

# Hysteretic Behavior of Methionine Adenosyltransferase III

METHIONINE SWITCHES BETWEEN TWO CONFORMATIONS OF THE ENZYME WITH DIFFERENT SPECIFIC ACTIVITY\*

Received for publication, March 29, 2000, and in revised form May 5, 2000  
Published, JBC Papers in Press, May 12, 2000, DOI 10.1074/jbc.M002730200

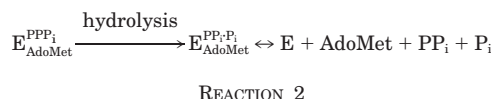
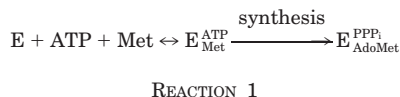
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**Methionine adenosyltransferase III (MATIII) catalyzes *S*-adenosylmethionine (AdoMet) synthesis and, as part of its reaction mechanism, it also hydrolyzes tripolyphosphate. Tripolyphosphatase activity was linear over time and had a slightly sigmoidal behavior with an affinity in the low micromolar range. On the contrary, AdoMet synthetase activity showed a lag phase that was independent of protein concentration but decreased at increasing substrate concentrations. Tripolyphosphatase activity, which appeared to be slower than AdoMet synthesis, was stimulated by preincubation with ATP and methionine so that it matched AdoMet synthetase activity. This stimulation process, which is probably the origin of the lag phase, represents the slow transition between two conformations of the enzyme that could be distinguished by their different tripolyphosphatase activity and sensitivity to *S*-nitrosylation. Tripolyphosphatase activity appeared to be the rate-determining reaction in AdoMet synthesis and the one inhibited by *S*-nitrosylation. The methionine concentration necessary to obtain half-maximal stimulation was in the range of physiological methionine fluctuations. Moreover, stimulation of MAT activity by methionine was demonstrated *in vivo*. We propose that the hysteretic behavior of MATIII, in which methionine induces the transition to a higher specific activity conformation, can be considered as an adaptation to the specific functional requirements of the liver.**

distributing role, the methylation cycle has additional functions (2, 3). It is used to clear most of the excess of blood methionine after a protein-rich meal. It uses the homocysteine produced in the cycle to synthesize cysteine and glutathione, which can then be exported to other organs. Thus, synthesis of AdoMet is a key reaction to regulate and control all these important metabolic pathways.

AdoMet is synthesized by the enzyme methionine adenosyltransferase (MAT; ATP:L-methionine *S*-adenosyltransferase, EC 2.5.1.6) in two consecutive reactions. The first reaction (Reaction 1) involves the synthesis of AdoMet and tripolyphosphate (PPP<sub>i</sub>) from ATP and methionine. In Reaction 2, tripolyphosphate is hydrolyzed to orthophosphate (P<sub>i</sub>) and pyrophosphate (PP<sub>i</sub>) before the release of the products. MAT is also able to hydrolyze exogenously added tripolyphosphate.



Although divergent sequences have been recently reported for archaea, MAT has been extremely well conserved through evolution, with 59% sequence identity being found between the human and *Escherichia coli* isozymes (4). In mammals, there are three isoforms of MAT that are encoded by two genes (5). MATI and MATIII are tetrameric and dimeric forms, respectively, of the same gene product, which is expressed in adult liver. The MATII isoform is encoded by a different gene and is expressed in extrahepatic tissues as well as in fetal liver. MATII is a heterotetramer consisting of two catalytic and two regulatory subunits. It is an intriguing and yet unsolved question why there are two different MAT isoforms in liver. They might be an adaptation to cope with the special metabolic requirements of the liver. However, despite the importance of hepatic MAT isoforms to understand their role in liver function, they are poorly characterized. A great variability in kinetic parameters, depending on the laboratory and experimental conditions used to purify and measure MAT activity, has been reported (6). Nevertheless, the predominant liver form, MATIII, seems to have lower affinity toward its substrates and a cooperative behavior, contrasting with the other mammalian enzymes. Based on the differential properties of hepatic MAT isoforms, it has been postulated that MATIII is the truly liver-specific isoform. MATI would, as MATII outside the liver, maintain the basal AdoMet levels required by cells, whereas MATIII would be responsible for other tasks such as methionine clearance.

Synthesis of *S*-adenosylmethionine (AdoMet)<sup>1</sup> plays an essential role in cellular metabolism. AdoMet is the major methyl donor in the cell and is a precursor of polyamine synthesis (1). Along with ATP, AdoMet is one of the metabolites most frequently used in intermediary metabolism. Synthesis of AdoMet is the first reaction of the methylation cycle (activated methyl cycle). In its last step, methionine and tetrahydrofolate, an essential cofactor for RNA and DNA synthesis, are regenerated. In the liver, due to its central metabolic processing and

\* This work was supported in part by grants from Comisión Interministerial de Ciencia y Tecnología, Spain (SAF 98/0132 and SAF 99/0038), Europharma, and Knoll. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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<sup>1</sup> The abbreviations used are: AdoMet, *S*-adenosylmethionine; DTT, dithiothreitol; GSNO, *S*-nitrosoglutathione; MATIII, methionine adenosyltransferase III; P<sub>i</sub>, orthophosphate; PP<sub>i</sub>, pyrophosphate; PPP<sub>i</sub>, tripolyphosphate; SAE, *S*-adenosylethionine.

A detailed kinetic characterization of enzymes is necessary to understand both their catalytic mechanism and their function in the cell. Here we report the partial characterization of the kinetic properties of MATIII. A hysteretic behavior has been found that could explain how the enzyme is regulated to accomplish its function in the liver.

#### EXPERIMENTAL PROCEDURES

**Materials**—Columns and chromatography media were purchased from Amersham Pharmacia Biotech except for the HPLC column, which was from Waters. Tissue culture media was obtained from Life Technologies, Inc. All other reagents were purchased from Sigma.

**Purification of MATIII**—MATIII isozyme was purified as described previously by Cabrero *et al.* (7) with some minor modifications. The livers of 20 male rats of about 250 g were removed and placed in 300 ml of ice-cold buffer containing 0.3 M sucrose, 0.1 mM EGTA, and 10 mM Tris/HCl, pH 7.4. Just before homogenization, benzamide, phenylmethylsulfonyl fluoride, and 2-mercaptoethanol were added to final concentrations of 1 mM, 0.1 mM, and 0.1% (v/v), respectively. The homogenate was clarified by centrifugation at 100,000 × *g* for 90 min. The supernatant was loaded onto a DEAE-Sepharose Fast Flow column, equilibrated in buffer A (10 mM MgSO<sub>4</sub>, 1 mM EDTA, and 10 mM Hepes, pH 7.5). The enzyme was eluted with a linear gradient of KCl in the same buffer. A single peak of AdoMet synthetase activity eluted at about 220 mM KCl. Fractions containing activity were pooled and loaded onto a phenyl-Sepharose Fast Flow column equilibrated in buffer A containing 200 mM KCl. After washing the column first with equilibration buffer and then with buffer A, MATIII was eluted with buffer A containing 50% (v/v) dimethyl sulfoxide. The eluate was dialyzed against buffer A and loaded onto a Blue-Sepharose Fast Flow column equilibrated in the same buffer. MATIII eluted in the flow-through. Fractions containing AdoMet synthetase activity were pooled and concentrated under nitrogen using an Amicon Ultrafiltration cell fitted with a YM30 membrane. The concentrated MATIII solution was cleared from particles and aggregates with a 0.22-μm filter, and then KCl and dithiothreitol (DTT) were added to final concentrations of 10 and 5 mM, respectively. The final MATIII solution was aliquoted, frozen in liquid nitrogen, and stored at -80 °C. The protein concentration was determined using the Bio-Rad Protein Microassay, with bovine serum albumin as the standard, based on the method of Bradford (8). The purity of the enzyme, estimated by an overloaded SDS-polyacrylamide gel electrophoresis, was over 95%. About 12 mg of protein were obtained in a typical purification.

**Activity Assays**—All assays were done at 37 °C. Enzyme assays were carried out in 100 μl containing 4 mM MgCl<sub>2</sub>, 250 mM KCl, 3.6 mM DTT, 50 mM Tris/HCl (pH 8), and 1.6 μg of MATIII. The assay was started by addition of the substrates to a final concentration of 2 mM, unless indicated otherwise in the figure legends. The substrates were ATP/MgCl<sub>2</sub> and methionine (2 mM each) to measure AdoMet synthetase activity, or triphosphosphate/MgCl<sub>2</sub> to measure triphosphatase activity. Both activities were monitored by measuring the release of P<sub>i</sub> by the method of Lanzetta *et al.* (9). DTT was omitted in *S*-nitrosylation experiments.

**Stimulation of Triphosphatase Activity**—Stimulation was carried out in 25 μl containing the same buffer composition as in the activity assays, 16 μg of MATIII, ATP/MgCl<sub>2</sub>, and methionine. At the indicated times at 37 °C, the mixture was diluted to 1 ml with the same buffer composition as before but containing triphosphatase as substrate. Aliquots of 100 μl were removed at different times to measure the released P<sub>i</sub>. The final assay conditions were the same as in the regular triphosphatase activity assay, except that it contained some ATP/MgCl<sub>2</sub> and methionine but never more than 50 μM each.

**MAT Activity Assay *in Vivo***—Hepatocytes were isolated from male rats as described previously (10). Isolated hepatocytes were suspended (2 million cells/ml) in minimal essential media (Life Technologies, Inc.) supplemented with 10 or 500 μM methionine, and incubated at 37 °C with a gas phase of 95% O<sub>2</sub> and 5% CO<sub>2</sub>. After 20 min, cells were washed and fresh media containing 2 mM ethionine was added. Aliquots of 1 ml were removed at the indicated times and added to tubes containing 72 μl of 6 M perchloric acid to disrupt the cells. Cell viability was measured by trypan blue exclusion at the beginning and at the end of the experiment. The amount of *S*-adenosylethionine (SAE) was determined by HPLC as described elsewhere (11). Identification and quantification of the SAE peak was done by comparison with standard solutions containing different amounts of SAE. The data was corrected by the percentage of viable hepatocytes, which was not altered by methionine. MAT content was assumed to be the same in both groups of

cells, because it has been shown that it does not change significantly in hepatocytes cultured up to 8 h (10). Similarly, MAT content is not affected by the presence of methionine in the media (up to 1 mM) during this period of time.<sup>2</sup>

***S*-Nitrosylation of MATIII**—The NO donor used was *S*-nitrosoglutathione (GSNO), which was prepared as described previously (12). *S*-Nitrosylation of the enzyme was carried out by incubation in the presence of the indicated concentrations of GSNO for 15 min at room temperature.

**Analysis of the Results**—The data of the substrate dependence of triphosphatase activity were fitted to the Hill equation, and its stimulation was fitted to a hyperbola.

A lag phase in product appearance can be described by

$$v(t) = \frac{dP_i}{dt} = v_f + (v_i - v_f)e^{-kt} \quad (\text{Eq. 1})$$

where  $v(t)$  is the velocity at any given time,  $v_i$  is the initial velocity,  $v_f$  is the final velocity (after the lag phase), and  $k$  is the apparent rate constant for the transition. The product accumulation data were fitted to the integrated form of the above equation. Because the best fit was usually obtained with near-zero values of  $v_i$ , this parameter was set to zero to have more robust fit parameters. Thus, the final linear velocity,  $v_f$ , and the apparent rate constant for the transition were obtained by fitting the accumulation of P<sub>i</sub> over time to the following expression.

$$P_i(t) = v_f \left[ t - \frac{1}{k}(1 - e^{-kt}) \right] \quad (\text{Eq. 2})$$

To measure MAT activity *in vivo*, we set two groups of cells that were incubated in the presence of low or high methionine concentration (see above). We propose a model where only MAT from cells incubated in low methionine shows a lag phase in product accumulation (see below). Thus, MAT activity in both groups of cells, expressed in terms of  $k_{\text{cat}}$  and substrate concentration (ethionine), will be described as follows. For low methionine,

$$\frac{dP_i}{dt} = S_{\text{in}}[k_f + (k_i - k_f)e^{-kT}] \quad (\text{Eq. 3})$$

and for high methionine,

$$\frac{dP_i}{dt} = S_{\text{in}}k_f \quad (\text{Eq. 4})$$

where  $k_i$  and  $k_f$  are the initial or final  $k_{\text{cat}}$  values, respectively, for the catalyzed global reaction, and  $S_{\text{in}}$  is the substrate concentration inside the cell. However, the intracellular substrate concentration is not constant over time. It increases gradually until it reaches the extracellular concentration. This transport process is given by the expression

$$\frac{dS_{\text{in}}}{dt} = (S_{\text{out}} - S_{\text{in}})k_T \quad (\text{Eq. 5})$$

where  $S_{\text{out}}$  is the extracellular substrate concentration and  $k_T$  is the rate constant for the transport process. The extracellular concentration of substrate can be considered constant, because the intracellular volume is negligible. The expression for the progress of product accumulation in cells incubated in low and high methionine is obtained by integration of Equations 3 and 5 or 4 and 5, respectively. The data from measurements of MAT activity *in vivo* were simultaneously fitted to the following equations.

For low methionine,

$$P_i(t) = S_{\text{out}} \left[ k_f t + \frac{(k_i - k_f)}{k}(1 - e^{-kt}) - \frac{k_f}{k_T}(1 - e^{-k_T t}) - \frac{(k_i - k_f)}{(k + k_T)}(1 - e^{-(k+k_T)t}) \right] \quad (\text{Eq. 6})$$

and for high methionine,

$$P_i(t) = S_{\text{out}} \left[ k_f t - \frac{k_f}{k_T}(1 - e^{-k_T t}) \right] \quad (\text{Eq. 7})$$

where only four parameters are needed to describe the behavior of cells incubated in low and high methionine.

<sup>2</sup> M. A. Avila J. M. Mato, unpublished results.

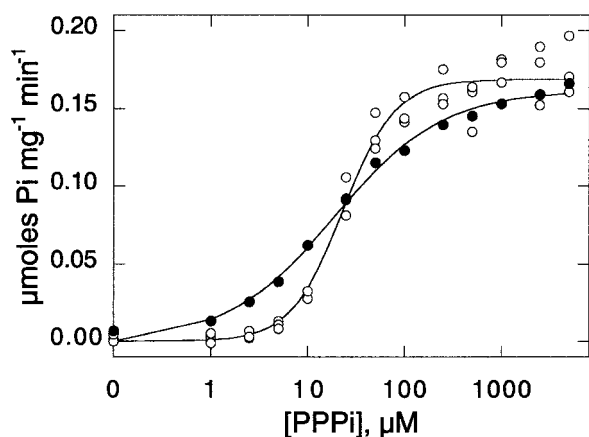


FIG. 1. **Kinetics of triphosphatase activity.** MATIII was incubated for 10 min at 37 °C with the indicated triphosphosphate (PPP<sub>i</sub>) concentrations in the absence (○) and in the presence (●) of 5 mM AdoMet. The *solid lines* represent the non-linear regression fit to the Hill equation. The parameters in the absence and in the presence of 5 mM AdoMet, respectively, are:  $S_{0.5}$ ,  $23 \pm 2 \mu\text{M}$ ;  $V_{\text{max}}$ ,  $169 \pm 3 \text{ nmol mg}^{-1} \text{ min}^{-1}$ ; Hill coefficient,  $1.6 \pm 0.2$ ; and  $S_{0.5}$ ,  $19 \pm 2 \mu\text{M}$ ;  $V_{\text{max}}$ ,  $162 \pm 3 \text{ nmol mg}^{-1} \text{ min}^{-1}$ ; Hill coefficient,  $0.8 \pm 0.1$ .

## RESULTS

**Characterization of the Triphosphatase Activity**—Triphosphatase activity was linear in time and with protein concentration, and no P<sub>i</sub> release was observed in the absence of MAT or in the presence of EDTA (not shown). The enzyme showed a slightly sigmoidal behavior with substrate concentration (Hill coefficient of 1.6) and an affinity in the low micromolar range ( $S_{0.5}$ , 23 μM) (Fig. 1). The maximum velocity is around  $170 \text{ nmol mg}^{-1} \text{ min}^{-1}$  ( $k_{\text{cat}}$  7.5 min<sup>-1</sup>). The presence of methionine or ATP in the incubation media had no effect on triphosphatase activity (not shown). However, AdoMet seemed to decrease cooperativity (Fig. 1), Hill coefficient from 1.6 to 0.8, without affecting significantly the affinity or the maximal activity.

**Characterization of the Lag Phase of the AdoMet Synthetase Activity**—The time course of the AdoMet synthetase activity showed a lag phase with a half time of about 2 min (Fig. 2). The steady-state activity, at 2 mM of each substrate, is about  $800 \text{ nmol mg}^{-1} \text{ min}^{-1}$  ( $k_{\text{cat}}$ , 35 min<sup>-1</sup>). This lag phase was not altered by preincubation of the enzyme with any of the substrates before addition of the other one (not shown). Similarly, the presence of AdoMet, at a concentration in the range of that present at the end of the lag phase (30 μM), had no effect on latency (Fig. 2). To check the possibility of a change in the oligomeric state, a time course experiment was performed using different MAT concentrations. No change in the lag phase or activity was observed (not shown). On the contrary, the lag phase depends on substrate concentration. The higher the substrate concentration, the shorter the lag phase (Fig. 3). Due to the lag phase, the kinetic characterization of the AdoMet synthetase activity is not trivial. Nevertheless, we estimated the  $S_{0.5}$  for ATP and methionine to be about 1 mM and 120 μM, respectively (not shown).

**Stimulation of Triphosphatase Activity by ATP and Methionine**—Triphosphatase activity was slower than AdoMet synthetase activity after the lag phase. Because the measured AdoMet synthetase activity is the combination of two consecutive reactions, AdoMet synthesis and hydrolysis of triphosphate, one would expect equal or faster triphosphatase activity than AdoMet synthetase activity. Comparison of the time course of both activities (Fig. 4) indicated that they were very similar initially. However, triphosphatase activity remained the same, whereas AdoMet synthetase activity

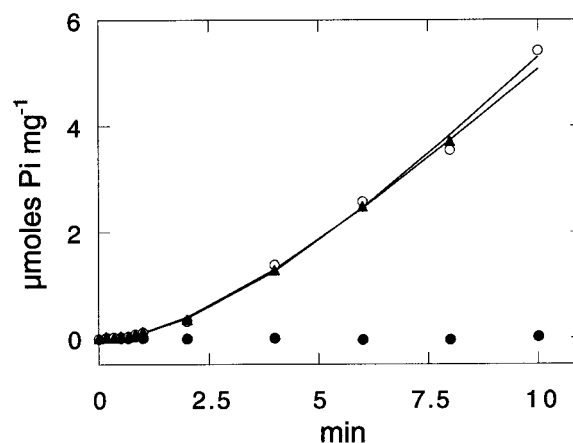


FIG. 2. **Lag phase in AdoMet synthetase activity.** MATIII was incubated at 37 °C in the presence of 2 mM ATP/MgCl<sub>2</sub> (●) or 2 mM each ATP/MgCl<sub>2</sub> plus methionine in the absence (○) or presence (▲) of 30 μM AdoMet. The *solid lines* represent the non-linear regression fit to Equation 2 under "Experimental Procedures." The parameters in the absence and in the presence of 30 μM AdoMet, respectively, are:  $v_p$ ,  $800 \pm 93 \text{ nmol mg}^{-1} \text{ min}^{-1}$ ;  $k$ ,  $0.28 \pm 0.07 \text{ min}^{-1}$ ; and  $v_p$ ,  $700 \pm 23 \text{ nmol mg}^{-1} \text{ min}^{-1}$ ;  $k$ ,  $0.35 \pm 0.03 \text{ min}^{-1}$ .

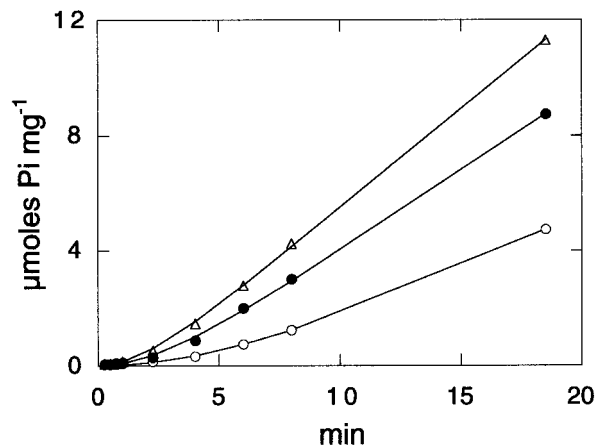


FIG. 3. **Effect of ATP and methionine concentrations on lag phase.** Accumulation of P<sub>i</sub> was measured at 37 °C in the presence of 1 mM (○), 1.5 mM (●), and 2 mM (△) ATP/MgCl<sub>2</sub> plus methionine (each). The *solid lines* represent the non-linear regression fit to Equation 2 under "Experimental Procedures." The parameters in the presence of 1, 1.5, and 2 mM substrate, respectively, are:  $v_p$ ,  $414 \pm 24 \text{ nmol mg}^{-1} \text{ min}^{-1}$ ;  $k$ ,  $0.13 \pm 0.02 \text{ min}^{-1}$ ;  $v_p$ ,  $563 \pm 10 \text{ nmol mg}^{-1} \text{ min}^{-1}$ ;  $k$ ,  $0.34 \pm 0.02 \text{ min}^{-1}$ ; and  $v_p$ ,  $687 \pm 7 \text{ nmol mg}^{-1} \text{ min}^{-1}$ ;  $k$ ,  $0.50 \pm 0.03 \text{ min}^{-1}$ .

increased with time (lag phase). This result suggests that ATP and/or methionine induces a stimulation of MAT that does not take place with triphosphate alone. To test this hypothesis, MAT was incubated in the presence of ATP and methionine prior to the measurement of triphosphatase activity. This is equivalent to the measurement of triphosphatase activity along different stages of the lag phase. It can be seen in Fig. 4 that triphosphatase activity was higher after preincubation. Under these experimental conditions, a 4-fold stimulation process, with a half time of 1.5 min, was observed (not shown). The increase in activity, similar to the ratio of steady-state AdoMet synthetase to non-stimulated triphosphatase activities as well as the half time suggest that the stimulation of triphosphatase activity and the lag phase of AdoMet synthetase activity are the same process. To stimulate the enzyme, both ATP and methionine were necessary, but ADP and methionine or AdoMet were unable to produce any significant effect (not shown).

The stimulation of MAT depended on the substrate concen-

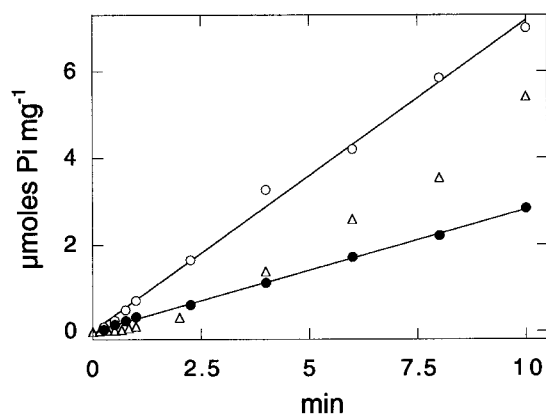


FIG. 4. **Stimulation of triphosphatase activity by ATP and methionine.** MATIII was incubated at 37 °C in the presence of buffer (●) or 2 mM ATP/MgCl<sub>2</sub> and methionine (○). After 1 min, MAT solutions were diluted 40-fold with reaction mixture containing 2 mM triphosphatase/MgCl<sub>2</sub>. At the indicated times the production of P<sub>i</sub> was measured. For comparative purposes, the AdoMet synthetase activity shown in Fig. 2 is also displayed (△).

tration. The  $S_{0.5}$  for ATP and methionine were  $\sim 760$  and  $120 \mu\text{M}$ , respectively (Fig. 5). The value for ATP, however, is not accurate because no saturation was achieved. The  $S_{0.5}$  values for MAT stimulation are very similar to those estimated for AdoMet synthetase activity. No significant cooperativity was observed. The stimulation of MAT could be due to the binding of substrates or to a subsequent step after it.

**Stimulation of AdoMet Synthetase in Vivo**—The stimulation process could be considered as a regulatory mechanism of MAT to control methionine concentration. Thus, we were interested in knowing if MAT stimulation takes place also *in vivo*. MAT was stimulated by incubating hepatocytes in high concentration of methionine. Non-stimulated cells were incubated in low methionine media. Then, MAT activity was monitored adding fresh media containing ethionine and measuring the intracellular *S*-adenosylethionine (SAE). Ethionine was used to measure MAT activity *in vivo* instead of methionine, because SAE synthesized by MAT is not metabolized (13). Thus, the amount of SAE is proportional to MAT activity, without contribution by AdoMet-consuming reactions. Although both groups of cells showed a lag phase to achieve a steady-state activity (Fig. 6), there was a longer delay in the cells incubated in low methionine. The time needed to equilibrate the intracellular ethionine concentration with the extracellular one should produce a lag phase. Three different processes could account for the data obtained: amino acid transport, steady-state activity, and, in the cells incubated in low methionine, stimulation of MAT. Four parameters describing these three processes were used to fit the data (Equations 6 and 7 under “Experimental Procedures”): amino acid transport rate constant ( $0.85 \text{ min}^{-1}$ ), initial activity ( $0.015 \text{ fmol cell}^{-1} \text{ min}^{-1}$ ), final activity ( $0.035 \text{ fmol cell}^{-1} \text{ min}^{-1}$ ), and MAT activation rate constant ( $0.07 \text{ min}^{-1}$ ).

**Effect of *S*-Nitrosylation on MAT Activity**—The activity of MAT has been recently shown to be regulated by *S*-nitrosylation. Incorporation of a single NO molecule in the residue of cysteine 121 results in a decrease in AdoMet synthetase activity (11, 12). Interestingly, as shown in Fig. 7, *S*-nitrosylation of the enzyme did not have any significant effect on basal triphosphatase activity. As expected, GSNO inhibited AdoMet synthetase activity (Fig. 7). Stimulation of triphosphatase activity, however, was lower in the presence of a NO donor than in its absence (Fig. 8). The half time of the lag phase of the residual AdoMet synthetase activity after *S*-nitrosylation was not significantly different to that in the absence of NO (not shown), suggesting that stimulation of triphosphatase ac-

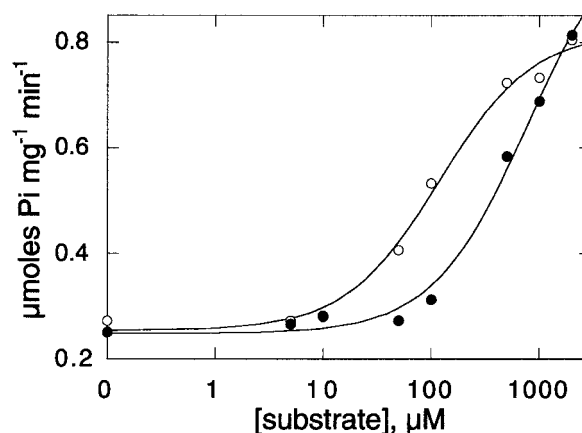


FIG. 5. **Dependence of the stimulation with the ATP/methionine concentration.** Stimulation of triphosphatase activity was performed incubating MATIII at 37 °C in the presence of the indicated ATP/MgCl<sub>2</sub> (●) or methionine (○) concentrations. The concentration of the non-variable substrate was kept at 2 mM. After 2 min, triphosphatase activity measurements were started by adding 40 volumes of reaction mixture containing 2 mM triphosphatase/MgCl<sub>2</sub>. The solid lines represent the non-linear regression fit to a hyperbola plus a basal activity. The parameters for ATP and methionine dependence, respectively, are: basal activity,  $249 \pm 12 \text{ nmol mg}^{-1} \text{ min}^{-1}$ ; increase,  $780 \pm 68 \text{ nmol mg}^{-1} \text{ min}^{-1}$ ;  $S_{0.5}$ ,  $763 \pm 181 \mu\text{M}$ ; and basal activity,  $254 \pm 14 \text{ nmol mg}^{-1} \text{ min}^{-1}$ ; increase,  $567 \pm 21 \text{ nmol mg}^{-1} \text{ min}^{-1}$ ;  $S_{0.5}$ ,  $120 \pm 20 \mu\text{M}$ .

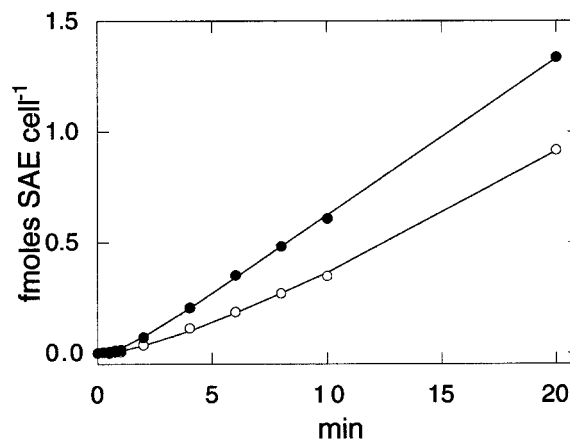


FIG. 6. **Stimulation of MAT *in vivo*.** Rat hepatocytes were incubated in media containing  $10 \mu\text{M}$  (○) or  $500 \mu\text{M}$  (●) methionine. After 20 min, both groups of cells were washed and fresh media, containing 2 mM ethionine and no methionine, was added. At the indicated times, cells were collected and their SAE content measured. The solid lines represent the non-linear regression fit to Equations 6 and 7 under “Experimental Procedures.” The parameters are: amino acid transport rate constant ( $0.85 \text{ min}^{-1}$ ), initial activity ( $0.015 \text{ fmol min}^{-1} \text{ cell}^{-1}$ ), final activity ( $0.035 \text{ fmol min}^{-1} \text{ cell}^{-1}$ ), and MAT activation rate constant ( $0.07 \text{ min}^{-1}$ ).

tivity was not affected by NO. To test whether NO affects directly the stimulated triphosphatase activity rather than the stimulation process, a series of experiments with different sequences of stimulation/*S*-nitrosylation were performed. Stimulation of triphosphatase activity was the same regardless the order in which *S*-nitrosylation was carried out (Fig. 8), indicating that NO has a direct effect on the stimulated activity.

**Rate-determining Step of the AdoMet Synthetase Reaction**—The possibility of measuring independently one of the two consecutive reactions catalyzed by MAT allows us to obtain information about the rate-determining reaction. Comparison of both activities can be done by plotting AdoMet synthetase

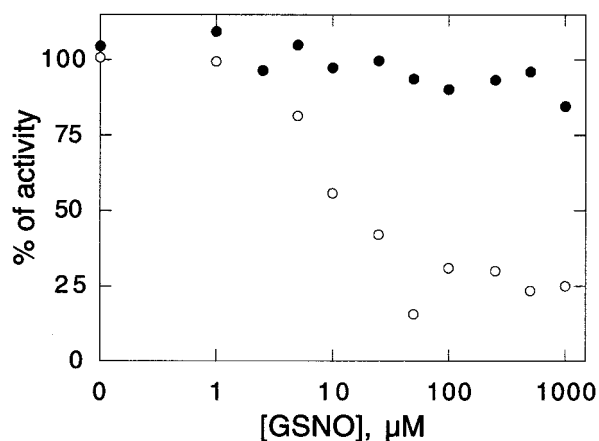


FIG. 7. Effect of NO on AdoMet synthetase and triphosphatase activities. MATIII was incubated in the presence of the indicated GSNO concentrations for 15 min at room temperature. After this time, triphosphatase/MgCl<sub>2</sub> (●) or ATP/MgCl<sub>2</sub> plus methionine (○) were added at a final concentration of 2 mM of each substrate. The amount of P<sub>i</sub> produced in 15 min at 37 °C was measured. Percentages of the activity without GSNO are shown.

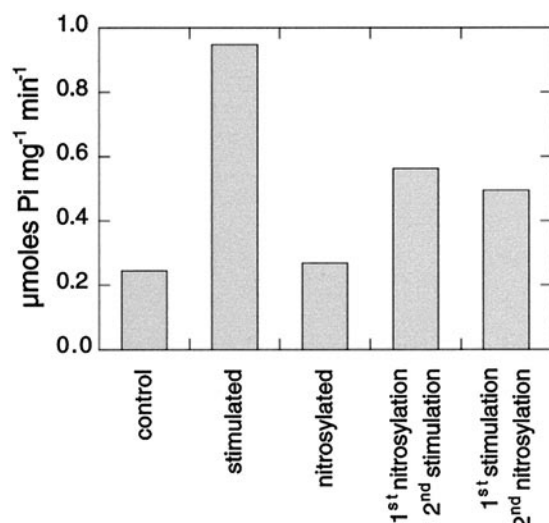


FIG. 8. Effect of NO on stimulation of triphosphatase activity. Triphosphatase activity was measured at 37 °C after different stimulation/nitrosylation protocols. Stimulation was done by incubating MATIII in the presence of 2 mM ATP plus methionine (each) for 5 min at 37 °C. S-Nitrosylation was achieved by exposing MATIII to 250 μM GSNO for 15 min at room temperature.

activity as a function of triphosphatase activity. If the synthesis reaction would be the rate-determining step, triphosphatase activity would be expected to be faster than the overall activity (AdoMet synthetase activity). Thus, a line of slope smaller than one should be obtained. On the contrary, if triphosphatase hydrolysis were the rate-determining step, both activities should be the same, and a line of slope one should be obtained. A line of slope greater than unity would indicate that triphosphatase activity measured with exogenous triphosphatase is slower than the hydrolysis reaction within the overall reaction and, thus, both activities would not be comparable. Such a plot is shown in Fig. 9. Under two different conditions, S-nitrosylated and non-S-nitrosylated MAT, lines with slopes close to unity were obtained, indicating that the rate-determining reaction is triphosphatase hydrolysis.

#### DISCUSSION

Our results indicate the presence of two MATIII isoforms, one with low triphosphatase activity that is insensitive to

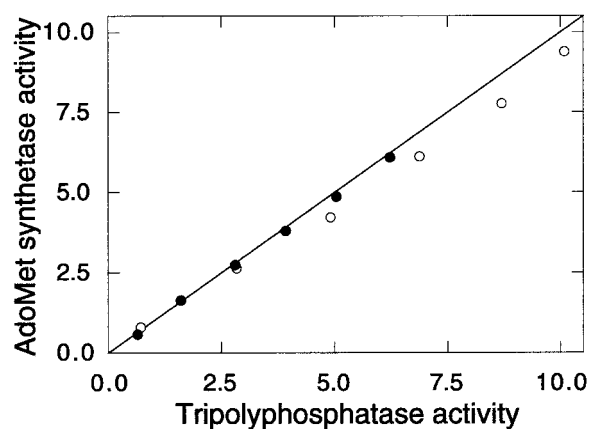


FIG. 9. Rate-determining reaction in the synthesis of AdoMet. Both AdoMet synthetase and triphosphatase activities were measured at 37 °C in non-nitrosylated (○) and nitrosylated (●) stimulated MAT, in the presence of 2 mM of each substrate. AdoMet synthetase activity is plotted versus triphosphatase activity. The identity line, expected if both activities were the same, is also shown. Activity units are in μmol mg<sup>-1</sup>.

NO, and another with high triphosphatase activity that is inhibited by NO. Hydrolysis of triphosphatase is the rate-determining step in the AdoMet synthetase reaction catalyzed by both MATIII isoforms. Interconversion of the two conformations is a slow process that originates a lag phase in AdoMet synthetase activity measurements. High methionine concentration triggers formation of the high activity form both *in vitro* and *in vivo*.

Although several laboratories have reported kinetic data for AdoMet synthetase activity, the properties and reaction mechanism of liver enzymes are far from clear or established. There is great variability in the reported kinetic parameters by different groups. For instance, the MATIII  $K_m$  value for methionine ranges from 215 μM (14) to 1–7 mM (7). Moreover, to our knowledge, no information about triphosphatase activity of liver enzymes has been published. Our results with MATIII differ from those previously reported (7, 14, 15), even though the estimated kinetic parameters for AdoMet synthetase activity are in the same range. Contrary to what has been reported (7, 14), in the presence of high ATP and methionine concentrations, AdoMet synthetase activity is not affected by low concentrations of AdoMet. However, triphosphatase activity showed a decrease in the Hill coefficient in the presence of high AdoMet concentrations as it has been reported for AdoMet synthetase activity in previous studies (14). The lag phase in AdoMet synthetase activity shown here has not been mentioned before, probably because it can be easily missed if not enough data are collected at incubation times shorter than 1 or 2 min. In our hands, the specific activity of MATIII, measured in 2 mM ATP and methionine, is similar to that of *E. coli* MAT (16), but about an order of magnitude higher than previously reported values for MATIII (7, 14, 15). This higher specific activity suggests that the enzyme we have purified is better preserved than are those with previous liver MAT preparations, probably due to the faster and better quality chromatographic equipment and media available nowadays.

There is a lag phase in the rate of AdoMet synthesis. When AdoMet synthetase activity is measured over time, there is a delay to reach the steady-state activity. If triphosphatase activity is measured along this lag phase, a parallel increase or stimulation of the activity is observed. The change in activity

only occurs when both MAT natural substrates are present. ATP or methionine by themselves do not have any effect, and the same is true for a combination of ADP and methionine. Tripolyphosphate alone does not stimulate its hydrolysis, because no lag phase in tripolyphosphatase activity can be detected. A lag phase in yeast MAT was also reported (17). It was shown that the AdoMet produced by the enzyme caused a stimulation of its own activity. However, in the case of the rat liver MATIII, AdoMet has no effect on either AdoMet synthetase or stimulation of tripolyphosphatase activity. Thus, the lag phase in both enzymes is probably due to different mechanisms. The presence of the lag phase complicates the kinetic studies of MAT activity, making it difficult to do measurements under initial velocity conditions. This is, probably, one of the reasons for the large variability of kinetic parameters found in the literature (reviewed in Ref. 6).

ATP and methionine act as a switch between two different MATIII isoforms. There are two possible explanations for the lag phase, accumulation of an intermediate in the catalytic pathway before the release of  $P_i$  or a change in activity during the time course of the experiment. Because the time needed to achieve the steady-state is longer than the time needed to complete one catalytic cycle, accumulation of an intermediate can be ruled out as the origin of the lag phase. Thus, latency should be explained by a slow transition between two states with different specific activities. These stable states with low and high activity, before and after the lag phase, were directly monitored by their tripolyphosphatase activity, which remained linear in time in all experiments. The existence of these two different MAT isoforms is also supported by their different sensitivity to NO. Only the high tripolyphosphatase activity form is inhibited by NO. The fact that the transition is speeded up by increasing substrate concentration favors a process in which binding induces a slow conformational change *versus* another in which a slow equilibrium between two species is shifted because of substrate binding to one of them (18). Because ADP can not substitute ATP eliciting the transition, it is probable that the slow step involves some catalytic step, not just binding, presumably the synthesis of the first AdoMet molecule.

The rate-determining reaction is hydrolysis of tripolyphosphate. AdoMet synthetase activity is the combination of two consecutive reactions, AdoMet synthesis and hydrolysis of tripolyphosphate. Thus, if the rate-determining step would be the synthetic reaction, the tripolyphosphatase activity measured with exogenous tripolyphosphate should be higher than AdoMet synthetase activity. At short incubation times, AdoMet synthetase activity is similar to tripolyphosphatase activity of non-stimulated MAT, whereas at longer incubation times AdoMet synthetase activity matches tripolyphosphatase activity of stimulated MAT. It seems that AdoMet synthetase activity parallels or follows the increase in tripolyphosphatase activity. Under our experimental conditions, maximal tripolyphosphatase and AdoMet synthetase activities were the same, within experimental error, indicating that the rate-determining reaction is tripolyphosphate hydrolysis. Moreover, the identical velocity of the two activities suggests that tripolyphosphatase activity measured with exogenous tripolyphosphate is equivalent to the reaction that takes place during the whole catalytic cycle.

NO inhibits the tripolyphosphatase activity of the more active MAT isoform but not its induction. AdoMet synthetase and tripolyphosphatase activities show different sensitivities toward NO, with the later apparently being resistant to inhibition. However, NO inhibits tripolyphosphatase activity when measured after the lag phase. There are two possible NO ac-

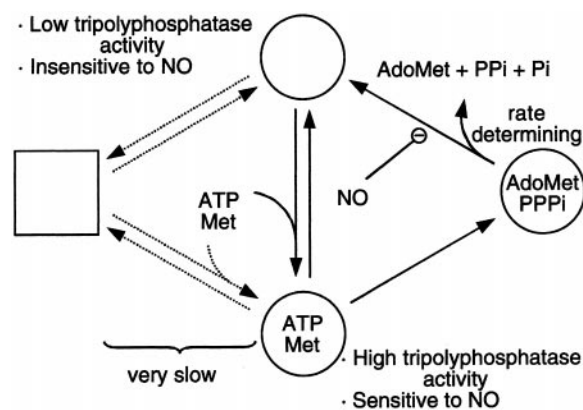


FIG. 10. Proposed model for MATIII action. See text for details.

tions to explain this pattern of inhibition: blocking or reducing the transition between isoforms or directly inhibiting the high tripolyphosphatase activity isoform. Our results support the later possibility, because the same tripolyphosphatase activity is measured when stimulation is carried out before or after *S*-nitrosylation. Moreover, the time course of the lag phase of the residual AdoMet synthetase activity is not altered by NO. Because MAT stimulation is induced by binding of ATP and methionine (although a subsequent step may be the slow process), *S*-nitrosylation does not appear to block the binding of these substrates, as we previously proposed (11). We can not rule out, though, the possibility of a decrease in affinity.

The hysteretic behavior may function as a regulatory mechanism of MAT activity *in vivo*. Availability of methionine is probably the determining factor in MAT activity. Its concentration in a liver cell is approximately 50–80  $\mu\text{M}$  (6). Under normal conditions, the low activity isoform would be the predominant MATIII species, and most of AdoMet synthesis would be carried out by MATI. However, after an increase in methionine concentration, *i.e.* after a protein-rich meal, conversion to the high activity MATIII would occur and methionine excess would be eliminated. The  $S_{0.5}$  for the stimulatory effect of methionine is 120  $\mu\text{M}$ , which is in the range of methionine concentration fluctuations. Such a mechanism would be very sensitive to changes in methionine concentration. This model predicts that a lag phase in MAT activity should be observed only at low methionine concentrations, because at higher concentrations the active isoform should already be present. Experiments carried out with hepatocytes showed a longer lag phase at low methionine concentration in the incubation media. Taking into account that transport of methionine into the cytoplasm is expected to introduce a delay, which could explain the short lag phase in high methionine, our results agree with the above model. Hysteretic behavior, defined as a slow response to changes in ligand binding, has been described for many important enzymes in metabolic regulation for a long time (reviewed in Ref. 19). We proposed that liver MATIII should be included among these enzymes and that this behavior is an important feature for its function as the key enzyme regulating the methionine cycle in the liver.

In conclusion, our results allow us to propose the mechanism depicted in Fig. 10. The key features of this mechanism are the following. There are two isoforms of rat liver MATIII. A form with low tripolyphosphatase activity that is not inhibited by NO and is present at low, physiological, methionine concentrations. Higher concentrations of methionine, in the presence of physiological concentrations of ATP, induce a slow transition to a conformation with high tripolyphosphatase activity that is inhibited by NO. The transition is not affected by NO. The

rate-determining reaction in the synthesis of AdoMet is hydrolysis of tripolyphosphate. The same regulation of MAT activity by methionine occurs *in vivo* and *in vitro*.

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