Nitric Oxide Inactivates Rat Hepatic Methionine Adenosyltransferase *In Vivo* by *S*-Nitrosylation

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We investigated the mechanism of nitric oxide (NO) action on hepatic methionine adenosyltransferase (MAT) activity using *S*-nitrosoglutathione (GSNO) as NO donor. Hepatic MAT plays an essential role in the metabolism of methionine, converting this amino acid into *S*-adenosylmethionine. Hepatic MAT exists in two oligomeric states: as a tetramer (MAT I) and as a dimer (MAT III) of the same subunit. This subunit contains 10 cysteine residues. In MAT I, *S*-nitrosylation of 1 thiol residue per subunit was associated with a marked inactivation of the enzyme (about 70%) that was reversed by glutathione (GSH). In MAT III, *S*-nitrosylation of 3 thiol residues per subunit led to a similar inactivation of the enzyme, which was also reversed by GSH. Incubation of isolated rat hepatocytes with *S*-nitrosoglutathione monoethyl ester (EGSNO), a NO donor permeable through the cellular membrane, induced a dose-dependent inactivation of MAT that was reversed by removing the NO donor from the cell suspension. MAT, purified from isolated rat hepatocytes, contained *S*-nitrosothiol groups and the addition of increasing concentrations of EGSNO to the hepatocyte suspension led to a progressive *S*-nitrosylation of the enzyme. Removal of the NO donor from the incubation media resulted in loss of most NO groups associated to the enzyme. Finally, induction in rats of the production of NO, by the administration of bacterial lipopolysaccharide (LPS), induced a fivefold increase in the *S*-nitrosylation of hepatic MAT, which led to a marked inactivation of the enzyme. Thus, the activity of liver MAT appears to be regulated *in vivo* by *S*-nitrosylation. (HEPATOLOGY 1998;28:1051-1057.)

Nitric oxide (NO) (nitrogen monoxide) exerts its direct effects by covalently modifying or oxidizing critical thiols or transition metals in proteins.[1,2] Consequently, the function of an increasing number of proteins has been demonstrated to be regulated *in vitro* by *S*-nitrosylation.[1,2] However, there is still little evidence showing the regulation of enzyme activity by reversible *S*-nitrosylation *in vivo* or in intact cells.[1,2] NO may be a physiological regulator of liver methionine adenosyltransferase (MAT), the enzyme that converts methionine, in the presence of adenosine triphosphate (ATP) into *S*-adenosylmethionine.[3,4] In mammals, it is now established that two genes encoding homologous, but different, MAT protein subunits are present.[5-7] One gene (MAT1A), expressed in the liver only, encodes a subunit found in two native MAT isoforms, which are either a homodimer (MAT III) or a homotetramer (MAT I) of this single subunit. The second gene (MAT2A) is expressed in all other mammalian tissues including fetal liver. The subunit it encodes is found in a native MAT isoform known as MAT II.

We have previously demonstrated that increased NO production in the liver, during septic shock or hypoxia, led to the inactivation of hepatic MAT, whereas inhibition of NO synthase, by N^G-nitro-L-arginine-methylester, resulted in markedly decreased hepatic MAT inactivation in response to hypoxia.[3,4] Moreover, the activity of MAT I and MAT III, purified from rat liver, was inactivated by incubation with NO donors (3-morpholinosydnonimine and *S*-nitroso-*N*-acetyl-penicillamine).[3] This inactivation of hepatic MAT by NO was reversed by millimolar amounts of glutathione (GSH).[3] The hepatic MAT subunit contains 10 cysteine residues.[6] We have also demonstrated that the enzymatic activity of a hepatic MAT mutant, in which the cysteine residue 121 was replaced by a serine, was resistant to NO inactivation.[3]

These results strongly suggest that hepatic MAT is regulated by reversible *S*-nitrosylation, but no direct evidence showing *S*-nitrosylation of MAT is yet available. However, although large amounts of NO (micromolar) are produced by the liver under pathological conditions (tumor necrosis factor α, interleukin-1, interferon gamma, bacterial lipopolysaccharide [LPS]),[8] the finding that physiological amounts (millimolar) of GSH reversed the action of NO on MAT activity[3] raises doubts about how NO would affect the activity of MAT *in vivo*. In this study, we demonstrate that liver MAT is *S*-nitrosylated both *in vitro* and *in vivo* and that LPS-mediated hepatic MAT inactivation involves the *S*-nitrosylation of the enzyme.

MATERIALS AND METHODS

Materials. Protease inhibitors (phenylmethylsulfonyl fluoride and benzamide), ATP, L-methionine, DL-dithiothreitol (DTT), LPS, and GSH were obtained from Sigma (St. Louis, MO); [2-³H] ATP (25.5 Ci/mmol) was from Amersham (Little Chalfont, England). The cation exchanger, AG 50W-X4 (200 to 400 mesh), was from Bio-Rad

Laboratories (Richmond, CA). DEAE fast-flow, phenyl Sepharose fast-flow, blue Sepharose fast-flow, Sephacryl S-300, DEAE sephacel, and phenyl Sepharose were from Pharmacia (Uppsala, Sweden). 2-Mercaptoethanol was from Merck AG (Darmstadt, Germany), and scintillation fluid Optiphase Hisafe 3 was from Wallac (Loughborough, UK). High-performance liquid chromatography column Aminopropyl Spherisorb for glutathione determination was from Waters (Bedford, MA).

Isolation and Incubation of Hepatocytes. Hepatocytes were isolated from normally fed Wistar albino rats (250 g) as described by Castaño et al.[9] Isolated hepatocytes were incubated as follows: 1 to 4 mL of cell suspension (2×10^6 cells/mL) were shaken in stopped 20-mL vials at 37°C in the presence of 10 mmol/L glucose. The gas phase was 95% O_2 and 5% CO_2. After 30 minutes of preincubation, *S*-nitrosoglutathione monoethyl ester (EGSNO) or buffer were added, and cells were incubated for the desired periods of time. The suspension of hepatocytes was poured after the indicated periods into precooled, centrifuged tubes and immediately centrifuged for 10 seconds. Cells were then washed twice in phosphate-buffered saline buffer before being homogenized by 3 cycles of freezing and thawing in 10 mmol/L Tris, 0.3 mol/L sucrose, and 1 mmol/L ethylene glycol-bis(β-aminoethyl ether)-*N,N*-tetraacetic acid (EGTA) (pH 7.5) containing 0.1% PMSF and 0.1% benzamidine. Cytosol was obtained by centrifugation at 100,000*g* at 4°C. The amount of hepatic MAT protein was determined by Western blot analysis as previously described.[10] Cell viability was determined before and after the incubation by the Trypan blue exclusion test. Only cell preparations with a viability greater than 85% were used. All animals were treated humanely, and study protocols were in compliance with our institution's guidelines for the use of laboratory animals.

LPS Treatment. Animals received intraperitoneal injections of LPS (20 mg/kg) or saline at 8 AM as previously reported.[3] Samples were collected 6 hours after the administration of LPS. Liver samples were removed and quickly freeze-clamped in liquid nitrogen. Livers were homogenized in 4 volumes of 10 mmol/L Tris/HCl (pH 7.5), containing 0.3 mol/L sucrose, 1 mmol/L EGTA, 0.1% phenylmethylsulfonyl fluoride, and 0.1% benzamidine. The homogenate was centrifuged for 20 minutes at 10,000*g* at 4°C; the supernatant was again centrifuged for 1 hour at 100,000*g*. Total MAT activity and MAT *S*-nitrosylation were analyzed in this last supernatant as described below.

Purification of MAT. MATI and MATIII were purified from the liver of 250-g Wistar albino rats according to the procedure previously described,[11] in the presence of protease inhibitors and reducing agents. All purified enzymes were stored at −70°C in 10 mmol/L HEPES, 10 mmol/L MgSO4, and 1 mmol/L ethylenediaminetetraacetic acid (EDTA) (pH 7.5) (buffer A) containing 20% glycerol and 10 mmol/L DTT. Recombinant liver MAT forms were overexpressed in *Escherichia coli* and purified according to Mingorance et al.[12]

MAT Activity Measurements. MAT activity assays were performed according to Cabrero et al.[13] at saturating concentrations of the substrates, 5 mmol/L methionine and 5 mmol/L ATP, in the absence of any reducing agent. Cytosol extracts, prepared as described above, were used directly for the enzymatic assay. Glycerol and DTT were removed from purified protein preparations by chromatography on a 1-mL DEAE column immediately before being used for activity measurements. Incubation with *S*-nitrosoglutathione (GSNO) was performed at 37°C for 15 minutes, and then enzymatic activity was assayed.

GSH and Oxidized Glutathione Measurements. GSH and oxidized glutathione (GSSG) content was measured by high-performance liquid chromatography according to the method described by Reed et al.[14] Briefly, 200 μL of the cytosolic fraction obtained as indicated above was immediately derivatized using 1-fluoro-2,4-dinitrobenzene. Samples were then chromatographed on a 3-aminopropyl-Spherisorb, 20 cm × 4.6-mm, 5-μm HPLC column, equilibrated in 80% methanol. Elution was performed with 0.5 mol/L sodium acetate in 64% methanol and followed at 365 nm. Analysis of the

chromatogram was performed with a Beckman System Gold software.

Preparation of GSNO and EGSNO. GSNO and EGSNO were prepared according to the method of Saville.[15] Briefly, equimolar concentrations of an aqueous solution of NaNO2 and a freshly prepared GSH or EGSH solution in 250 mmol/L HCl and 0.1 mol/L EDTA (pH 1.5) were mixed. The resulting mixture was incubated at room temperature for 5 minutes and then neutralized with NaOH.

Cytosolic Nitrite and Nitrate Measurements. Nitrite and nitrate were measured using a NOA 280 chemiluminescence detector following the method recommended by Sievers.[16] Cytosolic nitrite and nitrate were reduced to NO by incubation with glacial acetic acid containing 350 mmol/L INa or 1 N HCl containing 50 mmol/L VCl3, respectively. The resulting NO was measured by the chemiluminescence derived from its reaction with ozone, according with the following two reactions:

$$NO + O_3 \rightarrow NO_2^* + O_2; NO_2^* \rightarrow NO_2 + h\nu$$
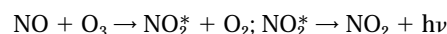
Determination of MAT S*-Nitrosylation.* Samples of purified MAT protein (0.2 mg/mL) were incubated with GSNO at 37°C for 15 minutes in the absence of any reducing agent. To remove the excess of GSNO, 200 μL of a DEAE resin equilibrated in 10 mmol/L HEPES, 10 mmol/L MgSO4, and 1 mmol/L EDTA (pH 7.5) (buffer A) were added to the assay mixture. The resin was washed twice with 10 volumes of buffer A with 100 mmol/L KCL (pH 7.5) before eluting the protein with the same buffer containing 200 mmol/L KCl. *S*-Nitrosylated proteins were incubated with 2.2 mmol/L HgCl2, and the amount of NO released was measured using a Sievers NOA 280 nitric oxide analyzer.[16] Standard curves were generated with known concentrations of GSNO. To measure the *S*-nitrosylation of MAT III in intact hepatocytes, the protein was purified from the cytosolic fraction of 4 mL of cell suspension (2×10^6 cells/mL) after incubation with the indicated concentrations of EGSNO. The cytosol was loaded into a 2-mL column of DEAE sephacel equilibrated in buffer A. The column was washed with 10 column volumes of buffer A containing 100 mmol/L KCl before elution with 2 mL of buffer A containing 200 mmol/L KCl. The enzyme was then chromatographed in a 1-mL Phenyl Sepharose column equilibrated in buffer A with 200 mmol/L KCl. The column was washed with 10 column volumes of buffer A with 200 mmol/L KCl, and then with 10 volumes of 10 mmol/l HEPES and 1 mmol/L EDTA (pH 7.5). Dimethyl sulfoxide is commonly used to elute MAT III from the phenyl Sepharose column.[11] Because dimethyl sulfoxide interfered with the NO detection, the resin containing MAT III protein was directly incubated with 2.2 mmol/L HgCl2, and the amount of NO released was measured using a Sievers NOA 280 nitric oxide analyzer. The purity of MAT III, eluted from the phenyl Sepharose column with 1 mL buffer A containing 50% dimethyl sulfoxide, was tested by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE).[17] To determine the recovery of *S*-nitrosylated MAT III purified by this procedure, a sample of MAT III purified from rat liver was reacted *in vitro* with 100 μmol/L GSNO as described above, and the amount of *S*-nitrosothiols (SNO) groups incorporated per microgram of protein determined in an aliquot. The rest of the *S*-nitrosylated enzyme was added to a freshly prepared homogenate of hepatocytes. MAT III was then purified and the amount of SNO groups determined as mentioned above. The recovery of SNO groups incorporated into MAT III was between 80% to 90%. To measure the *S*-nitrosylation of MAT III in liver, the protein was purified from the cytosolic fraction of about 0.2 g of liver and the amount of SNO groups incorporated per milligram of protein determined as described above.

RESULTS

S-Nitrosylation and Inactivation of Purified Rat Liver MAT by GSNO. *S*-Nitrosylation of purified rat liver MAT I and MAT III was directly measured after reaction with GSNO, a low-mass NO donor,[18] under conditions that led to inactivation of the
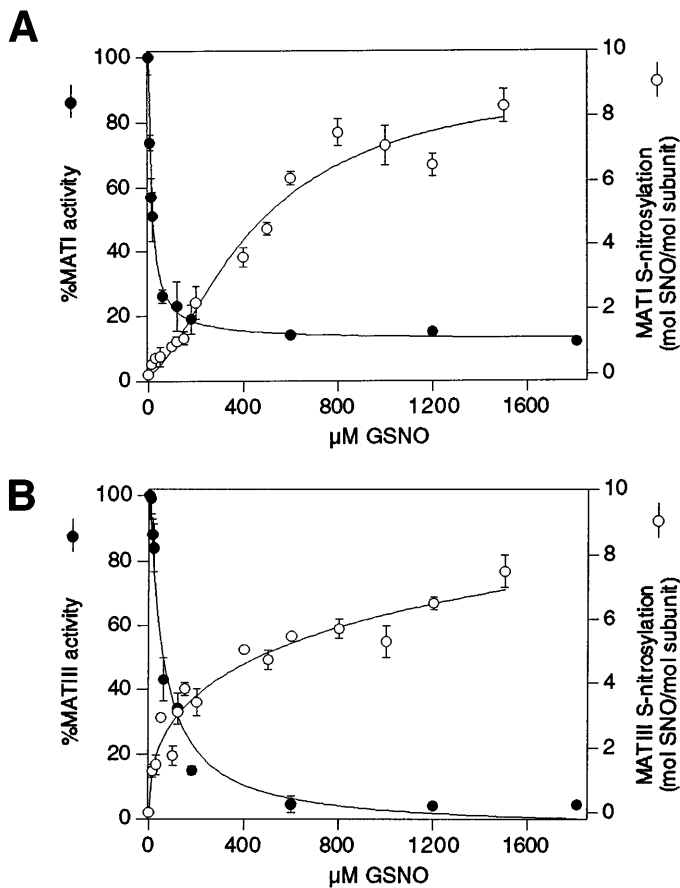
A

B

FIG. 1.   Effect of GSNO on hepatic MAT S-nitrosylation and inactivation. Purified liver MAT I (A) and MAT III (B) were reacted with various concentrations of GSNO and the formation of nitrosothiol (SNO) groups (○), and the activity of the enzyme (●) was determined as described in Materials and Methods. Values are expressed as means ± SEM of three experiments in triplicate using different preparations of the enzyme. One hundred percent activity was 39 ± 7 pmol/min/mg protein and 29 ± 3 pmol/min/mg protein for MAT I and MAT III, respectively.

enzyme (Fig. 1). In MAT I, 100 µmol/L GSNO induced the S-nitrosylation of about 1 thiol residue per subunit and led to 70% inactivation of the enzyme. In MAT III, 100 µmol/L GSNO also induced 70% inactivation, and this effect was accompanied by the S-nitrosylation of about 3 thiol residues per enzyme subunit. In the presence of 50 µmol/L GSNO, the inactivation of both MAT I and MAT III was about 50%. We have previously demonstrated that substitution of cysteine 121 by a serine residue prevented hepatic MAT inactivation by 3-morpholinosydnonimine (a NO donor) and OH radical.[3,19] As expected, the addition of 50 µmol/L GSNO to the wild-type recombinant enzyme, a mixture of MAT I and MAT III,[12] led to 50% reduction of the enzyme activity, whereas the substitution of cysteine 121 by a serine residue prevented the ability of GSNO to inactivate liver MAT (data not shown). Similarly, whereas incubation of the wild-type enzyme with 100 µmol/L GSNO led to the S-nitrosylation of 2.2 ± 0.2 thiol residues per subunit, in the mutant enzyme, only 1.2 ± 0.1 thiol residues per subunit were S-nitrosylated. S-Nitrosylation of up to 8 thiol residues per enzyme subunit was observed, with both MAT I and MAT III, at saturating concentrations of GSNO (Fig. 1). Under these conditions, both isoforms of liver MAT were almost completely inactivated.

The effect of GSNO on hepatic MAT activity was reversible. Thus, addition of 2 mmol/L GSH to purified MAT I or MAT III preincubated with 100 µmol/L GSNO (to reach an inactivation of about 70%) returned the activity to that of the untreated enzymes (Table 1). These results agree with previous findings showing that MAT inactivation by 3-morpholinosydnonimine was reversed by the addition of GSH.[3] The addition of GSH also induced the loss of SNO groups from purified MAT I and MAT III pretreated with GSNO (Table 1). Thus, addition of 2 mmol/L GSH to purified MAT I or MAT III pretreated with 100 µmol/L GSNO resulted in the loss of most of the NO groups incorporated into the enzymes. These results indicate that MAT S-nitrosylation is a reversible process and that conditions that resulted in loss of SNO groups reversed the inactivation of the enzyme.

MAT activity measurements were performed at saturating levels of methionine and ATP, the substrates of the reaction, indicating that GSNO reduced the V_{max} of both MAT I and MAT III. To determine whether GSNO also affected the affinity of the enzymes for their substrates, kinetics experiments were performed in the presence of 50 µmol/L GSNO (to reach an inactivation of about 50%). In MAT I, GSNO had no effect on the apparent $S_{0.5}$ values for methionine (170 µmol/L) and ATP (190 µmol/L). In contrast, the apparent $S_{0.5}$ values of MAT III for methionine and ATP increased in the presence of GSNO from 0.6 mmol/L to 1.4 mmol/L and from 0.9 mmol/L to 1.8 mmol/L, respectively.

S-*Nitrosylation and Inactivation of MAT in Isolated Rat Hepatocytes and in an* In Vivo *Model of Septic Shock.* GSNO, like GSH, is not readily permeable through the cell membrane. Consequently, we found that in isolated rat hepatocytes, the addition of GSNO (up to 10 mmol/L) had little or no effect on the intracellular concentration of nitrite and nitrate (NOx), the inactive metabolites of NO (data not shown). Accordingly, GSNO had no effect on MAT activity in isolated rat hepatocytes (data not shown). EGSH is a well-known permeable derivative of GSH, which, upon its entrance to the cell, is converted into GSH and ethanol.[20] We therefore tested the ability of EGSNO, the S-nitroso derivative of EGSH, to increase the intracellular concentration of NOx in isolated rat hepatocytes. We first studied the potency of EGSNO as a NO donor in vitro using MAT I and MAT III purified from rat liver, and observed that this molecule was as potent as GSNO in inducing MAT S-nitrosylation and inactivation (data not shown). The addition of EGSNO to isolated rat hepatocytes

TABLE 1.   Reversion by GSH of GSNO-Induced MAT Inactivation and S-Nitrosylation

Enzyme	Activity (% of control)		S-Nitrosylation (mol SNO/mol subunit)	
	−GSH	+GSH	−GSH	+GSH
MAT I (100 µmol/L GSNO)	25 ± 4.5	102 ± 5.4*	1.02 ± 0.19	0.20 ± 0.06*
MAT III (100 µmol/L GSNO)	32 ± 5.3	99 ± 4.6*	2.39 ± 0.20	0.24 ± 0.07*

NOTE. Purified MAT I and MAT III were incubated in the absence or presence of 100 µmol/L GSNO for 15 minutes. Samples were then incubated in the absence or presence of 2 mmol/L GSH for another 15 minutes. At the end of this period, MAT activity and S-nitrosylation were determined. Values are the means ± SEM of three independent experiments in triplicate.

*Values significantly different from the corresponding value in the absence of GSH ($P < .005$).

induced a dose-dependent intracellular accumulation of NOx, which led to a progressive MAT inactivation (Fig. 2A). The addition of 5 mmol/L EGSNO to the cell suspension induced an elevation of NOx levels of about eightfold, which led to 70% inactivation of MAT (Fig. 2A). Up to a concentration of 5 mmol/L EGSNO, added to the cell suspension, this molecule had no effect on the intracellular GSH levels (about 5 mmol/L) or in the ratio GSH/GSSG (about 100) (data not shown). However, at 10 mmol/L EGSNO, NOx levels increased about 25-fold (Fig. 2A), and the ratio of GSH/GSSG decreased to reach a value of about 10 (data not shown). The addition of 5 mmol/L EGSH to isolated rat hepatocytes had no effect on NOx levels and, as previously described for rat liver,[21] slightly increased MAT activity (data not shown). The reduction in MAT activity induced by EGSNO was not the consequence of an increased degradation or release of hepatic MAT, because no changes in the levels of this enzyme were detected by Western blotting (Fig. 2B). Moreover, no significant lysis of the hepatocytes was observed after incubation with the various doses of EGSNO mentioned in Fig. 2 (data not shown).

MAT III can be readily isolated in the absence of DTT in the buffers used for purification to avoid the loss of SNO groups in the enzyme, by using the procedure mentioned in Materials and Methods. The enzyme obtained with this protocol had a purity greater than 90% as judged by SDS-PAGE (Fig. 3B). On the contrary, no method is yet available that permits the rapid isolation of MAT I. When MAT I was purified by the conventional procedure described in Materials and Methods, the enzyme lost the SNO groups. Therefore, only S-nitrosylation of MAT III was directly measured in isolated rat hepatocytes after the addition of various concentrations of EGSNO to the cell suspension. MAT III purified from isolated rat hepatocytes contained SNO groups (10 ± 3 nmol of SNO
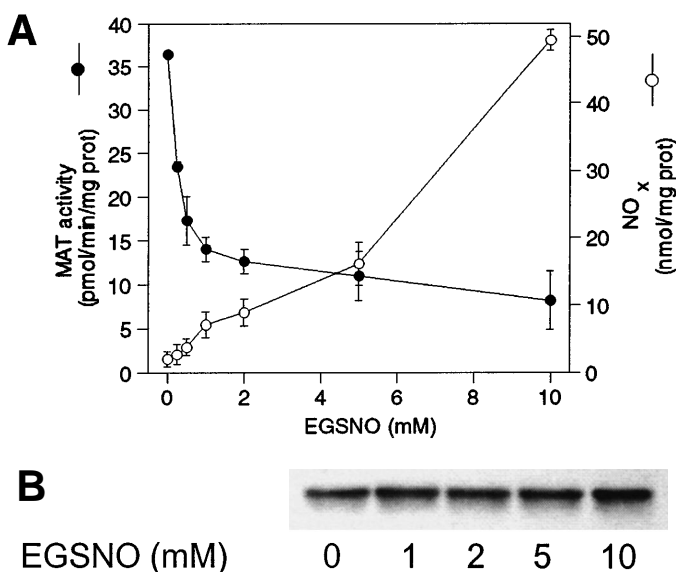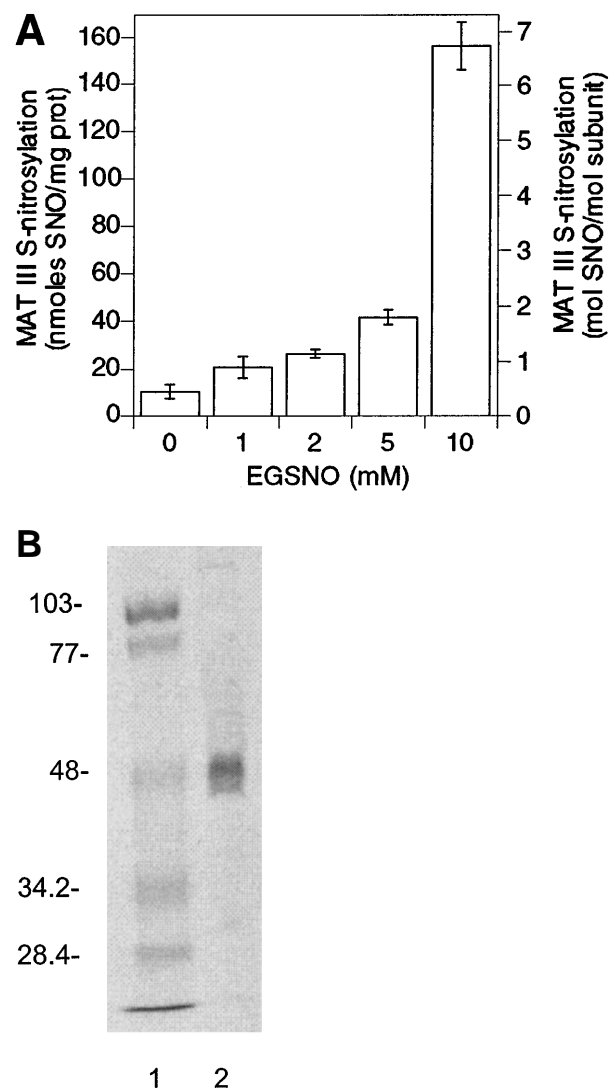

FIG. 3. S-Nitrosylation of MAT III isolated from hepatocytes treated with EGSNO. (A) MAT III was purified from hepatocytes exposed to various concentrations of EGSNO for 30 minutes, and the extent of S-nitrosylation was determined as described in Materials and Methods. Values are the means ± SEM of nine (0, 2, and 5 mmol/L EGSNO) or three (1 and 10 mmol/L EGSNO) independent experiments in triplicate. Values in the presence of EGSNO were significantly different from the control ($P < .01$). (B) SDS-PAGE analysis of MAT III purified from isolated rat hepatocytes. Lane 1: migration of the molecular-size markers; lane 2: migration of MAT III purified as described in Materials and Methods. Molecular weights are indicated.

FIG. 2. Effect of EGSNO on hepatocyte MAT activity and NOx levels. Hepatocytes were incubated with various concentrations of EGSNO. (A) Cytosolic NOx (○) levels and MAT (●) activity were determined after 30 minutes of incubation with the NO donor as described in Materials and Methods. Values are expressed as means ± SEM of three independent experiments in triplicate. (B) Hepatic MAT protein levels were determined by Western blot analysis in hepatocytes after 30 minutes of incubation in the presence of EGSNO.

per milligram MAT subunit; about 0.4 sites per MAT subunit) (Fig. 3A). EGSNO addition to the hepatocyte suspension induced a dose-dependent S-nitrosylation of MAT III (Fig. 3A). Thus, in the presence of 5 mmol/L EGSNO, the number of SNO groups incorporated into MAT III increased about fivefold (about 1.8 sites per MAT subunit), and the addition of 10 mmol/L EGSNO led to the S-nitrosylation of about 7 thiol groups per MAT subunit (Fig. 3A).

The reversibility of the inactivation of MAT by EGSNO in isolated hepatocytes was demonstrated by removing the NO donor from the incubation media and resuspension of the cells in fresh buffer without EGSNO (Fig. 4A). As mentioned above, a 60% to 70% inactivation of MAT was observed within 30 minutes after the addition of 5 mmol/L EGSNO. In
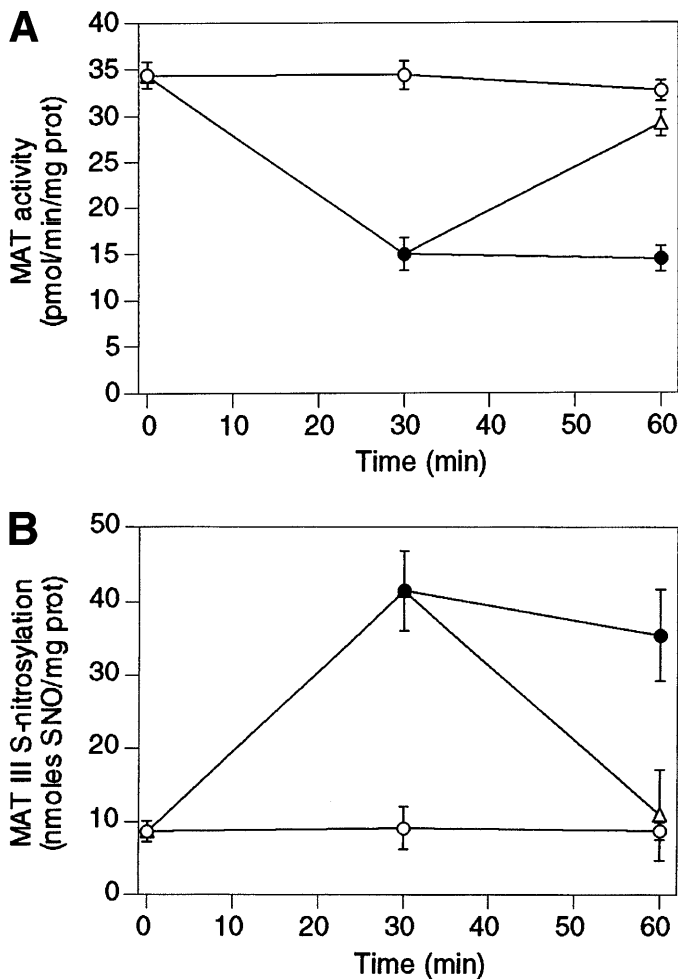
A

B

FIG. 4. Reversion of MAT inactivation and S-nitrosylation induced by EGSNO in isolated rat hepatocytes. Isolated rat hepatocytes were incubated in the absence (○) or presence (●) of 5 mmol/L EGSNO for 30 minutes. At the end of this period, EGSNO was removed from the media by centrifugation, and, after washing the cells twice, the hepatocytes were resuspended in fresh media and incubated again for another 30 minutes in the absence (△) or presence (●) of 5 mmol/L EGSNO. Total MAT activity (A) and MAT III S-nitrosylation (B) were determined as described in Materials and Methods at the times indicated in the figure. Values are expressed as means ± SEM of three independent experiments in triplicate. Values in the presence of EGSNO were significantly different from the corresponding values in the absence of this agent ($P < .005$).

the presence of EGSNO, this inactivation was maintained during the next 30 minutes. In contrast, removal of EGSNO, by taking off the incubation media by centrifugation and resuspension of the cells in fresh buffer without the NO donor, returned MAT activity close to that of untreated hepatocytes (Fig. 4A). S-Nitrosylation of MAT III was also measured after removal of EGSNO from the incubation media. As shown in Fig. 4B, elimination of the NO donor from the incubation media returned MAT S-nitrosylation to values similar to those of the untreated hepatocytes.

In vivo S-nitrosylation of rat liver MAT was also analyzed 7 hours after the intraperitoneal injection of LPS. In agreement with previous reports,[3,22,23] endotoxin administration to rats resulted in the expression in liver of inducible NO synthase and in the accumulation in serum of NOx (data not shown). Liver MAT III purified from control animals contained SNO groups (5 ± 3 nmol of SNO per milligram MAT subunit;

about 0.2 sites per MAT subunit) (Fig. 5). Administration of LPS to rats induced a fivefold increase in S-nitrosylation of MAT III (about 1 site per MAT subunit) (Fig. 5). Under the present conditions, the hepatic concentration of GSH and GSSG did not change significantly with LPS injection (data not shown). As previously reported,[3] hepatic MAT activity decreased by about 60% in animals treated with LPS (data not shown). These results demonstrate that NO inactivates hepatic MAT *in vivo* by S-nitrosylation in the presence of physiological concentrations of GSH.

DISCUSSION

Our previous work implicated NO as a regulator of liver MAT.[3,4] Thus, we have previously demonstrated that increased NO production in the liver, during septic shock or hypoxia, was associated with the inactivation of hepatic MAT, whereas inhibition of NO synthase, by N^G-nitro-L-arginine-methylester, resulted in markedly decreased hepatic MAT inactivation in response to hypoxia.[3,4] Moreover, the activity of MAT I and MAT III, purified from rat liver, was inactivated by incubation with the NO donors, 3-morpholinosydnonimine and S-nitroso-N-acetyl-penicillamine. Finally, elevated NO production in human cirrhosis, which is suggested by the finding of increased serum and urine nitrate,[24] is associated with reduced liver MAT activity[25,26] and impaired methionine metabolism.[27,28]

The present results indicate that NO appears to cause MAT inactivation by covalent modification by S-nitrosylation of specific thiol groups. The hepatic MAT subunit contains 10 cysteine residues. In MAT I, 100 µmol/L GSNO induced the modification of one site per subunit and was associated with an inactivation of about 70%. This agrees with the finding that substitution of cysteine 121 by serine, but not of any of the other nine thiol groups, protected liver MAT from the inactivation induced by 3-morpholinosydnonimine[3] or GSNO. In MAT III, three SNO groups per enzyme subunit were formed at 100 µmol/L GSNO, and this was also associated with the loss of about 70% of the enzyme activity. Because
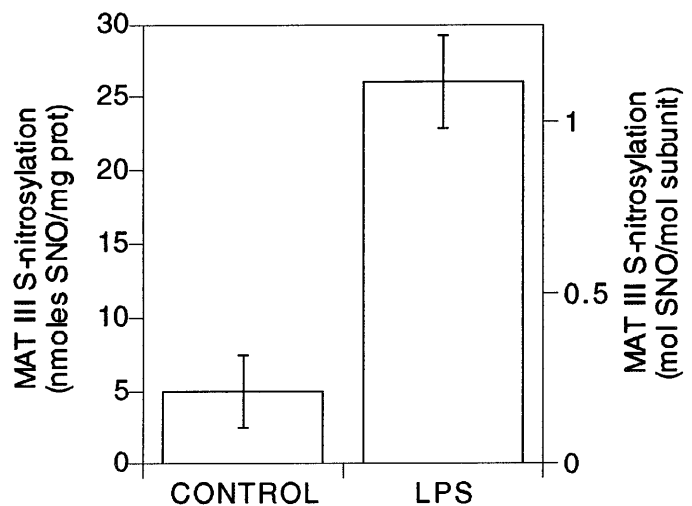

FIG. 5. Effect of LPS treatment on liver MAT III S-nitrosylation. Hepatic levels of MAT III S-nitrosylation were determined 7 hours after the intraperitoneal injection of LPS or saline as described in Materials and Methods. Values are expressed as means ± SEM of three independent experiments in triplicate. The value in the presence of LPS was significantly different from the control ($P < .001$).

MAT I is a tetramer and MAT III is a dimer of the same subunit, different accessibility to cysteine residues may account for these differences in *S*-nitrosylation between both forms. Thus, cysteine 105, which is in the interaction surface between dimers,[19] may be accessible to *S*-nitrosylation in MAT III, but protected in MAT I. The modification of these additional groups in MAT III might be related to the finding that *S*-nitrosylation not only affected the V_{max} of this enzyme, but also its affinity for the substrates, ATP and methionine. The finding that up to 8 sites per MAT subunit were *S*-nitrosylated, at saturating (millimolar) concentrations of GSNO, agrees with the observation that liver MAT contains 10 cysteine residues per subunit, 2 of which, probably residues 35 and 67, are forming an intrasubunit disulfide bridge.[29] It is important to point out that GSNO is an exemplary compound and not necessarily the physiological nitrosating agent in cells.

Inactivation and *S*-nitrosylation of purified MAT I and MAT III by micromolar GSNO was reversed by the addition of physiological amounts (millimolar) of GSH. This finding raises doubts about how NO would affect the activity of this enzyme *in vivo*. To clarify this point, we studied the effect of GSNO on MAT activity and *S*-nitrosylation in isolated rat hepatocytes. For these studies, we used EGSNO, a derivative of GSNO that is permeable through the cell membrane. Our results indicate that a relatively small increase (about five- to eightfold) in the hepatocyte NO content (measured as the cellular accumulation of NOx) was associated with a marked inactivation and *S*-nitrosylation of MAT. Moreover, removal of the NO donor from the media resulted in the reactivation of the enzyme and loss of the SNO groups. Up to a concentration of 5 mmol/L EGSNO in the incubation media, no significant changes in the intracellular GSH concentration or in the GSH/GSSG ratio was measured. The latter is important because GSSG has been previously shown to induce the inactivation of MAT I and MAT III purified from rat liver.[11] The present results indicate that *S*-nitrosylation and inactivation of hepatic MAT can occur *in vivo* in the presence of normal (millimolar) GSH levels. These results contrast with the observation that 2 mmol/L GSH led to the loss of NO groups from purified MAT I and MAT III and restored their activities to normal. The reasons for these differences between *in vivo* and *in vitro* experiments are not clear. Compartmentalization of GSH, which has been observed in various cell types,[30] may account for these differences.

Alterations of hepatic function have been described during septic shock,[31] a pathological condition triggered by LPS.[32] In sepsis, the liver is a target for LPS and a number of endotoxic shock effectors, such as proinflammatory cytokines.[33] We have evaluated the effect of LPS on hepatic MAT III *S*-nitrosylation. In agreement with previous reports,[3,22,23] intraperitoneal injection of LPS to rats resulted in the accumulation in serum of NOx and in the inactivation of hepatic MAT. Moreover, LPS treatment also induced a marked increase in MAT III *S*-nitrosylation in the presence of normal levels of GSH and GSSG. These results indicate that the mechanism by which LPS induces hepatic MAT inactivation *in vivo* involves *S*-nitrosylation of the enzyme.

We have previously demonstrated that hydrogen peroxide, via generation of a hydroxyl radical, also inactivates hepatic MAT by reversibly and covalently oxidizing the same enzyme site that NO does, i.e., cysteine 121.[19] Our findings therefore indicate that this cysteine residue can serve as a sensor of both nitrosative and oxidative signals. Cysteine 121 is localized at a "flexible loop" over the active site cleft of MAT.[19] We suggest that NO and OH may regulate MAT activity by fixing this "flexible loop" in a conformation where the access of ATP to the active site is impaired. In the resting state, the hepatocytes may produce small amounts of NO and OH, but the high intracellular concentration of GSH would protect cysteine 121 of MAT from being modified. In this state, hepatic MAT would be fully active and could convert all the available methionine into *S*-adenosylmethionine at the expenses of ATP. With hypoxia or septic shock, the hepatic production of NO and/or OH will be increased, switching MAT into a less-active conformation by *S*-nitrosylation or oxidation of cysteine 121. In this state, the consumption of ATP by MAT would be reduced. This would contribute to regulate the hepatic utilization of ATP, and therefore to prevent nicotinamide adenine dinucleotide depletion and mitochondrial denergization during the stress imposed by either oxygen- or nitrogen-reactive species. Indeed, overexpression of rat liver MAT cDNA in Chinese hamster ovary cells led to ATP and nicotinamide adenine dinucleotide depletion and increased the sensitivity of the cells to oxidative stress.[10] This mechanism would resemble that demonstrated in hemoglobin and cardiac calcium release channel, in which *S*-nitrosylation regulates the activity of these proteins.[34,35]

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