Recombinant expression of rat glycine N-methyltransferase and evidence for contribution of N-terminal acetylation to co-operative binding of *S*-adenosylmethionine

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An expression vector was constructed that produced rat glycine N-methyltransferase in *Escherichia coli*. Recombinant glycine N-methyltransferase was purified to homogeneity by DEAE-cellulose and gel-filtration chromatography, with a yield of more than 80 mg of pure enzyme from a 1 litre culture. HPLC of tryptic peptides and analysis of isolated peptides showed that the recombinant enzyme was structurally identical with the liver enzyme except for the absence of N-terminal blocking. The α-amino group of rat glycine N-methyltransferase is blocked by acetylation [Ogawa, Konishi, Takata, Nakashima and Fujioka (1987) Eur. J. Biochem. **168**, 141–151]. In contrast with the liver enzyme, which shows sigmoidal kinetics toward *S*-adenosylmethionine at all pH values tested [Ogawa and Fujioka (1982) J. Biol. Chem. **257**, 3447–3452], the recombinant enzyme exhibited hyperbolic kinetics at low pH and sigmoidal rate behaviour at high pH. The Hill coefficient increased with increasing pH and a pK_a of 8.11 was obtained in this transition. The values of V_{max} and K_m for glycine were not different between the two enzymes. These results suggest that elimination of the positive charge at the N-terminal end either by acetylation or deprotonation is required for co-operative behaviour.

INTRODUCTION

Glycine N-methyltransferase (GNMT; EC 2.1.1.20) catalyses the conversion of *S*-adenosylmethionine (AdoMet) and glycine to *S*-adenosylhomocysteine (AdoHcy) and sarcosine [1]. As are many enzymes involved in methionine metabolism, GNMT is found predominantly in the livers of mammals [1,2]. The product sarcosine, which has no known physiological role, is degraded to glycine and CO_2 by sarcosine oxidase in mitochondria. Thus by the coupled action of GNMT and sarcosine oxidase, AdoMet can be converted to AdoHcy without the consumption of glycine. From this fact, together with the abundance [2–4] and regulatory properties [5–8] of the enzyme, the physiological role of GNMT is considered to be in regulating the hepatic pool of AdoMet. In addition to acting as a methyltransferase, GNMT was demonstrated to be a major folate-binding protein of rat liver [9], and more recently to bind polycyclic aromatic hydrocarbons such as benzo[*a*]pyrene and 3-methylcholanthrene [10].

GNMT is unique among AdoMet-dependent methyltransferases in that it is an oligomeric protein as opposed to the monomeric structure of most methyltransferases. The enzymes from various sources including rabbit, human, rat and pig livers are all tetramers consisting of identical subunits of molecular mass 32.5 kDa and show an extensive sequence similarity. With the rat and rabbit enzymes, it is known that the N-terminus is blocked by acetylation. Reflecting their structural similarity, all enzymes exhibit similar kinetic patterns; sigmoidal rate behaviour with respect to AdoMet and hyperbolic kinetics with respect to glycine are observed [11]. Detailed studies with the rat enzyme showed that the sigmoidal kinetics was due to the co-operative binding of AdoMet to the active site residing on each subunit, and that the removal of a short N-terminal segment from each subunit deprived the enzyme of this property [12].

To facilitate investigations on the mechanism of the catalytic action of GNMT and its nature as a binding protein, it is desirable to develop a procedure to overexpress GNMT in bacteria. Here we report the construction of a plasmid that produces a large amount of recombinant enzyme in *Escherichia coli*. The recombinant enzyme lacks the N-terminal acetyl group and shows no co-operativity at neutral pH. This enables us to conclude that the acetylation of the α-amino group of GNMT contributes to the co-operative binding of AdoMet to each subunit.

MATERIALS AND METHODS

Materials

Male Wistar rats weighing approx. 200 g were purchased from Sankyo Labo Service (Tokyo, Japan). Biochemical reagents were obtained from the indicated sources: adenosine deaminase, AdoMet (chloride salt), ampicillin and molecular mass marker proteins (Sigma); DEAE-cellulose (DE52) (Whatman); lysozyme (Seikagaku Kogyo, Tokyo, Japan); peptone, yeast extract and isopropyl β-D-thiogalactoside (IPTG) (Wako Pure Chemicals, Osaka, Japan); trypsin treated with 1-chloro-4-phenyl-3-L-toluene-*p*-sulphonamidobutan-2-one (Tos-Phe-CH_2Cl-trypsin) (Worthington); Sephacryl S-200 (Pharmacia); acylamino-acid-

Abbreviations used: AdoMet, *S*-adenosyl-L-methionine; AdoHcy, *S*-adenosyl-L-homocysteine; GNMT, glycine N-methyltransferase; IPTG, isopropyl β-D-thiogalactoside; Tos-Phe-CH_2Cl-trypsin, trypsin treated with 1-chloro-4-phenyl-3-L-toluene-*p*-sulphonamidobutan-2-one.

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releasing enzyme (*N*-acylaminoacyl-peptide hydrolase; Takara Shuzo, Kyoto, Japan), DNA-modifying enzymes (Takara Shuzo and Toyobo, Osaka); collodion bag type 13200 (Sartorius, Göttingen, Germany). Before use, AdoMet was purified by passage through a C_{18} cartridge (Sep-Pak; Millipore/Waters, MA, U.S.A.) as described previously [13]. Sodium iodoacetate (Nacalai Tesque, Kyoto, Japan) was recrystallized from hot chloroform. Recombinant rat AdoHcy hydrolase was prepared as described [14]. Other reagents were of the highest grade available from commercial sources and were used without further purification.

Plasmid construction

Plasmid pCWOri+ was a gift from Dr. Amy Ross (Institute of Molecular Biology, University of Oregon, Eugene, OR, U.S.A.). The initial portions of *E. coli* mRNA species are usually rich in A and U, and the expression of foreign DNA species in the bacterium is often facilitated by making the relevant regions rich in A or T [15]. Thus we introduced silent mutations into codons 2–5 [i.e. from the native sequence 5′-GTGGACAGCGTG to the mutated sequence 5′-GTTGATAGTGTT (the changes introduced are underlined)] via PCR mutagenesis [16–18]. Plasmid pCWOri+ has an *Nde*I restriction site (CA|TATG, where | indicates an *Nde*I cutting site) coincident with the initiation ATG codon and multicloning sites of *Xba*I, *Sal*I, *Pst*I and *Hin*dIII downstream of the *Nde*I site. A foreign DNA is to be inserted between the *Nde*I site and any of the cloning sites. GNMT cDNA has no *Nde*I site coincident with the initiation ATG codon and also no usable restriction site in the 3′ non-coding region (see Figure 1a). Therefore an *Nde*I site and a *Hin*dIII site were created by PCR mutagenesis. For this purpose, oligonucleotides 5′-TGGTTGATAGTGTTTACCGTACCCGC-3′ (the underlined sequence is part of the *Nde*I site) and 5′-CGATAAGCTTAGGGTGGGAGCCG-3′ (the underlined sequence is the *Hin*dIII site) were synthesized as the 5′ and 3′ primers respectively. The latter sequence is derived from the native antisense-strand sequence corresponding to positions 11–31 downstream of the TGA stop codon (5′-CGATGGTCTTAGGGTGGGAGCCG-3′) [19]. With these two primers the GNMT cDNA containing silent mutations was amplified by PCR. The PCR product was then digested with *Hin*dIII to produce a *Hin*dIII-cut site at the 3′ end. Before ligation of the DNA, pCWOri+ was digested with *Nde*I and the *Nde*I site was filled with the Klenow enzyme. This linearized plasmid was further digested with *Hin*dIII to remove the original insert (*cheW* gene), and the resulting plasmid was ligated to the modified GNMT cDNA. The construct was designated pCWgnmt(Rat) (see Figure 1b).

Enzyme assay

The GNMT activity was assayed spectrophotometrically as described [12]. The assay mixture contained, in 2 ml of 50 mM potassium phosphate, pH 7.2, 0.1 mM AdoMet, 10 mM glycine, 60 μg of recombinant AdoHcy hydrolase and 1.4 units of adenosine deaminase. The reaction was started by the addition of GNMT, and the decrease in absorbance at 265 nm due to the conversion of adenosine to inosine was followed at 30 °C. The difference in molar absorption coefficient between adenosine and inosine at 265 nm was taken as 7.6×10^{-3} $M^{-1} \cdot cm^{-1}$ [20].

Bacterial culture

E. coli strain JM109 carrying pCWgnmt(Rat) was cultured aerobically at 37 °C in 250 ml of 2YT medium (containing 2.5 g of yeast extract, 4 g of polypeptone and 1.25 g of NaCl) with 6.25 mg of ampicillin. When the cell turbidity measured at 600 nm reached an attenuance of approx. 0.4, IPTG was added to a final concentration of 1 mM and culture was continued for an additional 15 h. Cells were harvested by centrifugation; the cell pellet was washed once with 10 mM Tris/HCl (pH 7.5)/0.15 M NaCl and kept frozen at −70 °C until use.

Purification of recombinant GNMT

All operations were performed at 0–4 °C unless otherwise stated. The frozen cells from a 250 ml culture were suspended in 25 ml of 50 mM Tris/HCl (pH 8.0)/2 mM EDTA/10 mM 2-mercaptoethanol containing 12.5 μg of leupeptin (TEML buffer) and 25 mg of lysozyme was mixed with the suspension. After 15–30 min the viscous suspension was frozen at −70 °C, thawed by immersion of the container in running water, and sonicated for approx. 30 s at 200 W. The cell debris was removed by centrifugation for 15 min at 10000 ***g*** and the supernatant obtained was put on a column of DE52 (2 cm × 7 cm) prewashed with 10 mM Tris/HCl, pH 8.0, and the column was washed with 80 ml of TEML buffer. To the pass-through fraction, which contained GNMT, triturated $(NH_4)_2SO_4$ (19 g/100 ml) was added. After standing for 30 min, the mixture was centrifuged for 30 min at 10000 ***g*** and the supernatant was again treated with $(NH_4)_2SO_4$ (15 g/100 ml). After 1 h the precipitate was recovered by centrifugation and dissolved in a minimal volume of TEML buffer. The solution was then applied to a column of Sephacryl S-200 (3.2 cm × 97 cm) equilibrated and eluted with 20 mM Tris/HCl (pH 7.4)/1 mM EDTA/10 mM 2-mercaptoethanol/0.15 M NaCl. Fractions (approx. 10 ml each) with high enzyme activity were pooled and the enzyme was concentrated by precipitation with $(NH_4)_2SO_4$. After dialysis against 10 mM potassium phosphate (pH 7.2)/1 mM EDTA/1 mM dithiothreitol, the concentrated enzyme solution was put on a column of DE-52 (2 cm × 2 cm) equilibrated with the dialysis buffer. GNMT was not retained by the resin under these conditions and appeared in the flow-through fractions. Fractions of 50 drops were collected; the fractions showing enzyme activity were pooled. The enzyme was concentrated in a collodion bag under reduced pressure, and the concentrate was stored at −20 or −70 °C in portions of approx. 1 ml.

Purification of rat liver GNMT

Rat liver GNMT was purified to homogeneity as described previously [4].

HPLC peptide mapping and determination of N-terminal sequence

Liver and recombinant GNMT (200 μg of each) were carboxymethylated and subjected to tryptic digestion as described [14]. The tryptic peptides were analysed by HPLC on a TSK ODS-120T column (4.6 mm × 250 mm) (Tosoh, Tokyo) [14]. The amino acid sequence was determined by automated Edman degradation on a Shimadzu PPSQ-10 gas-phase sequencer (Kyoto). The blocked N-terminal peptide from liver GNMT was first treated with acylamino acid-releasing enzyme followed by Edman degradation as described [11].

Limited proteolysis with trypsin

Liver and recombinant GNMT were each treated with Tos-Phe-CH_2Cl-trypsin (ratio of substrate to enzyme 1000:1, w/w) for 2–2.5 h in 0.1 M Tris/HCl, pH 8.0, containing 1 mM AdoMet

[12]. The progress of digestion was followed by SDS/PAGE (see Figure 5) and the reaction was terminated by the addition of a 5-fold molar excess of leupeptin over trypsin. The truncated enzyme lacking the N-terminal eight residues were isolated by HPLC as described [12].

Other analytical methods

SDS/PAGE was performed by the method of Laemmli [21], with a 12.5% (w/v) gel. Samples were denatured by boiling for 1.5 min in 0.2 M Tris/HCl (pH 6.8)/10% (v/v) glycerol/5% (v/v) 2-mercaptoethanol/Bromophenol Blue and run on a gel 1 mm thick at 175 V. After electrophoresis the gel was stained with 0.25% Coomassie Brilliant Blue/10% (v/v) acetic acid/40% (v/v) methanol for 5 min and destained in 7% (v/v) acetic acid with gentle rocking. Spectrophotometric and absorbance measurements were made with a Hitachi 320 recording spectrophotometer. Protein concentrations were determined by the method of Lowry et al. [22], with BSA as the standard.

RESULTS

Expression and purification of recombinant GNMT

An expression vector was constructed in pCW plasmid by introducing rat liver GNMT cDNA that had silent mutations in codons 2–5 (Figure 1). *E. coli* cells transformed with this plasmid were cultured in 2YT medium containing IPTG, and disrupted by treatment with lysozyme followed by sonication. The GNMT activity was found in the soluble fraction. The extract of cells cultured in the absence of IPTG showed no activity. 'Terrific broth' [23], a very rich medium used for the high-yield expression of various enzymes, was less effective than 2YT medium in producing recombinant GNMT. Recombinant GNMT was purified to homogeneity by a simple procedure including gel filtration on Sephacryl S-200 and negative absorption on DEAE-cellulose. Figure 2 shows results of SDS/PAGE of the sample at each step of purification. The purified preparation gave only one band at 32 kDa. The 32 kDa band was found only in cells treated with IPTG (Figure 2, lanes 2 and 3). The recombinant enzyme behaved identically with the liver enzyme on the gel-filtration column and was eluted at 130 kDa (results not shown). These results indicate that recombinant GNMT is a tetramer of identical subunits with a molecular mass indistinguishable from that of the liver enzyme. A thick band seen at 17 kDa in the electrophoretogram of the crude extract (lanes 2 and 3) is due to the lysozyme added for cell lysis, and densitometric analysis indicated that GNMT represented approx. 15% of the soluble protein of *E. coli*. The procedures described above permitted us to obtain more than 80 mg of homogeneous enzyme from a 1 litre culture. The purified preparation was unstable on storage at 4 or −70 °C unless thiol compounds such as 2-mercaptoethanol or dithiothreitol were added.

(a)

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          1   2   3   4   5   6   7   8   9
    (Met)Val Asp Ser Val Tyr Arg Thr Arg Ser
CAGG ATG GTG GAC AGC GTG TAC CGT ACC CGC TCC

291 292
Thr Gly End
ACA GGC TGA GCCTGGCTCCGGCTCCCACCCTAAGACCATCGCCTA
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(b)

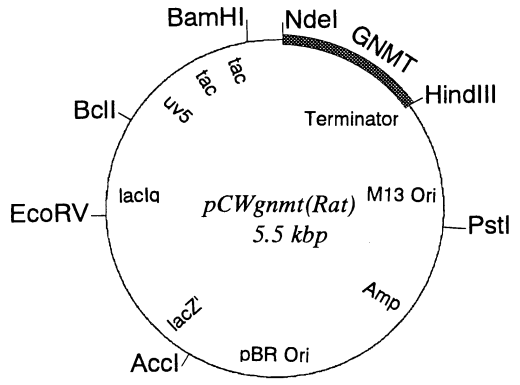

Figure 1 Nucleotide sequences of the 5′ (upper) and 3′ (lower) regions of rat liver GNMT, and translated amino acids (a), and construction of vector pCWgnmt(Rat) expressing rat liver GNMT (b)

The sequence data are from [19]. The N-terminal valine residue is acetylated. The underlined sequence was used to create a *Hind*III restriction site. The original plasmid, pCWOri+, is described in [24]. The inserted *cheW* gene was removed by digestion with *Nde*I and *Hind*III, and the modified sequence of liver GNMT cDNA was ligated to the resulting plasmid as described in the Materials and methods section.

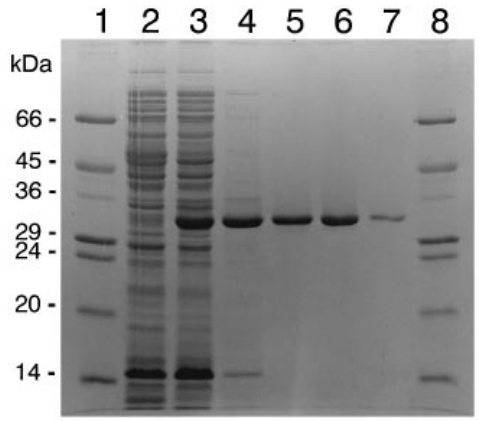

Figure 2 SDS/PAGE of recombinant GNMT at each step of purification

Lane 1, molecular mass marker proteins (from top to bottom: BSA, egg albumin, glyceraldehyde-3-phosphate dehydrogenase, carbonic anhydrase, trypsinogen, trypsin inhibitor, α-lactalbumin); lane 2, crude extract of cells cultured in the absence of IPTG (75 μg); lane 3, crude extract of cells cultured in the presence of IPTG (75 μg); lane 4, eluate from the first DEAE-cellulose column [$(NH_4)_2SO_4$ concentrate] (20 μg); lane 5, eluate from a Sephacryl S-200 column (5 μg); lane 6, eluate from the second DEAE-cellulose column (5 μg); lane 7, rat liver GNMT (1.4 μg); lane 8, molecular mass marker proteins. The gel was stained with Coomassie Brilliant Blue.

Chemical characterization of recombinant GNMT

Figure 3 compares the elution profiles from a reverse-phase HPLC column of the tryptic peptides prepared from the liver and recombinant GNMTs after carboxymethylation. The elution patterns of peptides were identical except for one peak in each profile. This was confirmed by chromatography of an equimolar mixture of the liver and recombinant peptide preparations (results not shown). The peptides showing different behaviour on HPLC (peak at 29.5 min in the liver sample and peak at 23.5 min in the recombinant sample; indicated by arrows) were isolated and analysed. The liver peptide was refractory to Edman degradation.

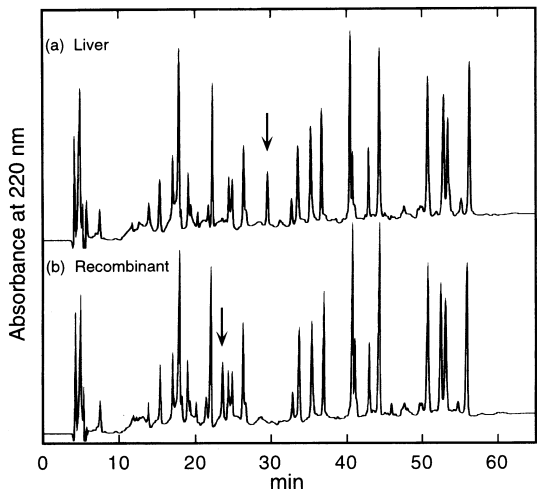

Figure 3 HPLC profiles of tryptic peptides from rat liver and recombinant GNMT

Peptides (approx. 12 μg) were separated on TSK ODS-120T with a linear gradient from 0.1% trifluoroacetic acid/0% acetonitrile to 0.09% trifluoroacetic acid/80% (v/v) acetonitrile at a flow rate of 0.7 ml/min. Arrows show peaks with different retention times in the two chromatograms.

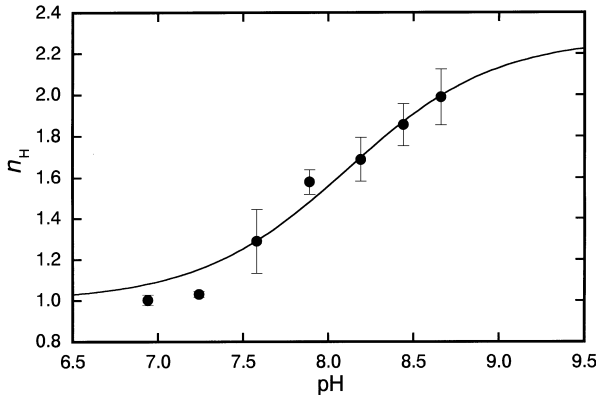

Figure 4 Co-operativity of recombinant GNMT as a function of pH

Initial velocity measurements with AdoMet as the variable substrate were made at constant high levels of glycine in 50 mM potassium phosphate at the pH values indicated. Values of Hill coefficient (h; shown as n_H) were determined from Hill plots of log[AdoMet] against $\log v/(V-v)$, where v and V represent the initial and maximum velocities respectively. The curve is drawn by a least-squares fit to the equation $h = 1+(h_0-1)K_a/([H]+K_a)$, where h_0 is the maximum Hill coefficient, [H] is the hydrogen ion concentration and K_a is the acid dissociation constant. Error bars represent S.E.M. for three determinations.

Table 1 Kinetic parameters of liver and recombinant GNMTs determined at pH 7.2

Initial velocity measurements were made in 50 mM potassium phosphate, pH 7.2, at 30 °C. $[S]_{0.5}$ for AdoMet was determined at a constant glycine concentration of 10 mM, and K_m for glycine and V were determined at 0.1 mM AdoMet. Values are means ± S.E.M. for three determinations.

	AdoMet			
Enzyme	$[S]_{0.5}$ (μM)	h	K_m (glycine) (mM)	V (μmol/min per mg)
Liver	61.2 ± 5.7	2.05 ± 0.04	0.23 ± 0.07	0.99 ± 0.08
Recombinant	22.3 ± 1.5	1.07 ± 0.03	0.20 ± 0.05	1.06 ± 0.10

Because liver GNMT has acetylvaline at the N-terminus [19], this was assumed to be the N-terminal peptide. To confirm its identity, the peptide was first treated with acylamino acid-releasing enzyme, and the resulting peptide was subjected to Edman degradation. The peptide successively released phenylthiohydantoin derivatives of Asp, Ser, Val, Tyr and Arg, consistent with the N-terminal sequence from residues 2–6. The peptide from the recombinant enzyme, in contrast, was susceptible to Edman degradation, and its sequence was determined as Val-Asp-Ser-Val-Tyr-Arg. Thus the recombinant enzyme also has no N-terminal methionine residue. The results of peptide analysis establish that the recombinant enzyme is structurally identical with the liver enzyme except that its N-terminus is not blocked.

Comparative catalytic properties of liver and recombinant GNMT

Table 1 compares the kinetic parameters of liver and recombinant GNMT determined at pH 7.2. Except for the difference in the kinetic behaviour towards AdoMet, both enzymes displayed similar values for V_{max} and K_m for glycine. GNMTs from various mammalian livers all exhibit sigmoidal rate behaviour towards AdoMet and hyperbolic kinetics towards glycine [4,11]. The co-operativity observed with AdoMet is virtually pH independent, and a Hill coefficient of approx. 2.1 is obtained [4]. The kinetic pattern of recombinant GNMT for AdoMet, however, varied with pH; the hyperbolic kinetics observed at low pH became sigmoidal as pH was raised. The kinetic pattern for glycine remained hyperbolic at all pH values. Figure 4 shows a plot of Hill coefficient (h) as a function of pH. The value increased with increasing pH. The shape of the curve is that of a typical titration curve, and a pK_a value (± S.D.) of 8.11 ± 0.14 and a maximal Hill coefficient of 2.28 ± 0.15 were calculated by the least-squares method. The pK_a value observed was that expected for the dissociation of the α-amino group of a protein or peptide, which suggests that deprotonation of the N-terminal amino group is responsible for the appearance of co-operativity.

Previous investigations have shown that the peptide bonds between Arg-6 and Thr-7 and between Arg-8 and Ser-9 of rat liver GNMT are very susceptible to trypsinolysis, and that the enzyme lacking the N-terminal eight residues no longer shows co-operativity [12]. Recombinant GNMT was also susceptible to tryptic digestion. A 2 h incubation with trypsin in the presence of AdoMet, as described in the Materials and methods section, was sufficient to convert all the enzyme (subunit molecular mass 32 kDa) to an enzyme with a subunit molecular mass of 31 kDa (Figure 5). The latter enzyme was isolated by HPLC and was shown to be a tetrameric enzyme lacking the N-terminal eight residues in each subunit. The truncated recombinant enzyme showed no co-operativity at low or high pH and had exactly the same kinetic constants as the truncated enzyme from liver GNMT (for the values of kinetic constants see [12]). This confirms the results of peptide analysis that the recombinant enzyme differs from the liver enzyme only in having no N-terminal acetyl group, and also suggests that there is no conformational difference between the two truncated enzymes. The fact that removal of the N-terminal eight residues results in an enzyme that shows no co-operativity even at high pH indicates that the co-operativity is not caused by simply deprotonating any α-amino acid but also requires the presence of the short N-terminal segment.

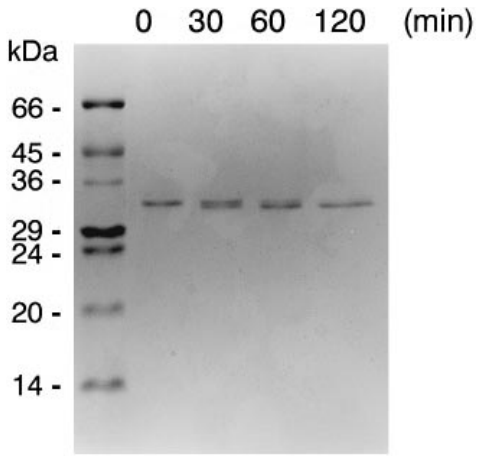

Figure 5 Time course of tryptic digestion of recombinant GNMT

Recombinant GNMT was incubated with Tos-Phe-CH_2Cl-trypsin as described in the Materials and methods section. At 0, 30, 60 and 120 min of incubation, aliquots (2 μg of protein) were removed, mixed with leupeptin and subjected to SDS/PAGE. The first lane shows molecular mass maker proteins. The gel was stained with Coomassie Brilliant Blue.

DISCUSSION

In the present study we constructed an expression vector that produced a large amount of rat GNMT in *E. coli*. The plasmid used was pCW, which had double *tac* promoter cassettes and a potent *TrpA* transcription terminator. This plasmid was originally used to express the bacterial genes involved in chemotaxis [24], and thereafter to express bovine [17] and human [18] microsomal steroid 17α-hydroxylase (P450c17). Initially, Barnes et al. [17] failed to produce bovine P450c17 in *E. coli* with a pCW plasmid carrying the wild-type cDNA. Successful production was achieved by modifying the cDNA. They changed the second codon from TGG (Trp) to GCT (Ala), a preferred second codon for expression of the *lacZ* gene, and introduced silent mutations in codons 4–7 by changing the last nucleotide of these codons to A or T. It is known that this region of *E. coli* mRNA species is rich in A and U [15]. We used a similar approach to express GNMT cDNA. Because the second codon of GNMT cDNA is GTG (Val) (Figure 1a) and amenable to silent mutation, we changed each of the last nucleotides of codons 2–5 to T. The resulting DNA, when ligated to pCW plasmid, was effective in producing a large amount of recombinant GNMT. It is not known whether the nucleotide changes are absolutely required or whether the use of this plasmid with powerful promoters alone is sufficient for the efficient expression of GNMT cDNA. However, it should be noted that previous attempts to produce the enzyme by using the native cDNA and plasmids containing the promoter of the *lac* gene or T7 RNA polymerase gene universally failed; no enzyme could be obtained even when the cDNA was placed downstream of the promoter keeping an ideal distance of seven nucleotides between the Shine–Dalgarno sequence and the initiation ATG codon. By use of the procedures described here we obtained more than 80 mg of pure recombinant GNMT from a 1 litre culture by a three-step purification procedure. This contrasts with a time-consuming purification of liver GNMT, which requires a 1000-fold purification by a multi-step procedure including CM-Sephadex and hydroxyapatite chromatography [4].

Like the liver enzyme, recombinant GNMT is a tetramer of identical subunits. HPLC of the tryptic peptides and analysis of the isolated peptides indicate that the enzyme is structurally identical with the liver enzyme except that it has free rather than acetylated valine at N-terminus. Thus, in *E. coli*, the N-terminal methionine residue is removed from the enzyme by post-translational modification, but acetylation of the new terminal residue does not occur. The same is true with guanidinoacetate methyltransferase [25] and AdoHcy hydrolase [14]; the N-termini of the liver enzymes are blocked but those of the recombinant enzymes produced in *E. coli* are not.

Rat liver GNMT shows sigmoidal kinetics with respect to AdoMet and hyperbolic kinetics with respect to glycine at all pH values tested [4]. The enzyme binds substrates in an obligatory order with AdoMet as the first substrate; the co-operative rate behaviour is due to the co-operative binding of AdoMet to each subunit [12]. In contrast, the kinetic behaviour of the recombinant enzyme toward AdoMet is hyperbolic at low pH and sigmoidal at high pH values, with a transition pK_a of 8.11. Although it is possible that the absence of an N-terminal acetyl group leads to improper polypeptide folding, resulting in different kinetic behaviour, the available evidence suggests that liver and recombinant GNMTs are conformationally similar. They have essentially the same values of V_{max} and K_m for glycine (Table 1) and are equally susceptible to trypsinolysis. Also, they show exactly the same catalytic properties after N-terminal truncation. Thus it would be reasonable to conclude that elimination of the positive charge of the N-terminal amino group is responsible for the co-operative binding of AdoMet and that the co-operativity of liver GNMT at low pH is due to the prevention of protonation by acetylation. Removal of the N-terminal eight residues by proteolysis deprives the enzyme completely of the co-operative behaviour. The importance of the N-terminal region in the co-operative binding of AdoMet is corroborated by the crystal structure of GNMT. The crystal structure of recombinant GNMT complexed with AdoMet and a competitive inhibitor acetate has been determined recently at 2.2 Å resolution [26]: four nearly spherical subunits are arranged to form a flat square tetramer with a large hole in the centre. The active site is near the centre of each subunit. The N-terminal 20 residues protrude from the main body of each subunit and interact with each other in the central region of the tetrameric structure. Residues 2–7 of subunit A (the identical subunits are designated A, B, C and D) are tightly connected to residues 7–2 of subunit D by six consecutive anti-parallel β-sheet hydrogen bonds, and residues 9–20 form a U-shaped loop that hangs into the active site of subunit B. Glu-15 is located at the bottom of this loop. The N-terminal residue seems to be placed near Arg-8 of subunit D. Although the three-dimensional structure of the free enzyme is not known, it would be reasonable to assume that similar interactions of the N-terminal segments exist in the free enzyme, and a conformational change caused by binding of AdoMet to one subunit is transmitted to other subunits through the N-terminal network, to facilitate binding. The presence of a positive charge at the N-terminus would weaken or abolish this interaction between subunits, thereby eliminating the co-operativity.

It is reported that a majority (70–80 %) of enzymes are oligomers, of which approx. 32 % exhibit co-operativity [27]. The N-terminal region is often related to co-operativity. The best known example is haemoglobin; replacement of Glu-6 of the β-chain with valine results in a loss of co-operativity of O_2 binding and causes sickle-cell anaemia [28–30]. The Arg-4 or Lys-3 substitution mutant in the N-terminal basic domain of bacteriophage T4 gene 32 protein shows no co-operative binding with a single-stranded DNA [31,32]. In chicken smooth-muscle tropomyosin [33] and *Pseudomonas* ornithine transcarbamoylase

[34], the N-terminal sequence is shown to be important for co-operative substrate binding. In GNMT, although the precise mechanism must await further investigation, it is clear that elimination of the positive charge of the α-amino group is critical for the co-operativity of AdoMet binding. To our knowledge this is the first example in which co-operative substrate binding is caused by N-terminal acetylation.

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