Biochemical Basis for the Dominant Inheritance of Hypermethioninemia Associated with the R264H Mutation of the *MAT1A* Gene

A MONOMERIC METHIONINE ADENOSYLTRANSFERASE WITH TRIPOLYPHOSPHATASE ACTIVITY*

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Methionine adenosyltransferase (MAT) catalyzes the synthesis of *S*-adenosylmethionine (AdoMet), the main alkylating agent in living cells. Additionally, in the liver, MAT is also responsible for up to 50% of methionine catabolism. Humans with mutations in the gene *MAT1A*, the gene that encodes the catalytic subunit of MAT I and III, have decreased MAT activity in liver, which results in a persistent hypermethioninemia without homocystinuria. The hypermethioninemic phenotype associated with these mutations is inherited as an autosomal recessive trait. The only exception is the dominant mild hypermethioninemia associated with a G-A transition at nucleotide 791 of exon VII. This change yields a *MAT1A*-encoded subunit in which arginine 264 is replaced by histidine. Our results indicate that in the homologous rat enzyme, replacement of the equivalent arginine 265 by histidine (R265H) results in a monomeric MAT with only 0.37% of the AdoMet synthetic activity. However the tripolyphosphatase activity is similar to that found in the wild type (WT) MAT and is inhibited by PP$_i$. Our *in vivo* studies demonstrate that the R265H MAT I/III mutant associates with the WT subunit resulting in a dimeric R265H-WT MAT unable to synthesize AdoMet. Tripolyphosphatase activity is maintained in the hybrid MAT, but is not stimulated by methionine and ATP, indicating a deficient binding of the substrates. Our data indicate that the active site for tripolyphosphatase activity is functionally active in the monomeric R265H MAT I/III mutant. Moreover, our results provide a molecular mechanism that might explain the dominant inheritance of the hypermethioninemia associated with the R264H mutation of human MAT I/III.

Methionine adenosyltransferase (MAT,[1] EC 2.5.1.6.) is a key

metabolic enzyme that catalyzes the synthesis of the most important biological alkylating agent, *S*-adenosylmethionine (AdoMet) (1). The synthesis of AdoMet occurs in a two-step reaction. In the first step AdoMet and PPP$_i$ are synthesized from the substrates methionine and ATP. Subsequently the tripolyphosphate generated is hydrolyzed to PP$_i$ and P$_i$ before the products of the reaction are released (2, 3). The function of the tripolyphosphatase activity in the overall reaction catalyzed by MAT is still under discussion (4). In mammalian tissues three forms of MAT have been described that are the products of two distinct genes (5–8). The gene *MAT2A* encodes a 396-amino acid catalytic subunit expressed in extrahepatic tissues, as well as in fetal liver and hepatocarcinoma, that associates with a regulatory β subunit to form MAT II (6, 9). The gene *MAT1A* encodes a 395-amino acid subunit, expressed in adult liver, that organizes into dimers, MAT III, and tetramers, MAT I (5, 10–14). The reason for the presence of these two different isoenzymes in liver has not been yet elucidated, although it may be an adaptation to the metabolic requirements of the liver. The liver has the highest specific activity of MAT, which agrees with the observation that up to 85% of all methylation reactions and as much as 50% of methionine catabolism occur in this tissue (15). Based upon the different kinetic properties of MAT I and MAT III isoforms, MAT III has been considered the liver-specific enzyme. While MAT I, similarly to MAT II in extrahepatic tissues, may maintain the basal AdoMet levels required by cells, MAT III would be responsible for the clearance of methionine after a load of this amino acid.

Two decades or more ago, it was observed that several newborn children, screened for hypermethioninemia as an indicator of homocystinuria due to a deficiency in cystathionine β-synthase activity, presented a persistent hypermethioninemia with normal plasma levels of homocystine and tyrosine and without severe liver disease (16–19). Enzymatic studies demonstrated that the origin of this isolated persistent hypermethioninemia was a severe depletion of MAT activity in liver, whereas the activity of MAT II in erythrocytes, lymphocytes, and fibroblasts of these individuals was normal (16, 17, 19, 20). Clinical and metabolic features in more than 30 such patients have been described (20–23). The characterization of the human *MAT1A* gene led to the detection of 17 different mutations that cause the previously reported depletion of MAT activity in liver (24–28), designated, therefore, as MAT I/III deficiency.

[1] The abbreviations used are: MAT, methionine adenosyltransferase; AdoMet, *S*-adenosylmethionine; DTT, dithiothreitol; P$_i$, orthophosphate; PP$_i$ pyrophosphate; PPP$_i$, tripolyphosphate; WT, wild type; PAGE, polyacrylamide gel electrophoresis.

Segregation and mutation analysis revealed that in most of the individuals with *MAT1A* mutations the hypermethioninemic phenotype is transmitted as an autosomal recessive trait (22–25, 28). However, a dominant inherited form of this abnormality has been reported in five families (21, 22, 26, 28–30). In each such family a G-A transition at nucleotide 791 was detected in one *MAT1A* allele. This change results in a MAT in which arginine 264 is replaced by histidine (26). Crystallographic studies of *Escherichia coli* and recombinant rat liver MAT show that the equivalent arginine 244 or 265, respectively, is located in the interface between the two subunits of the dimeric enzyme and is involved in a salt bridge with glutamic 42, or the homologous 58 in the rat enzyme, of the symmetric subunit, which contributes to the stabilization of the oligomeric state of MAT (31, 32, 40). Moreover, arginine 244 contributes to each active site and is located in the immediate vicinity of the polyphosphate group of ADP (4, 31). It has been shown that replacement of arginine 264 by histidine in human MAT inactivates the enzyme. Moreover, it has been proposed that this mutation hinders normal oligomeric formation (26). However, substitution of arginine 244 by leucine or histidine in *E. coli* MAT resulted in an inactive enzyme, which, in contrast, remains tetrameric with no apparent changes in the secondary structure (4). To understand the biochemical basis of the dominant inheritance of the phenotype associated with the R264H mutation of human MAT I/III, we have purified and characterized the homologous rat R265H MAT I/III mutant. Our data indicate that the active site for the tripolyphosphatase activity is functionally active in the monomeric R265H MAT I/III mutant and provide a molecular mechanism that might explain the dominant inheritance of the hypermethioninemia associated with the R264H mutation in human MAT I/III.

EXPERIMENTAL PROCEDURES

Materials—Columns and chromatography media were from Amersham Pharmacia Biotech. AdoMet was from Boehringer Ingelheim (Knoll). All other reagents were from Sigma.

Site-directed Mutagenesis—A 1.2-kilobase fragment containing the rat *MAT1A* coding region (33) was subcloned into a pET vector. The resulting plasmid includes a 5′-sequence that encodes for 6 histidine residues and a thrombin cleavage site in frame with the rat liver *MAT1A* coding region. Mutants were obtained by inverse polymerase chain reaction according to the procedure of Pérez-Mato *et al.* (34). The mutants were identified by sequencing the complete MAT cDNA.

Expression and Purification of His-tagged WT and MAT I/III Mutants—WT and MAT I/III mutants were overexpressed in *E. coli* BL21(DE3) as described previously (33). Recombinant His-tagged WT and MAT I/III mutant proteins were purified from the bacterial cytosolic extracts by affinity chromatography on a Ni^{2+}-Sepharose column equilibrated in 50 mM Tris/HCl, pH 8, 0.5 M NaCl, 75 mM imidazole. Elution was performed using a linear gradient from 75 to 500 mM imidazole in the same buffer. His-tagged MAT I/III proteins elute from the Ni^{2+} column at 250 mM imidazole. Protein purity was always more than 95% as estimated by SDS-PAGE (35). Imidazole was removed by dialysis against 10 mM Tris/HCl, pH 7.5, 150 mM KCl, 5 mM DTT. No changes in the fluorescence, CD spectra, oligomeric state, and enzymatic activity were observed after digestion of the recombinant proteins with thrombin (data not shown). Therefore all the assays were carried out using the His-tagged recombinant proteins.

Association between WT and R265H MAT I/III Mutant Subunits—To study the formation of heterodimers *in vivo*, bacteria were cotransformed with the plasmid pSSRL (12) to produce the WT MAT subunit and a pET vector with the coding region of the R265H MAT mutant to produce a His-tagged protein. His-tagged proteins were purified from the cytosolic extracts of cotransformed bacteria following the procedure indicated above. The molecular mass of the purified protein was estimated by size exclusion chromatography. To assess the subunit composition, the recombinant protein was denatured by incubation with 5 M urea in 50 mM Tris/HCl, pH 7.5, 150 mM KCl, 10 mM $MgCl_2$, 5 mM DTT. Then, the mixture was chromatographed on a 1-ml Ni^{2+}-Sepharose column equilibrated with 50 mM Tris/HCl, pH 8, 0.5 M NaCl, 75 mM imidazole. After loading the sample, the column was washed

with 10 column volumes of the same buffer. Elution was carried out with 5 column volumes of 50 mM Tris/HCl, pH 8, 0.5 M NaCl, 0.5 M imidazole, and 0.5-ml fractions were collected. Protein elution was monitored by measuring the absorbance at 280 nm. The protein-containing fractions were pooled, and aliquots were analyzed by size exclusion chromatography and SDS-PAGE (35). Heterodimer formation was also tested *in vitro* in two ways. In the first, MAT III purified from rat liver was incubated with equimolar concentration of the R265H mutant His-tagged protein (0.2 mg/ml) in 50 mM Tris/HCl, pH 7.5, 150 mM KCl, 10 mM $MgCl_2$, 5 mM DTT for 30 min at 25 °C. Alternatively, MAT III purified from rat liver was denatured by incubation with 5 M urea in 50 mM Tris/HCl, pH 7.5, 150 mM KCl, 10 mM $MgCl_2$, 5 mM DTT, and then refolded by 20-fold dilution (the final concentration of WT MAT subunits was 0.6 μM) in the same buffer containing a 3-fold molar excess of His-tagged R265H MAT I/III mutant subunit, followed by incubation for 30 min at 25 °C. The two mixtures were then re-purified on a Ni^{2+} column as indicated above. Imidazole was removed from the retained fractions using a 5-ml Hi-trap desalting cartridge. Protein was then denatured by adding urea to a final concentration of 5 M and chromatographed again on a 1-ml Ni^{2+}-Sepharose column equilibrated with 50 mM Tris/HCl, pH 8, 0.5 M NaCl, 75 mM imidazole. Flow-through and imidazole eluted fractions were analyzed, in both experiments, by SDS-PAGE (35).

Purification of MAT from Rat Liver—MAT III was purified from rat liver according to the procedure described previously (36). Protein purity was more than 95% as estimated by SDS-PAGE (35).

Size Exclusion Chromatography—Protein samples were analyzed using a Superdex 200 HR 10/30 column equilibrated with 50 mM Tris/HCl, pH 7.5, 150 mM KCl, 10 mM $MgCl_2$ in an AKTA fast protein liquid chromatography (Amersham Pharmacia Biotech). After sample injection, proteins were isocratically eluted at a flow rate of 0.8 ml/min. Fractions of 0.2 ml were collected. Protein elution was monitored by measuring the absorbance at 280 nm. The elution volume (in ml) of the standard proteins were: tiroglobulin (669 kDa), 8.1; ferritin (440 kDa), 10.22; catalase (232 kDa), 12.11; ovalbumin (43 kDa), 14.73; chymotripsin A (25 kDa), 18; ribonuclease A (13.7 kDa), 19. According to the elution volume of the standard proteins, the estimated molecular mass of the R265H MAT I/III rat mutant protein was 41 kDa. AdoMet synthetic as well as tripolyphosphatase activities were measured in the collected fractions.

Enzymatic Activity Measurements—Activity assays were performed in a final volume of 100 μl, at 37 °C for 10 min in 50 mM Tris/HCl, pH 8, 4 mM $MgCl_2$, 250 mM KCl, 3.6 mM DTT. 2 mM ATP/$MgCl_2$ and methionine or 2 mM tripolyphosphate were used as substrates to determine AdoMet synthetase or tripolyphosphatase activities, respectively. Both activities were monitored by following the formation of inorganic phosphate according to the method of Lanzetta (37). The effect of NO on the tripolyphosphatase activity of the R265H mutant was determined in the absence of DTT. Activation of the tripolyphosphatase activity by substrates was studied by enzyme preincubation with 2 mM methionine and 2 mM ATP.

RESULTS

Oligomeric State of the R265H MAT I/III Mutant Protein—The molecular mass and catalytic properties of a purified R265H MAT I/III mutant protein have been studied by size exclusion chromatography. The elution volume of the R265H MAT was 16 ml (Fig. 1), which corresponds to a molecular mass of 41 kDa according to the elution profile of standard proteins. This result suggests that this mutant protein is a monomeric MAT.

Kinetics of the Monomeric R265H MAT I/III Mutant—To test the enzymatic activity of the R265H MAT I/III mutant protein, fractions were collected, and AdoMet synthetic activity and tripolyphosphatase activity were measured. Interestingly, the analysis of the R265H fractions revealed that, although no AdoMet synthetic activity was detected, a tripolyphosphatase activity peak was measured at the same elution volume as the absorbance peak (Fig. 1). This result indicates that tripolyphosphate hydrolysis can be catalyzed by a single MAT subunit. In contrast, AdoMet synthetic activity requires at least a dimeric enzyme, since this active site is constituted between two monomers.

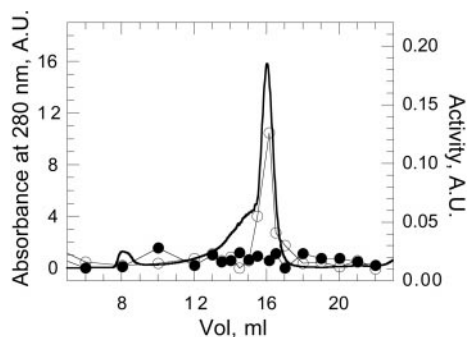The catalytic properties of the R265H MAT mutant were

FIG. 1. **Analysis by size exclusion chromatography of the R265H MAT I/III mutant.** The molecular mass of the His-tagged R265H MAT I/III mutant, that had been purified by Ni^{2+}-Sepharose column chromatography, was estimated by size exclusion chromatography on a Superdex 200 HR 10/30 column. —, absorbance profile at 280 nm. Fractions of 0.2 ml were collected and tripolyphosphatase (*open circles*) and AdoMet (*closed circles*) activities were measured as described under "Experimental Procedures." The estimated molecular mass of the R265H MAT mutant is 41 kDa.

then further studied. MAT and tripolyphosphatase activities of the MAT mutant R265H were measured at 2 mM of methionine and ATP or tripolyphosphate (Fig. 2). Both activities were determined as the accumulation of inorganic phosphate (P$_i$) after incubation at 37 °C for different periods of time. Our data indicate that while the V_{max} for the tripolyphosphatase activity of the R265H mutant was very similar to that determined for a WT enzyme, AdoMet synthetic activity of this MAT variant was decreased by more than 99% (Table I). Replacement of arginine 265 by serine instead of histidine resulted in a monomeric MAT protein (data not shown) with more than 99% reduction of the AdoMet synthetic activity and a 5-fold decrease in the tripolyphosphatase activity (Table I), suggesting that the positive charge of the residue at position 265 is involved in the hydrolysis of PPP$_i$. Tripolyphosphatase activity of the R265H MAT I/III mutant was dependent on Mg^{2+} concentration, but K$^+$ was not required (not shown). No change of tripolyphosphatase specific activity was observed by increasing the protein concentration (0.026–0.345 mg/ml). Additionally, to verify that the monomer does not reassociate under assay conditions, a gel filtration molecular mass determination in a column equilibrated with 2 mM PPP$_i$ was performed. Under these conditions the estimated molecular mass of the R265H MAT mutant was 41.6 kDa (not shown). These two lines of evidence indicate that the monomeric state of this MAT mutant is maintained after incubation with PPP$_i$, and therefore, the tripolyphosphatase activity of the R265H MAT mutant is not a consequence of subunit dimerization. In contrast to WT protein, preincubation with methionine and ATP did not stimulate the tripolyphosphatase activity (not shown). R265H tripolyphosphatase activity is specific for PPP$_i$. Less than 2% of the hydrolytic activity measured in the presence of PPPi was observed when ATP, PP$_i$, or metatripolyphosphate were used as substrates (Table II). No significant changes of enzymatic activity or K_m were detected when the tripolyphosphatase activity of the R265H MAT I/III mutant was tested in the presence of ATP (Fig. 3). However, a decrease of the enzymatic activity from 128 to 67 nmol min^{-1} mg^{-1} was observed when the tripolyphosphatase activity was measured in the presence of 2 mM PP$_i$, a classical inhibitor of the tripolyphosphatase activity of MAT (Fig. 3). Since the Hill coefficient was always close to 1.0, the experimental data were fitted to the Michaelis-Menten equation. The K_m for PPP$_i$ was increased from 84 to 143 μM when the tripolyphosphatase activity was measured in the presence of PP$_i$. NO regulates hepatic MAT activity through specific interaction with cysteine residue 121 (33, 38). To analyze the effect of NO on the tri-
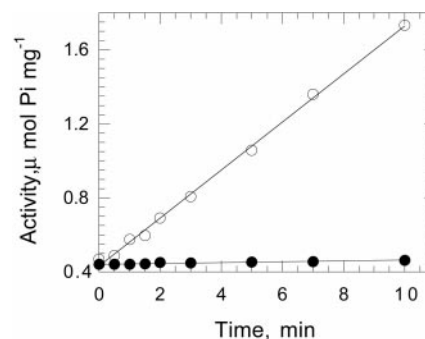

FIG. 2. **AdoMet synthetic and tripolyphosphatase activity of the R265H MAT I/III mutant.** Tripolyphosphatase (*open circles*) and MAT (*closed circles*) activities of a purified R265H MAT I/III mutant were measured by following the formation of P$_i$ after incubation with the substrates for different periods of time. Tripolyphosphatase and MAT activity values were 129 and 2 nmol min^{-1} mg^{-1}, respectively.

TABLE I
Enzymatic activity and estimated K_m values for PPP_i of mutants and wild type recombinant rat liver MAT

	$V_{max}^{ATP,Met}$	V_{max}^{PPPi}	K_m^{PPPi}
	nmol min^{-1} mg^{-1}		μM
WT MAT	588 ± 63	156 ± 21	20 ± 5
R265H MAT	2 ± 0.23	128 ± 13.7	83 ± 6
R265S MAT	1 ± 0.12	29 ± 7.4	122 ± 5

TABLE II
Substrate specificity of the monomeric MAT mutant R265H
Substrate concentration was 2 mM.

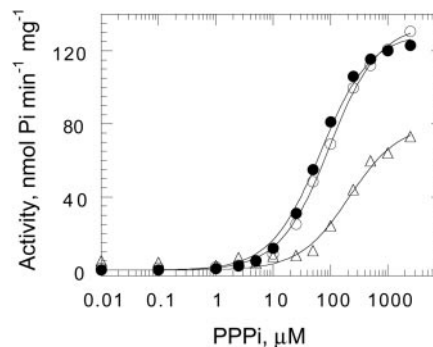|	Enzymatic activity
	nmol min^{-1} mg^{-1}
PPP$_i$	128 ± 13
ATP	1.2 ± 0.73
PP$_i$	1.9 ± 0.68
MP3	0.4 ± 0.22

FIG. 3. **Inhibition of the tripolyphosphatase activity of the R265H MAT I/III mutant by PP$_i$.** The inhibition of tripolyphosphatase activity of the R265H MAT I/III mutant by ATP and PP$_i$ was examined. Tripolyphosphatase activity of the R265H mutant was measured in the absence (*open circles*) and in the presence of 5 mM ATP (*closed circles*) or 5 mM PP$_i$ (*triangles*). The experimental data were fitted to the Michaelis-Menten equation. Values of V_{max} and K_m for PPP$_i$ were 128 nmol min^{-1} mg^{-1} and 83 μM, respectively, in the absence of any inhibitor; 67 nmol min^{-1} mg^{-1} and 143 μM, respectively, in the presence of PP$_i$; and 129 nmol min^{-1} mg^{-1} and 78 μM, respectively, in the presence of ATP.

polyphosphatase activity of the R265H MAT I/III mutant, the enzyme was nitrosylated by incubation with 250 μM nitrosylated glutathione before determining its capacity to hydrolyze tripolyphosphate. No variations of the activity were observed after nitrosylation of this enzyme. The enzymatic activities were 125 and 129 nmol P$_i$ min^{-1} mg^{-1}for the non-nitrosylated and nitrosylated forms, respectively.

FIG. 4. ***In vivo* association between WT and His-tagged R265H MAT mutant subunits.** Proteins were obtained and purified as described under "Experimental Procedures." Hetero-oligomers were chromatographed on a Ni^{2+}-Sepharose column before (*A*) or after (*B*) denaturation with 5 M urea. The presence of MAT protein in the flow-through fraction of the affinity column was indicative of association between WT and His-tagged mutant subunits, which were eluted with 500 mM imidazole. Proteins were identified by SDS-PAGE. *FT*, flow-through fraction from the Ni^{2+}-Sepharose column; *R*, fraction retained on the Ni^{2+}-Sepharose column; *Mr*, molecular mass standards (bovine serum albumin, 81 kDa; ovalbumin, 47.7 kDa; carbonic anhydrase, 34.6 kDa).

Association between WT and R265H Mutant MAT I/III Subunits—The capacity of the R265H MAT I/III mutant to form hetero-oligomers with a WT subunit has been examined both *in vivo* and *in vitro*, taking advantage of the N-terminal His-tag of the mutant protein. If such an interaction occurred, mutant and WT subunits would combine in a hetero-oligomeric form that would be retained on a Ni^{2+}-Sepharose column. When MAT purified from bacteria overexpressing WT and R265H MAT subunit is chromatographed on a Ni^{2+}-Sepharose column, all the protein binds to the column, as shown by SDS-PAGE analysis of the flow-through and the 0.5 M imidazole eluted fraction (Fig. 4*A*). However, when the enzyme is unfolded with urea before being reanalyzed by Ni^{2+} chromatography, the protein re-distributes between the flow-through and retained fractions (Fig. 4*B*). Therefore, the original protein must be a hetero-oligomer constituted by WT and His-tagged mutant subunits.

Hetero-oligomeric formation was also demonstrated *in vitro*. When MAT III was incubated with equimolar concentrations of R265H mutant, the Ni^{2+} column did not retain any MAT III subunit, suggesting that there was no association between the mutant and the rat liver protein after 30-min incubation (Fig. 5*A*). Alternatively, hetero-oligomer formation was attempted under conditions more nearly approaching those that might exist *in vivo* by refolding a urea-denatured MAT III in the presence of the R265H mutant. After purification and urea denaturation of the resulting His-tagged protein, the flow-through and Ni^{2+}-retained fraction of a second Ni^{2+}-Sepharose chromatography were analyzed by SDS-PAGE. Protein was detected in both fractions (Fig. 5*B*), indicating that the His-tagged purified protein was a hetero-oligomer consisting of refolded MAT III and mutant (His-tagged) subunits.

Hybrid MAT Characterization—To estimate the molecular mass of the hetero-oligomers resulting from cotransformed bacteria, size exclusion chromatography was performed, 0.4-ml fractions were collected, and AdoMet synthetic and tripolyphosphatase enzymatic activities were measured. The estimated molecular mass of MAT III purified from rat liver, which was used as a control, was 91 kDa, according to the dimeric state of this protein. Peaks of AdoMet synthetic and tripolyphosphatase activities were detected when fractions of MAT III were analyzed (Fig. 6*A*). The molecular mass of the purified hetero-oligomeric MAT was of 90 kDa, indicating its dimeric nature. Similarly to MAT III, the protein-containing fractions had tripolyphosphatase activity, but, in contrast, no AdoMet synthetic activity was detected (Fig. 6*B*). Therefore, MAT purified by Ni^{2+} chromatography from cotransfected bacteria is a heterodimer with an impaired AdoMet synthetic capacity, although its tripolyphosphatase activity is maintained. The absence of MAT activity in the heterodimeric WT R265H MAT might explain the dominant inheritance of the phenotype as-

FIG. 5. ***In vitro* association between MAT III purified from rat liver and recombinant His-tagged R265H mutant subunits.** Association between purified MAT III and R265H MAT mutant subunits was analyzed under native conditions (*A*) or by refolding of MAT III in the presence of mutant subunits (*B*). The obtained protein mixtures were then denatured with 5 M urea and chromatographed on a Ni^{2+}-Sepharose column. The presence of MAT in the flow-through fraction of the affinity column was indicative of association between MAT III and His-tagged mutant subunits, which were eluted with 500 mM imidazole. Proteins were identified by SDS-PAGE. *FT*, flow-through fraction from the Ni^{2+}-Sepharose column; *R*, fraction retained on the Ni^{2+}-Sepharose column; *Mr*, molecular mass standards (standard proteins were the same as in Fig. 4).
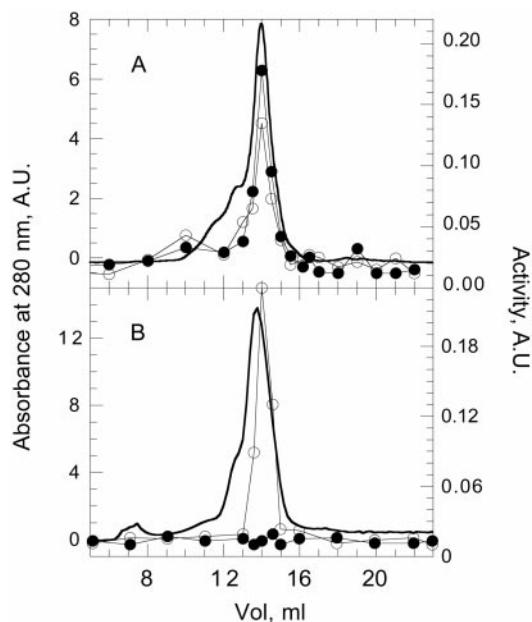

FIG. 6. **Size exclusion chromatography of MAT III and hetero-oligomeric MAT.** The molecular mass of the hetero-oligomeric MAT was estimated by size exclusion chromatography of the protein purified by Ni^{2+}-Sepharose chromatography. —, absorbance profile at 280 nm. Fractions of 0.4 ml were collected, and tripolyphosphatase (*open circles*) and AdoMet synthetic (*closed circles*) activities were measured as described under "Experimental Procedures." *A*, rat liver MAT III. *B*, hetero-oligomeric MAT. The estimated molecular mass for MAT III and the hetero-oligomer was 90 kDa.

sociated with the R264H mutation described elsewhere (26). To further assess the subunit composition of the heterodimeric MAT, the flow-through and retained fractions resulting from a Ni^{2+}-Sepharose chromatography of a denatured heterodimer were analyzed by size exclusion chromatography. The imidazole-eluted protein had a molecular mass of 41 kDa and maintains the tripolyphosphatase activity, but no synthesis of AdoMet was detected (Fig. 7*A*). The molecular mass, inability to oligomerize, and the absence of AdoMet synthetic activity indicate that this enzyme is the His-tagged R265H MAT mutant. However, the protein obtained in the flow-through had a molecular mass of 90 kDa, and both the tripolyphosphatase and AdoMet synthetic activities were recovered (Fig. 7*B*). These findings confirm that this protein is a non-His-tagged WT MAT subunit that refolds and oligomerizes to get the native, fully active conformation. Additionally, refolding of WT MAT subunits in the presence of R265H MAT mutant results in the formation of WT R265H heterodimers (not shown). It has been shown previously that the tripolyphosphatase activity of MAT III is activated by preincubation of the enzyme with the
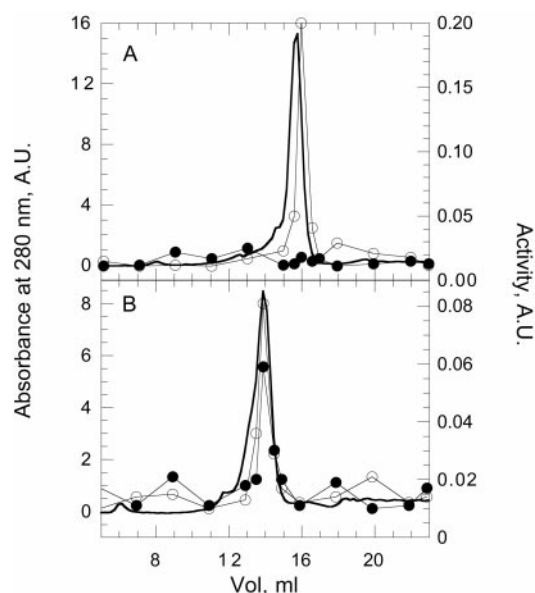
Fɪɢ. 7. **Size exclusion chromatography of heterodimeric MAT subunits.** Heterodimeric MAT purified from *E. coli* expressing WT and His-tagged R265H mutant was denatured in 5 ᴍ urea, and the resulting subunits were separated by chromatography on a Ni^{2+}-Sepharose column. The flow-through and retained fractions were analyzed by size exclusion chromatography on a Superdex 200 HR 10/30 column equilibrated without urea. *A*, retained fraction; *B*, flow-trough. —, absorbance profile at 280 nm. Fractions of 0.4 ml were collected, and tripolyphosphatase (*open circles*) and AdoMet synthetic (*closed circles*) activities were measured as described under "Experimental Procedures." The estimated molecular mass of the retained fraction and flow-through was 41 and 90 kDa, respectively.

substrates methionine and ATP (36). To test whether activation occurs also upon preincubation of the WT R265H heterodimeric MAT, the protein was pretreated with 2 mᴍ methionine and 2 mᴍ ATP, and tripolyphosphatase activity was then measured. No activation was observed after incubation of the heterodimer with methionine and ATP. The activity values were 132 and 134 nmol P_i min^{-1} mg^{-1} for the non-preincubated and preincubated forms, respectively. The absence of activation of the heterodimeric MAT might be explained by a deficient or non-productive interaction with the substrates. This hypothesis would agree with the previous result, indicating that the synthesis of AdoMet is impeded in the heterodimer.

DISCUSSION

Methionine adenosyltransferase catalyzes the synthesis of AdoMet, the main alkylating agent in living cells (1). In the liver, MAT is also responsible for the catabolism of up to 50% of the dietary methionine (15). Humans with mutations in the gene *MAT1A* have decreased MAT activities in the liver, resulting in persistent hypermethioninemia without homocystinuria (22, 23). The hypermethioninemic phenotype associated with all mutations tested to date is inherited as an autosomal recessive trait, the only known exception being the hypermethioninemia due to the G-A transition at nucleotide 791 of exon VII (21–30). This change results in a mutant *MAT1A*-encoded protein in which arginine 264 has been replaced by histidine (26). To investigate the biochemical basis of the dominant inheritance of hypermethioninemia of individuals with this mutation, we have studied the enzyme kinetics and the oligomerization capacity of a purified MAT protein containing the homologous R265H mutation encoded by rat *MAT1A*.

Our results indicate that the molecular mass of the R265H MAT I/III rat mutant protein is 41 kDa, establishing its monomeric state. Although previous studies using cell extracts also

suggested that the equivalent R264H MAT I/III mutant protein cannot form homo-oligomers (26, 39), it has been reported that the tetrameric conformation of the *E. coli* MAT is not altered by the replacement of the equivalent arginine 244 by histidine (4). Our results demonstrate that the R265H MAT I/III rat mutant has impaired ability to form homo-oligomers. Arginine 265, similarly to arginine 244 in the *E. coli* MAT, is involved in a salt bridge formation with symmetrical glutamic 58 (31, 32), which is important for dimerization (40). Therefore, the absence of this arginine might compromise the dimerization capacity of this MAT mutant.

The synthetic reaction catalyzed by MAT occurs through two consecutive steps. AdoMet and PPP_i are first synthesized from methionine and ATP; PPP_i is subsequently hydrolyzed to PP_i and P_i to allow product release from the active site of the enzyme (2, 3). The function of the tripolyphosphatase activity of MAT is still under discussion (4, 41). MAT activity of the R265H MAT I/III mutant was less than 1% of the activity of the WT enzyme, in agreement with previous data, which indicate that the active site of a dimeric MAT is configured by amino acid residues from both subunits (31, 32, 40). However, the tripolyphosphatase activity was not modified by this mutation, indicating that the active site for this activity is fully functional. Therefore, the hydrolytic site for the tripolyphosphatase activity must be configured by residues held on a single subunit. Tripolyphosphatase activity of this MAT mutant is similar to that determined for a WT enzyme in the resting, less active state (36). Additionally, we found that tripolyphosphatase activity of the R265H MAT I/III mutant is not regulated by NO, which further agrees with the mutant enzyme being in the resting state. We have shown previously that tripolyphosphatase activity of MAT III is stimulated by preincubation with methionine and ATP (36). However, no activation of the tripolyphosphatase activity was found when R265H MAT was preincubated with methionine and ATP, suggesting that the regulation of this activity by the natural substrates of the enzyme has been lost. The absence of AdoMet synthetic activity, and the failure of methionine and ATP to activate the tripolyphosphatase activity, suggest that binding of the substrates is impaired. Substitution of arginine 265 by serine instead of histidine resulted in a 5-fold decrease of tripolyphosphatase activity. Thus, it seems that, although the presence of a positive charge at position 265 is involved, it is not an absolute requirement for the PPP_i hydrolytic activity. This result might be explained by previous evidence, which indicates the positive charge of the equivalent arginine 244 in the *E. coli* MAT is responsible for the correct orientation of the PPP_i in the active site of the enzyme (4, 42).

Tripolyphosphatase activity of the R265H MAT I/III mutant is specific of PPP_i, and depends on the presence of Mg^{2+} in the assay mixture, but K^+ is not required. These findings suggest that while Mg^{2+} is directly involved in the binding of PPP_i (31), K^+, which binds to the interface between monomers (40), might contribute to the stabilization of a functional conformer of dimeric MAT, which is not accessible to the monomeric R265H mutant. This mutant showed no cooperativity when its tripolyphosphatase activity was assayed with different PPP_i concentrations. Tripolyphosphatase activity of WT MAT is not altered by the presence of saturating concentrations of ATP (36). This finding might be explained by assuming that ATP and PPP_i bind to different sites, or, alternatively, binding of the PPP_i moiety of ATP might be sterically restricted in the dimeric enzyme. To further assess this question, we studied the effect of ATP on the tripolyphosphatase activity of the monomeric R265H MAT mutant. We found that ATP had no effect on the tripolyphosphatase activity of the R265H MAT I/III mutant.

Characterization of the R265H MAT Mutant

Since accessibility should not be compromised in the monomer, we propose that ATP and PPP_i have different binding sites. In contrast, PP_i, a classical inhibitor of MAT tripolyphosphatase activity, induces a 1.5- and 2.3-fold decrease of the enzymatic activity and affinity of the enzyme, respectively. Similarly to *E. coli* MAT (43), PP_i and PPP_i might compete for the same binding site. Our data suggest that the active site of MAT has two coordinated subsites: the synthetic site, configured by amino acid residues from both subunits (31, 32), is responsible for the binding of methionine and ATP and performs the AdoMet synthetic reaction. The hydrolytic site, configured by amino acid residues from one single subunit, accounts for the binding of PPP_i and performs the PPP_i hydrolytic reaction.

Dominant inheritance of the hypermethioninemia of humans carrying the R264H mutation in *MAT1A* might be explained by our findings, which indicate that replacement in the rat homologue of arginine 265 by histidine produces a monomeric MAT that interacts with the WT MAT subunit, *in vivo* and *in vitro*, resulting in a hybrid enzyme with impaired AdoMet synthetic activity. Evidence suggesting the capacity of the MAT mutant R264H to associate with WT MAT was first proposed for the human enzyme by Chamberlin *et al.* (26) on the basis of MAT activity recovered in COS cell extracts after cotransfection with two vectors expressing human R264H mutant and WT MAT, respectively. We have demonstrated that MAT enzyme purified by Ni^{2+}-Sepharose chromatography from *E. coli* cotransformed with plasmids expressing rat R265H mutant and WT MAT is a hetero-oligomer, which is formed by mutant and WT subunits. In contrast, when MAT III purified from rat liver was incubated with the recombinant rat R265H MAT I/III mutant, no association was observed under the experimental conditions used in our studies. This apparent discrepancy might be explained by assuming that the dissociation constant for the dimer is sufficiently low to prevent MAT III-mutant subunit exchange under our experimental conditions. Since monomers generally fold to nearly the final conformation before the association step (44), association between MAT subunits may occur at a late intermediate step of the folding process. Indeed, we found that a hybrid MAT, containing WT and mutant subunits, was formed when a urea-unfolded MAT III purified from rat liver was refolded in the presence of the R265H MAT I/III mutant. The presence of urea might overcome the proposed kinetic restrictions, allowing subunit exchange either by increasing the dimer K_d or by altering the protein conformation.

To estimate the oligomeric state of the hetero-oligomeric MAT species, size exclusion chromatography studies were performed, and fractions were collected to determine their catalytic capacity. The calculated molecular mass of MAT III was 91 kDa in agreement with its dimeric structure (5), and AdoMet synthetic as well as tripolyphosphatase activities were found when the protein-containing fractions were assayed. The hetero-oligomeric MAT purified from *E. coli* expressing WT and R265H MAT mutant has a molecular mass of 90 kDa. Since this MAT was previously retained on a Ni^{2+}-Sepharose column, and the R265H MAT I/III mutant has lost the ability to form homo-oligomers, the 90-kDa protein must correspond to a heterodimer containing one R265H mutant (His-tagged) and one WT subunit. The heterodimer might be stabilized by the salt bridge interaction between arginine 265 in the WT subunit and glutamic 58 in the mutant subunit. The heterodimeric MAT maintains the tripolyphosphatase activity, but no synthesis of AdoMet was determined after incubation with methionine and ATP. This lack of AdoMet synthetic activity might be a consequence of a non-native association between monomers, resulting in an altered active site and, consequently, in a deficient substrate binding or catalytic capacity of the heterodimer.

This hypothesis is further supported by the finding that, in contrast to WT enzyme (36), tripolyphosphatase activity of the R265H-WT MAT is not activated when the enzyme is preincubated with methionine and ATP. The ability of the monomeric R265H MAT I/III mutant to associate with a WT subunit, resulting in a hybrid WT R265H MAT without AdoMet synthetic activity, might explain the dominant inheritance of the hypermethioninemia associated with the human R264H MAT I/III mutation. Additionally, denaturation of heterodimeric MAT by incubation with 5 M urea results in two forms of the enzyme, which were separated by Ni^{2+} chromatography, and showed different molecular mass and enzymatic activities upon refolding. The retained fraction was a monomer (41 kDa) with tripolyphosphatase activity, and the flow-through was a dimer (90 kDa) with both tripolyphosphatase and AdoMet synthetic activity as determined by size exclusion chromatography. According to their properties, these two forms of MAT must be identified as refolded His-tagged, monomeric R265H mutant and dimeric, fully active WT MAT. These findings indicate that urea unfolding of MAT is a reversible process and further support that MAT purified from *E. coli* expressing WT and R265H MAT mutant is a heterodimer constituted by WT and mutant subunits.

In conclusion, our data provide a molecular explanation for the dominant inheritance of the persistent hypermethioninemia associated with the human R264H MAT I/III mutation. We demonstrate that the equivalent R265H mutation in rat MAT I/III results in a monomeric MAT, which can associate with the WT enzyme to form a dimeric R265H-WT MAT lacking AdoMet synthetic activity. We have also shown that the active site for the tripolyphosphatase activity is functionally active in the monomeric R265H MAT I/III mutant. Our data suggest that the active site of MAT has two coordinated subsites: the synthetic site, configured by amino acid residues from both subunits, that performs the AdoMet synthetic reaction, and the hydrolytic site, configured by amino acid residues from one single subunit, that performs the PPP_i hydrolytic reaction.

REFERENCES

1. Cantoni, G. (1953) *J. Biol. Chem.* **204,** 403–416
2. Mudd, S. H. (1962) *J. Biol. Chem.* **237,** 1372–1375
3. Mudd, S. H. (1963) *J. Biol. Chem.* **238,** 2156–2163
4. Reczkowski, R. S., Taylor, J. C., and Markham, G. D. (1998) *Biochemistry* **37,** 13499–13506
5. Cabrero, C., Puerta, J., and Alemany, S. (1987) *Eur. J. Biochem.* **170,** 299–304
6. Kotb, M., and Kredich, N. M. (1985) *J. Biol. Chem.* **260,** 3923–3930
7. Okada, G., Teraoka, H., and Tsukada, K. (1981) *Biochemistry* **20,** 934–940
8. Sullivan, D. M., and Hoffman, J. L. (1983) *Biochemistry* **22,** 1636–1641
9. Horikawa, S., Sasuga, J., Shimizu, K., Ozasa, H., and Tsukada, K. (1990) *J. Biol. Chem.* **265,** 13683–13686
10. Horikawa, S., Ishikawa, M., Ozasa, H., and Tsukada, K. (1989) *Eur. J. Biochem.* **184,** 497–501
11. Horikawa, S., and Tsukada, K. (1991) *Biochem. Int.* **25,** 81–90
12. Alvarez, L., Asuncion, M., Corrales, F., Pajares, M. A., and Mato, J. M. (1991) *FEBS Lett.* **290,** 142–146
13. Alvarez, L., Corrales, F., Martin-Duce, A., and Mato, J. M. (1993) *Biochem. J.* **293,** 481–486
14. Tsukada, K., and Okada, G. (1980) *Biochem. Biophys. Res. Commun.* **94,** 1078–1082
15. Mudd, S. H., and Poole, J. R. (1975) *Metabolism* **29,** 707–720
16. Gaull, G. E., and Tallan, H. H. (1974) *Science* **186,** 59–60
17. Finkelstein, J. D., Kyle, W. E., and Martin, J. J. (1975) *Biochem. Biophys. Res. Commun.* **66,** 1491–1497
18. Gout, J. P., Serre, J. C., Dieterlen, M., Antener, I., Frappat, P., Bost, M., and Beaudoing, A. (1977) *Arch. Fr. Pediatr.* **34,** 416–423
19. Gaull, G. E., Tallan, H. H., Lonsdale, D., Przyrembel, H., Schaffner, F., and von Bassewitz, D. B. (1981) *J. Pediatr.* **98,** 734–741
20. Gahl, W. A., Finkelstein, J. D., Mullen, K. D., Bernardini, I., Martin, J. J., Backlund, P., Ishak, K. G., Hoofnagle, J. H., and Mudd, S. H. (1987) *Am. J. Hum. Genet.* **40,** 39–49
21. Blom, H. J., Davidson, A. J., Finkelstein, J. D., Luder, A. S., Bernardini, I., Martin, J. J., Tangerman, A., Trijbels, J. M., Mudd, S. H., Goodman, S. I., and Gahl, W. A. (1992) *J. Inherit. Metab. Dis.* **15,** 188–197
22. Mudd, S. H., Levy, H. L., Tangerman, A., Boujet, C., Buist, N., Davidson-Mundt, A., Hudgins, L., Oyanagi, K., Nagao, M., and Wilson, W. G. (1995) *Am. J. Hum. Genet.* **57,** 882–892
23. Mudd, S. H., Levy, H. L., and Kraus, J. P. (2001) in *The Metabolic and Molecular Bases of Inherited Disease* (Scriver, C. R., Beaudet, A. L., Sly,

W. S., Valle, D., Childs, B., and Vogelstein, B., eds) McGraw-Hill, New York, in press

24. Ubagai, T., Lei, K. J., Huang, S., Mudd, S. H., Levy, H. L., and Chou, J. Y. (1995) *J. Clin. Invest.* **96,** 1943–1947

25. Chamberlin, M. E., Ubagai, T., Mudd, S. H., Wilson, W. G., Leonard, J. V., and Chou, J. Y. (1996) *J. Clin. Invest.* **98,** 1021–1027

26. Chamberlin, M. E., Ubagai, T., Mudd, S. H., Levy, H. L., and Chou, J. Y. (1997) *Am. J. Hum. Genet.* **60,** 540–546

27. Hazelwood, S., Bernardini, I., Shotelersuk, V., Tangerman, A., Guo, J., Mudd, H., and Gahl, W. A. (1998) *Am. J. Med. Genet.* **75,** 395–400

28. Chamberlin, M. E., Ubagai, T., Mudd, S. H., Thomas, J., Pao, V. Y., Nguyen, T. K., Levy, H. L., Greene, C., Freehauf, C., and Chou, J. Y. (2000) *Am. J. Hum. Genet.* **66,** 347–355

29. Nagao, M., and Oyanagi, K. (1996) in the *39th Meeting of the Japanese Society of Inherited Metabolic Disease*, November 14–16, Abstract 61, Tokyo, Japan

30. Nagao, M., and Oyanagi, K. (1997) *Acta Paediatr. Jpn.* **39,** 601–606

31. Takusagawa, F., Kamitori, S., and Markham, G. D. (1996) *Biochemistry* **35,** 2586–2596

32. Takusagawa, F., Kamitori, S., Misaki, S., and Markham, G. D. (1996) *J. Biol. Chem.* **271,** 136–147

33. Mingorance, J., Alvarez, L., Sanchez-Gongora, E., Mato, J. M., and Pajares, M. A. (1996) *Biochem. J.* **315,** 761–766

34. Pérez-Mato, I., Castro, C., Ruiz, F. A., Corrales, F. J., and Mato, J. M. (1999) *J. Biol. Chem.* **274,** 17075–17080

35. Laemmli, U. K. (1970) *Nature* **227,** 680–685

36. Sánchez del Pino, M. M., Corrales, F. J., and Mato, J. M. (2000) *J. Biol. Chem.* **275,** 23476–23482

37. Lanzetta, P. A., Alvarez, L. J., Reinach, P. S., and Candia, O. A. (1979) *Anal. Biochem.* **100,** 95–97

38. Avila, M. A., Mingorance, J., Martinez-Chantar, M. L., Casado, M., Martin-Sanz, P., Bosca, L., and Mato, J. M. (1997) *Hepatology* **25,** 391–396

39. Chamberlin, M. E., Ubagai, T., Pao, V. Y., Pearlstein, R. A., and Chou, J. Y. (2000) *Arch. Biochem. Biophys.* **373,** 56–62

40. Gonzalez, B., Pajares, M. A., Hermoso, J. A., Alvarez, L., Garrido, F., Sufrin, J. R., and Sanz-Aparicio, J. (2000) *J. Mol. Biol.* **300,** 363–375

41. McQueney, M. S., Anderson, K. S., and Markham, G. D. (2000) *Biochemistry* **39,** 4443–4454

42. Reczkowski, R. S., and Markham, G. D. (1999) *Biochemistry* **38,** 9063–9068

43. Markham, G. D., Hafner, E. W., Tabor, C. W., and Tabor, H. (1980) *J. Biol. Chem.* **255,** 9082–9092

44. Creighton, T. E. (1993) *Proteins Structure and Molecular Properties* (Creighton, T. E., ed) pp. 261–328, W. H. Freeman & Co., New York