Creation of a functional *S*-nitrosylation site in vitro by single point mutation

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Abstract Here we show that in extrahepatic methionine adenosyltransferase replacement of a single amino acid (glycine 120) by cysteine is sufficient to create a functional nitric oxide binding site without affecting the kinetic properties of the enzyme. When wild-type and mutant methionine adenosyltransferase were incubated with *S*-nitrosoglutathione the activity of the wild-type remained unchanged whereas the activity of the mutant enzyme decreased markedly. The mutant enzyme was found to be *S*-nitrosylated upon incubation with the nitric oxide donor. Treatment of the *S*-nitrosylated mutant enzyme with glutathione removed most of the *S*-nitrosothiol groups and restored the activity to control values. In conclusion, our results suggest that functional *S*-nitrosylation sites can develop from existing structures without drastic or large-scale amino acid replacements.

Key words: Methionine adenosyltransferase; *S*-Nitrosylation

1. Introduction

Three methionine adenosyltransferases (MATs) which differ with respect to tissue distribution and kinetic properties regulate the synthesis of *S*-adenosylmethionine (AdoMet) from methionine and ATP in mammals [1–3]. Two of these isoforms (MAT I and MAT III) are a homotetramer (MAT I) and a homodimer (MAT III) of the same α subunit (43.7 kDa) and are expressed exclusively in the adult rat liver [1–3]. A third isoform (MAT II) is present in fetal hepatocytes, extrahepatic tissues, liver cancer cells and, in small quantities, in adult liver [1–3]. MAT II is thought to consist of two α and two β subunits [1–3]. The hepatic and extrahepatic α subunits are very similar and display sequence identities of about 85% [4]. The β subunit is unrelated to the α subunit with respect to peptide maps and immunoreactivity and has been proposed to have a regulatory function [5].

S-Nitrosylation of liver MAT isoforms (MAT I and MAT III) is thought to be an important factor in the regulation of hepatic AdoMet synthesis [6–9]. Nitric oxide (NO) groups from small-mass NO donors are introduced into a specific thiol group (cysteine 121) of liver MAT inactivating the enzyme [7,8]. The *S*-nitrosylation site of liver MAT has been recently identified and consists of two basic (arginine 357 and arginine 363) and one acidic (aspartate 355) amino acid thought to be surrounding the target thiol (cysteine 121) [8]. This acid-base catalyzed SNO/SH exchange reaction is further regulated by the cellular glutathione (GSH) content and the reactivities of the NO donors [7–9]. Cysteine 121 is localized at a 'flexible loop' over the active site cleft of MAT [10–12]. This loop seems to adopt two conformations, open and closed, and in the closed conformation is thought to prevent the entrance of the substrates to the active site [11]. Cysteine 121 is not essential for activity, since substitution of this amino acid by serine has no effect on hepatic MAT activity [13]. Cysteine 121 is present in all cloned and sequenced liver-specific enzymes (human, rat and mouse liver), but is absent in all other cloned and sequenced MATs including *Mycoplasma genitalium*, *Escherichia coli*, *Saccharomyces cerevisiae*, *Arabidopsis thaliana*, and mammalian MAT II, where in the corresponding position there is a glycine residue [10,13,14]. The 'flexible loop' is exceptionally well conserved and except for this difference in position 121 and a conservative change in position 131 (valine for isoleucine) the rest of the sequence (around 20 amino acids) is identical in both MAT I/III and MAT II α subunits [10,13,14]. Consequently, it would be interesting to determine whether a functional *S*-nitrosylation site in the MAT II α subunit could be constructed simply by replacement of glycine 120 (which corresponds to cysteine 121 in liver MAT) by cysteine (G120C). Expression of the human MAT II α subunit in *E. coli* has been shown to yield a catalytically active enzyme [15]. Here we report that the mutation G120C does not affect the K_m or V_{max} of MAT II but instead yields an enzyme that upon incubation with *S*-nitrosoglutathione (GSNO) is *S*-nitrosylated and inactivated. These results suggest that functional *S*-nitrosylation sites can develop from existing conformations without drastic or large-scale amino acid replacements.

2. Materials and methods

2.1. Site-directed mutagenesis

Human lymphocyte MAT gene containing the complete coding region for MAT II [15] was subcloned into the pCAL-n-EK plasmid (Stratagene) which allows fusion of the calmodulin binding peptide (CBP) purification tag to the N-terminus of the MAT II coding sequence which is removable with enterokinase. Five MAT II missense mutations were introduced by PCR site-directed mutagenesis [8,16]. These mutations consisted of the substitution of the residues histidine 9, alanine 60, alanine 68, glycine 120 and alanine 376 by cysteine

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Abbreviations: AdoMet, *S*-adenosylmethionine; CBP, calmodulin binding peptide; GSH, glutathione; GSNO, *S*-nitrosoglutathione; MAT, methionine adenosyltransferase; NO, nitric oxide; SNO, nitrosothiol

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(H9C, A60C, A68C, G120C and A376C). All mutants were identified by sequencing of the complete MAT II cDNA.

2.2. Purification and characterization of wild-type and MAT II mutants

Expression of the MAT II wild-type protein and the mutants was carried out using the BL21(DE3 pLys) *E. coli* strain as described previously [8,13]. A bacterial lysate was obtained by incubation in lysis buffer (50 mM Tris-HCl pH 8.0, 150 mM KCl, 1 mM imidazole, 1 mM $MgSO_4$, 2 mM $CaCl_2$, 10 mM β-mercaptoethanol, 0.1 mM PMSF and 0.1 mM benzamidine) with 1 mg/ml lysozyme for 30 min followed by five sonication cycles of 30 s. For the enzyme purification 150 ml of the cytosolic fraction obtained from 2.5 l cultures was loaded on a chromatography column containing 15 ml of calmodulin affinity resin (Stratagene), equilibrated in the previously described lysis buffer. The resin was washed with 10 column volumes of lysis buffer and the sample was eluted with 10 column volumes of elution buffer (50 mM Tris-HCl pH 8.0, 150 mM KCl, 2 mM EGTA and 10 mM β-mercaptoethanol) and 10 ml fractions were collected. All human MAT II activity appeared in fractions 2–7, while *E. coli* activity eluted in the flowthrough fraction. The CBP tag was finally cleaved using 10 units of enterokinase (Stratagene) per milligram of CBP fusion protein. Protein purity was greater than 95% in all cases as determined by SDS-PAGE. Protein concentration was measured using the Bio-Rad protein assay kit based on the Bradford assay [17].

2.3. Gel filtration chromatography of WT and G120C recombinant MAT II

Gel filtration chromatography was carried out on a Superdex 200 HR 10/30 column equilibrated in buffer (10 mM HEPES, 10 mM $MgCl_2$, 1 mM EDTA and 200 mM KCl pH 7.4) at a flow rate of 0.8 ml/min. Protein elution was performed using the same buffer and followed by recording the absorbance at 280 nm. The apparent molecular weights of the recombinant proteins were determined using cytochrome *c* (M_r 12 400), carbonic anhydrase (M_r 29 000), bovine serum albumin (M_r 66 000), alcohol dehydrogenase (M_r 150 000) and β-amylase (M_r 200 000) as marker proteins.

2.4. MAT activity measurements

MAT activity was measured as described previously by Cabrero et al. [18] using 10 μM methionine and 5 mM ATP, in the absence of any reducing agent. The effect of GSNO on MAT activity was tested by preincubating the enzyme with the NO donor at 37°C for 15 min and removing the excess of GSNO by DEAE chromatography [7,8].

2.5. Determination of MAT S-nitrosylation

MAT *S*-nitrosylation was measured according to the procedure described by Ruiz et al. [7]. Purified recombinant wild-type MAT II and G120C mutant (0.4 mg/ml) were incubated in the presence of GSNO at 37°C for 15 min. The excess of GSNO was removed by DEAE chromatography. *S*-Nitrosylated proteins were incubated with 2.2 mM $HgCl_2$ and the amount of NO released was measured by chemiluminescence derived from its reaction with ozone using a Sievers NOA 280 nitric oxide analyzer. Standard curves were obtained with known GSNO concentrations.

2.6. Preparation of GSNO

GSNO was prepared according to Ruiz et al. [7]. Equimolar concentrations of an aqueous solution of $NaNO_2$ and freshly prepared GSH in 250 mM HCl and 0.1 M EDTA, pH 1.5 were mixed. The resulting solution was incubated at room temperature for 5 min and then neutralized with NaOH. Only freshly prepared solutions of GSNO were used.

3. Results

We observed previously that in a model structure of liver MAT one residue of aspartic acid (Asp-355) and two residues of arginine (Arg-357, Arg-363) are all found in the vicinity of the sulfur group of cysteine 121 [8]. Replacement of the acidic (Asp-355) or basic (Arg-357, Arg-363) amino acids surrounding cysteine 121 leads to a marked reduction in the ability of NO to *S*-nitrosylate and inactivate hepatic MAT [8]. We therefore speculated that the *S*-nitrosylation of protein thiol

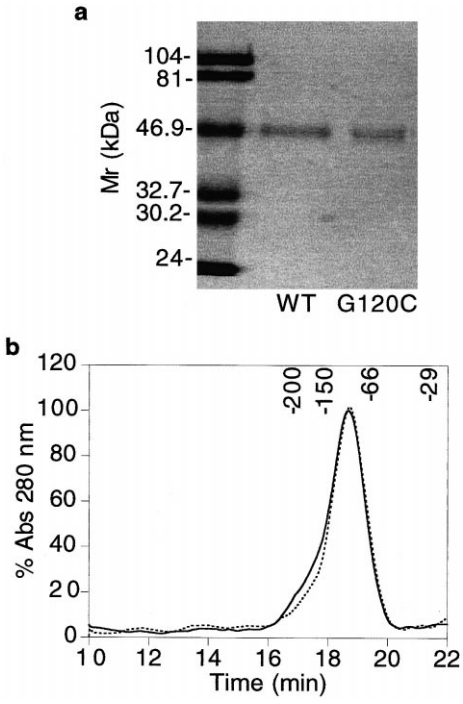

Fig. 1. Characterization of wild-type and G120C mutant MAT II. a: MAT II purified from *E. coli* transformed with wild-type (WT) or mutant (G120C) cDNA was analyzed by SDS-PAGE as described in Section 2. Molecular size markers are indicated on the left. b: MAT II purified from *E. coli* transformed with wild-type (solid line) or mutant (dashed line) cDNA was analyzed by FPLC gel filtration as described in Section 2. FPLC gel filtration was used to demonstrate that wild-type (WT) and mutant (G120C) recombinant MAT II are dimeric enzymes. Molecular size markers are indicated at the top.

residues is governed by the basic and acidic amino acids surrounding the target thiol. In MAT II the aspartic acid (Asp-354) and both arginines (Arg-356, Arg-362) are present but in position 120 (the equivalent of position 121 in liver MAT) there is a glycine instead of a cysteine. Consequently, we prepared a MAT II mutant where glycine 120 was replaced by cysteine (G120C) and determined the ability of GSNO to inactivate and nitrosylate the wild-type and mutant enzymes.

Wild-type and G120C mutant human recombinant extrahepatic MAT were efficiently purified from *E. coli* extracts. SDS-PAGE revealed that associated proteins did not significantly contaminate the purified proteins (Fig. 1a). FPLC gel filtration was used to demonstrate that wild-type and G120C recombinant extrahepatic MAT form homodimers composed of an α subunit of M_r about 46 000 (Fig. 1b). The wild-type and mutant enzymes have a K_m for methionine and ATP of around 15 μM and 250 μM respectively and V_{max} of around 24 nmol *S*-adenosylmethionine/min/mg protein. When wild-type and G120C MAT II were incubated with various concentrations of GSNO the activity of the wild-type enzyme remained unchanged but the activity of the mutant enzyme decreased up to 70% (Fig. 2). GSNO-induced G120C MAT inactivation was due to an increase in the K_m of the enzyme for methionine and ATP without significantly changing the V_{max}. Thus, the K_m of the enzyme for methionine increased from 15.6 ± 4.1 μM in the absence of GSNO to 38.7 ± 10.7 μM in the presence of 100 μM GSNO. Similarly, the K_m for

ATP was 264 ± 22 μM and 408 ± 27 μM in the absence or presence of 100 μM GSNO respectively.

S-Nitrosylation of purified wild-type and G120C MAT II proteins was determined by photolysis chemiluminescence [7,8]. Upon incubation with 50 μM GSNO the content of SNO measured in G120C MAT II (1.23 ± 0.25 mol SNO/mol subunit) was much higher than the content of SNO of the wild-type (0.15 ± 0.12 mol SNO/mol subunit). Fig. 3 shows that in G120C there is a close inverse correlation between the extent of *S*-nitrosylation and loss of activity induced by GSNO ($r = 0.98$, $P < 0.001$). Incorporation of 1.2 mol of SNO/mol of MAT II subunit led to about 70% inactivation of mutant MAT II. Maximal *S*-nitrosylation and inactivation were obtained within 10 min of incubation with GSNO (data not shown). We have previously shown that the effect of GSNO on hepatic MAT inactivation was reversible upon incubation with GSH [7]. Consistently, incubation of G120C MAT II pretreated with 50 μM GSNO (to reach an inactivation of about 70% and an incorporation of 1.23 ± 0.25 mol SNO/mol subunit) with 5 mM GSH reduced the NO content to 0.2 ± 0.12 mol SNO/mol subunit and returned the enzyme activity to control values.

Liver MAT contains 10 cysteine residues per subunit [4]. Five of these cysteine residues are conserved in the extrahepatic enzyme whereas the other five cysteines are replaced by: histidine 9, alanine 60, alanine 68, glycine 120 and alanine 376 [13,15]. We have replaced histidine 9 (H9C) and alanine 376 (A376C) by cysteine and observed that the addition of 100 μM GSNO had no effect on the activity of these mutant enzymes. The replacement of alanine 60 or alanine 68 by cysteine did not yield active enzymes. These controls provide evidence that the effects on MAT II *S*-nitrosylation and activity of changing glycine 120 to cysteine are specific.

4. Discussion

NO modifies the properties and functions of numerous proteins including ion channels, transcription factors, ras, a variety of enzymes and hemoglobin [19,20]. Recent evidence indicates that these alterations are achieved by *S*-nitrosylation of specific cysteine residues or by introduction of NO groups into transition metals present in the target proteins [19,20]. Studies with a variety of proteins [20] have suggested that protein *S*-nitrosylation is an acid-base catalyzed SNO/SH exchange reaction where the target cysteine residue is localized close to basic and acidic amino acids. We have recently shown that the *S*-nitrosylation site of liver MAT consists of two basic and one acidic amino acid which are thought to be surrounding the target cysteine [8]. Little is known, however, about how a *S*-nitrosylation site can evolve from existing structures. In MAT II the acidic (Asp-354) and basic (Arg-356, Arg-362) amino acids are conserved. We show here that replacement of a single amino acid (Gly-120) by cysteine can result in a functional NO binding site without affecting the kinetic parameters of the enzyme. Whereas wild-type MAT II activity was insensitive to GSNO, in the mutant enzyme the incorporation of about 1 mol SNO/mol of subunit led to a marked inactivation of the enzyme which could be reversed by incubation with millimolar GSH. In G120C MAT II, *S*-nitrosylation increased the K_m for methionine and ATP of the enzyme. This unprecedented observation suggests that simple changes in protein sequence may provide one mechanism for the evolution of *S*-nitrosylation sites.

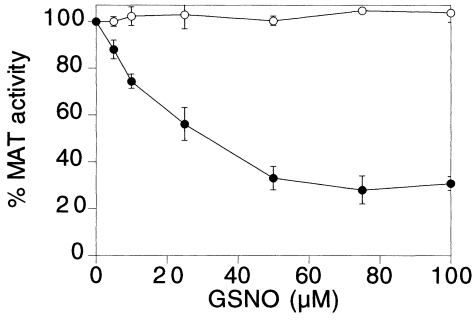

Fig. 2. Effect of GSNO on wild-type and G120C mutant MAT II activity. MAT II purified from *E. coli* transformed with wild-type (○) or mutant (●) cDNA was incubated in the presence of various concentrations of GSNO (5–100 μM) for 10 min at 37°C. The enzyme activity was then determined as described in Section 2. MAT activity is expressed as the percent of activity remaining after treatment with GSNO. 100% activity was 11.0 ± 1.5 and 11.8 ± 2.6 nmol/min/mg protein for the wild-type and mutant G120C MAT II respectively. Values are the mean ± S.E.M. of three different enzyme preparations.

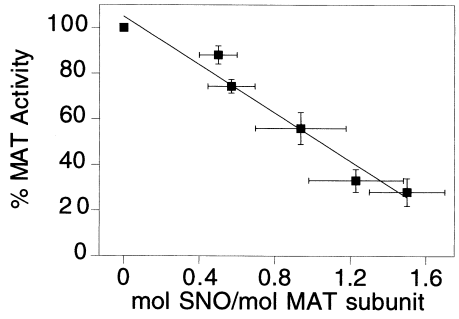

Fig. 3. Correlation between extent of MAT II *S*-nitrosylation and loss of activity. MAT II purified from *E. coli* transformed with mutant cDNA was incubated in the presence of various concentrations of GSNO (5–100 μM) for 10 min at 37°C. The enzyme activity and the formation of SNO groups were then determined as described in Section 2. MAT activity is expressed as the percent of activity remaining after treatment with GSNO. Values are the mean ± S.E.M. of three different enzyme preparations ($r = 0.98$; $P < 0.001$).

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