obtained for fraction 43-U of urea-denatured MAT I.

new and has been observed in some cell-surface or secreted proteins [31], however, to the best of our knowledge no description of such a role for cytosolic enzymes has been made to date. Finally, in addition to the importance of this finding for the stability of the protein oligomers, our results provide evidence of a new example of a disulfide-containing cytosolic protein.

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