Rat liver guanidinoacetate methyltransferase

Proximity of cysteine residues at positions 15, 90 and 219 as revealed by site-directed mutagenesis and chemical modification

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Cys-90 of rat liver guanidinoacetate methyltransferase is a very reactive residue, and chemical modification of this residue results in a large decrease in activity [Fujioka, Konishi & Takata (1988) *Biochemistry* 27, 7658–7664]. To understand better the role of Cys-90 in catalysis, this residue was replaced with alanine by oligonucleotide-directed mutagenesis. The mutant is active and has kinetic constants similar to those of wild-type, indicating that Cys-90 is not involved in catalysis and substrate binding. The u.v.-absorption, fluorescence and c.d. spectra are also unchanged. Reaction of the mutant with an equimolar amount of 5,5'-dithiobis-(2-nitrobenzoic acid) or 2-nitro-5-thiocyanobenzoic acid results in an almost quantitative disulphide cross-linking between Cys-15 and Cys-219. The same treatment effects disulphide bond formation between Cys-15 and Cys-90 in wild-type [Fujioka, Konishi & Takata (1988) *Biochemistry* 27, 7658–7664]. Since the mutant and wild-type enzymes appear to have similar secondary and tertiary structures, these results suggest that Cys-15, Cys-90 and Cys-219 of the methyltransferase occur spatially close together. The mutant cross-linked between Cys-15 and Cys-219 and the wild-type cross-linked between Cys-15 and Cys-90 show very similar spectroscopic properties. Although treatment of the mutant and wild-type enzymes with equimolar concentrations of 5,5'-dithiobis-(2-nitrobenzoic acid) causes a large loss of enzyme activity in each case, kinetic analyses with the modified enzymes suggest that cross-linking of Cys-15 with Cys-90 or Cys-219 does not abolish activity and does not result in a large change in the Michaelis constants. Incubation of the mutant enzyme with excess 2-nitro-5-thiocyanobenzoic acid leads to modification of Cys-207 in addition to Cys-15 and Cys-219. Retention of considerable enzyme activity in the modified enzyme indicates that Cys-207 is also not an essential residue.

INTRODUCTION

Guanidinoacetate methyltransferase (EC 2.1.1.2; GAMT) catalyses the *S*-adenosylmethionine (AdoMet)-dependent methylation of guanidinoacetate to form creatine. The enzyme occurs in the livers of vertebrates [1], and has been purified to homogeneity from rat [2] and pig liver [3]. The enzymes from both sources are monomeric proteins of relatively small size, and have similar enzymic properties [2–5]. The cDNA for rat liver GAMT has been cloned, sequenced and expressed in *Escherichia coli*. The cDNA-derived primary structure indicates that rat GAMT consists of 235 amino acid residues, relatively rich in aromatic acids, and possesses five half-cystine residues [4]. The catalytically active enzyme has no disulphide bonds [6]. The three-dimensional structure of the enzyme has not been determined, and nothing is known about the active-site region. At present, the only implication that can be made about the tertiary structure is the proximity of Cys-15 and Cys-90. This comes from the observation that these cysteine residues readily form a disulphide bond on incubation of the enzyme with 1 equivalent of 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) [6].

The disulphide bond formation between Cys-15 and Cys-90 deprives the enzyme of a large portion (approx. 90%) of activity. Cys-15 and Cys-90 also react rapidly and concurrently with iodoacetate, again leading to a large loss of activity [6]. Thus, either one or both of Cys-15 and Cys-90, if not essential for activity, may be thought to occur in the region crucial for catalytic functioning of the enzyme. Because of the concurrent

modification of these cysteine residues by DTNB and iodoacetate, it has not been possible to determine the role of the individual residue in catalysis. Also, their high reactivity and the large loss of activity associated with their modification make it difficult to study the functional roles of other cysteine residues. In the present study, we attempted to examine these points by using the mutants in which each of Cys-15 and Cys-90 is replaced by alanine. By oligonucleotide-directed mutagenesis of a pUC plasmid that contained the coding sequence of rat GAMT [4], we constructed a plasmid expressing the Cys-90→Ala mutant (C90A) in *E. coli* in good yield. However, we were not able to clone the plasmid for Cys-15→Ala mutation. A mutant plasmid that substitutes serine for Cys-15 could be obtained, but the altered protein was produced very poorly in *E. coli*; SDS/PAGE of bacterial crude extract showed only a faint band having the M_r of GAMT protein. Thus, although the initial aim was met with limited success, the studies with C90A revealed some interesting aspects on the enzyme structure and the roles of thiol groups. The present paper reports that Cys-90 has no catalytic or structural role, and presents evidence that Cys-15, Cys-90 and Cys-219 of GAMT occur in close proximity in the three-dimensional structure. It is also shown that Cys-207 and Cys-219 are not essential for activity.

MATERIALS AND METHODS

Materials

Wild-type recombinant rat GAMT was produced in *E. coli*

Abbreviations used: GAMT, guanidinoacetate methyltransferase; WT, wild-type recombinant rat guanidinoacetate methyltransferase; C90A, recombinant rat guanidinoacetate methyltransferase in which Cys-90 is replaced by alanine; AdoMet, *S*-adenosyl-L-methionine; AdoHcy, *S*-adenosyl-L-homocysteine; DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid); NTCB, 2-nitro-5-thiocyanobenzoic acid; TNB, 2-nitro-5-mercaptobenzoic acid; SSC, standard saline citrate (0.15 M-NaCl/15 mM-sodium citrate buffer, pH 8.0).

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JM109 (*recA1*, *endA1*, *gyrA96*, *thi*, *hsdR17*, *supE44*, *relA1*, λ^- , $\Delta(lac-proAB)F'$: *traD36*, *proAB*⁺, *lacI*⁺, Δ AM15) [7] transformed with plasmid pUCGAT9-1 that contained the coding region of rat GAMT cDNA linked to the *lac* promoter, and purified as described previously [4]. The enzyme lacks the *N*-terminal acyl group present in the rat liver enzyme, but, except for this difference, exhibits the same functional and physical properties as the liver enzyme. Before experiments, the enzyme was dialysed exhaustively against 20 mM-potassium phosphate buffer, pH 7.2, containing 1 mM-EDTA to remove the dithiothreitol that had been added during purification. Molar concentrations of the enzyme were calculated on the basis of M_r 26140 [4]. Protein concentrations were determined by the method of Lowry *et al.* [8] with purified recombinant GAMT as the standard. *S*-Adenosylhomocysteine (AdoHcy) hydrolase was purified from rat liver as described previously [9]. The enzymes and reagents used for recombinant DNA experiments were obtained from Takara Shuzo, Kyoto, Japan, and Toyobo, Tokyo, Japan. NTCB and calf intestinal adenosine deaminase were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A., and α -chymotrypsin was from Worthington Biochemical Corp., Freehold, NJ, U.S.A. DTNB was purchased from Nacalai Tesque, Kyoto, Japan, and the amino acid calibration mixture and phenyl isothiocyanate were from Wako Pure Chemical Industries, Osaka, Japan. *N*-Ethyl[2,3-¹⁴C]maleimide (4 mCi/mmol) and iodo[2-¹⁴C]acetic acid (55 mCi/mmol) were from Amersham International, Amersham, Bucks., U.K., and K¹⁴CN (49 mCi/mmol) was from Du Pont-New England Nuclear, Boston, MA, U.S.A. 2-Nitro-5-[cyano-¹⁴C]thiocyanobenzoic acid (¹⁴C]NTCB) was prepared from DTNB and K¹⁴CN as described by Degani & Patchornik [10]. AdoMet (chloride salt; Sigma Chemical Co.) was purified by passage through a C₁₈ cartridge (Sep-Pak; Waters Associates, Milford, MA, U.S.A.) as described previously [11]. Iodoacetic acid was recrystallized from hot chloroform. Other chemicals were of the highest purity available from local commercial sources and were used without further purification.

Site-directed mutagenesis

Conversion of Cys-90 of GAMT into alanine was carried out by oligonucleotide-directed mutagenesis of plasmid pUCGAT9-1 by using a synthetic 23-mer oligonucleotide, 3'-TAATAACTTCGGTTGCTACCCCA-5'. The underlined letters represent the base replacements. The construction of the mutant plasmid was performed essentially as described previously [4]. Briefly, the mutagenic oligonucleotide was annealed to the single-stranded pUCGAT9-1 DNA obtained by use of helper bacteriophage M13KO7, and double-stranded DNA was prepared with DNA polymerase and T4 DNA ligase in the presence of dNTP mix and ATP. *E. coli* JM109 was transformed with the double-stranded DNA, and the cells carrying the mutant plasmid were screened by selective colony hybridization [12]. For this the filters were incubated in 5 × SSC containing 20% formamide and the ³²P-labelled mutagenic oligonucleotide for 2 h at 37 °C, and washed three times with 0.5 × SSC at 54 °C for 10 min.

Enzyme assay

The GAMT activity was determined spectrophotometrically by a coupled assay [6]. The product of the methyltransferase reaction, AdoHcy, was converted into inosine by the consecutive action of AdoHcy hydrolase and adenosine deaminase, and the decrease in absorbance at 265 nm that accompanies the conversion was monitored continuously. The standard assay mixture contained 20 μ M-AdoMet, 0.5 mM-guanidinoacetate, and sufficient amounts of AdoHcy hydrolase and adenosine deaminase in 2.0 ml of 50 mM-potassium phosphate buffer, pH 8.0.

Chemical modification reactions

Solutions of DTNB and NTCB in appropriate buffers were prepared fresh before each experiment. The concentrations of DTNB and NTCB were determined spectrophotometrically: DTNB, $\epsilon_{324} = 1.778 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$ [13]; NTCB, $\epsilon_{293} = 8.0 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$ [10]. The reaction of WT or C90A with DTNB was carried out in 0.1 M-Tris/HCl buffer, pH 8.0, containing 1 mM-EDTA. The reaction with NTCB was performed in 0.1 M-potassium phosphate buffer, pH 8.0, containing 1 mM-EDTA to avoid the slow reaction between Tris and NTCB. All incubations were at 30 °C. The concentrations of 2-nitro-5-mercaptobenzoate (TNB) anion released from DTNB and NTCB were calculated by using $\epsilon_{412} = 1.415 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$ [13].

Labelling of cysteine residues modified by treatment with DTNB and NTCB

The DTNB- or NTCB-modified enzyme was separated from small molecules by gel filtration over Sephadex G-25 or dialysis against 50 mM-potassium phosphate buffer, pH 6.8, containing 0.5 mM-EDTA. To avoid oxidation of unchanged thiol groups, the buffer solutions were prepared with O₂-free water that had been boiled for 20 min and then bubbled with N₂ while cooling. The modified enzyme was concentrated to approx. 1 mg/ml by ultrafiltration on Ultracent-10 (Tosoh, Tokyo, Japan) and treated with 5 mM-*N*-ethylmaleimide at 30 °C to block the unchanged thiol groups. The reaction with *N*-ethylmaleimide was carried out first in the absence of denaturant for 10 min and then in the presence of 4 M-guanidinium chloride for 30 min. The *N*-ethylmaleimide-treated enzyme was dialysed against 50 mM-potassium phosphate buffer, pH 6.8, then against water, and freeze-dried. The freeze-dried sample was dissolved in buffered 6 M-guanidinium chloride, pH 8.0, containing dithiothreitol (5-fold molar excess over thiol groups) and incubated for 3 h at 37 °C. Iodo[¹⁴C]acetate (500 c.p.m./nmol) in 5-fold molar excess over dithiothreitol was then added, and the mixture was incubated for an additional 1 h to label the regenerated thiol groups. After incubation, excess radiolabelled iodoacetate was destroyed with 2-mercaptoethanol. After dialysis as described above, the ¹⁴C-labelled sample was freeze-dried.

For labelling the cysteine residues not modified by NTCB, the NTCB-treated enzyme was processed as above except that *N*-ethyl[¹⁴C]maleimide was used instead of unlabelled *N*-ethylmaleimide and non-radioactive iodoacetate instead of iodo[¹⁴C]acetate.

Chymotryptic digestion and separation of peptides

The freeze-dried protein sample was dissolved in 0.1 M-NH₄HCO₃ and digested with α -chymotrypsin (substrate/proteinase ratio 100:1, w/w) at 37 °C overnight. The chymotryptic-digest peptides were separated and purified on a TSK ODS 120T column (0.46 cm × 25 cm) (Tosoh) with a Hitachi 638-30 liquid chromatograph equipped with an SIC integrator 7000A. Chromatographic conditions are described in the Figure legends. The effluent was monitored for absorbance at 220 nm. Purified peptides were identified by their amino acid compositions. All of the carboxymethylcysteine-containing chymotryptic-digest peptides have been sequenced previously [6].

Amino acid analysis

Samples were hydrolysed in 5.7 M-HCl containing 0.1% (v/v) phenol and 10 mM-dithiothreitol for 24 h at 108 °C. Amino acid composition was determined based on reverse-phase separation of phenylthiocarbamoyl derivatives as described previously [14]. The phenylthiocarbamoyl derivative of *S*-(1,2-dicarboxyethyl)-L-cysteine appeared just behind the derivative of glutamic acid.

Determination of thiol groups

The contents of thiol groups of native and modified enzymes were determined by the reaction with DTNB under denaturing conditions. After exhaustive dialysis of the protein sample against O_2 -free 0.1 M-sodium phosphate buffer, pH 7.5, containing 1 mM-EDTA, its thiol content was determined as described by Riddles *et al.* [13].

Other analytical procedures

SDS/PAGE was performed according to the procedure of Laemmli [15]. Protein bands were detected by staining with Coomassie Brilliant Blue. Spectrophotometric and absorbance measurements were made with a Hitachi 320 recording spectrophotometer and fluorescence measurements with a Farrand MK-2 spectrofluorimeter. C.d. spectra were recorded with a Jasco J-500C spectropolarimeter.

RESULTS

Expression, purification and properties of mutant enzyme C90A

Cell-free extracts of *E. coli* transformed with the Cys-90→Ala mutant (C90A) plasmid and grown in the presence of isopropyl β -thiogalactopyranoside exhibited the GAMT activity. SDS/PAGE showed a heavy protein band having the M_r of GAMT (M_r 26140). The protein was not found in the extract of uninduced cells (results not shown). C90A could be purified to homogeneity by the procedure used for purification of WT; C90A behaved exactly as WT during purification, which involved

Fig. 2. Rechromatography of the peptides eluted at 39.5 min in Fig. 1

The materials eluted at around 39.5 min were chromatographed on a TSK ODS-120T column with an acetonitrile gradient in 5 mM-ammonium acetate buffer, pH 6.8. Acetonitrile gradient: 0–16% between 10 and 15 min; 16–80% between 15 and 60 min. (a) WT; (b) C90A.

Fig. 1. H.p.l.c. profile of chymotryptic peptides derived from (a) WT and (b) C90A

The enzymes were *S*-carboxymethylated with iodoacetate after treatment with dithiothreitol [16] and digested with chymotrypsin as described in the Materials and methods section. About 30 nmol of peptides was fractionated on a TSK ODS-120T column with a linear gradient of acetonitrile in 0.05% trifluoroacetic acid. The concentration of acetonitrile was varied from 0 to 48% over a period of 50 min starting at 10 min. The flow rate was 0.8 ml/min. The arrow (peptide 1) indicates the peptide unique to C90A.

$(NH_4)_2SO_4$ fractionation, Sephadex G-100 chromatography and DEAE-cellulose chromatography [4]. The chromatographic behaviour on the Sephadex column indicates that C90A consists of a single polypeptide chain, as does WT.

The presence of amino acid substitution at the intended position was confirmed by analysis of chymotryptic-digest peptides derived from the *S*-carboxymethylated C90A and WT. Fig. 1 compares the elution patterns for peptides from C90A and WT in reverse-phase h.p.l.c. Close examination reveals that the two profiles differ in only two respects; the chymotryptic digest from C90A has a peak at 39 min that is not found in the digest from WT, and a 39.5 min peak that is thinner than the corresponding peak of WT. It is known that the 39.5 min peak of WT is a composite of several peptide peaks, of which one is the carboxymethylcysteine-containing peptide comprising residues 87–95 [6]. Therefore it is likely that conversion of Cys-90 into alanine results in a peptide that is eluted earlier at 39 min. Thus the 39.5 min peptides derived from the two enzymes were further analysed by rechromatography on the same h.p.l.c. column with a different solvent system. As shown in Fig. 2, the sample from C90A lacked a peptide (peptide 2), which represented residues 87–95 (Table 1). Amino acid analysis of the peptide unique to C90A (peptide 1, Fig. 1) showed a result that was compatible with residues 87–95 in which Cys-90 was changed to alanine (Table 1). Also, titration of C90A with DTNB under denaturing conditions gave a value of 3.98 mol of thiol groups/mol of enzyme.

Table 2 shows kinetic constants of the mutant and wild-type enzymes. C90A had a K_m value for AdoMet 1.7-fold greater than that of WT, but the values of K_m for guanidinoacetate and k_{cat} were similar for the two enzymes. The fluorescence spectrum

Table 1. Amino acid composition data for chymotryptic-digest peptides from WT and C90A

Amino acid	Peptide ...	Amino acid composition (mol of residue/mol of peptide)						
		1	2	3a	3b	3c	4a	4b
Asx		1.7	1.8	1.0	1.3	1.0	1.1	1.2
Glx		0.8	1.2			1.1		
Cys			1.2*	0.9*	1.0*	1.0*	0.9†	1.0†
Ser							1.1	0.9
Gly		1.0	1.1			2.1		
Arg				0.9	1.2			
Thr							1.1	0.9
Ala		0.9		2.2	2.2	2.3		
Pro				2.0	2.2	2.2		
Tyr				1.0	2.0			
Val		1.0	1.1	1.2	1.1			
Ile		2.1	1.8					
Leu				1.0	0.7		1.3	1.3
Phe		1.0	1.0			1.0		
Position		87–95‡	87–95	212–221	212–222	9–19§	168–173§	168–173§

* S-Carboxymethylcysteine.

† S-(1,2-Dicarboxyethyl)cysteine.

‡ Conversion of Cys-90 into Ala-90 is assumed.

§ Presence of C-terminal tryptophan is assumed.

Table 2. Kinetic constants of C90A and WT

Initial-velocity measurements were made at pH 8.0 and 30 °C by a coupled assay as described in the Materials and methods section. Values of kinetic constants were determined by fitting the initial-velocity data to the Michaelis–Menten equation by the least-squares method.

Enzyme	K_m (AdoMet) (μM)	K_m (GAA*) (μM)	k_{cat} (min^{-1})
C90A	$4.51 \pm 0.40^\dagger$	$24.62 \pm 2.49^\ddagger$	4.52 ± 0.41
WT	$2.74 \pm 0.12^\dagger$	$22.73 \pm 2.03^\ddagger$	4.91 ± 0.44

* GAA, guanidinoacetate.

† Determined in the presence of 1.0 mM-guanidinoacetate.

‡ Determined in the presence of 56 μM -AdoMet.

Table 3. Reaction of C90A with graded amounts of DTNB

C90A (13 μM) was treated successively with 1.0, 0.5, 0.5 and 1.0 equivalent of DTNB. After each addition, reaction was allowed to go to completion, with monitoring of the absorbance change at 412 nm. Values are corrected for small volume changes.

DTNB added (mol/mol of enzyme)	TNB released (mol/mol of enzyme)	Activity remaining (%)
1.0	1.88	12
1.5	2.43	3
2.0	2.82	N.D.*
3.0	2.99	

* N.D., not detected.

(emission maximum at 327 nm when excited at 280 nm) and the u.v.-absorption spectrum of C90A were identical in shape and intensity with those of WT (spectra not shown). C90A and WT also showed very similar far-u.v. and near-u.v. c.d. spectra (cf. Fig. 5).

Reaction of C90A with DTNB

Incubation of C90A with a 12-fold molar excess of DTNB at pH 8.0 in the absence of denaturant caused a rapid appearance of 3.12 mol of TNB/mol of enzyme. The reaction was complete within 10 min at 30 °C, and no enzyme activity was detected after the reaction. As with WT [6], the rate and extent of reaction of C90A with DTNB were not affected by the presence of AdoMet or guanidinoacetate. The DTNB-modified enzyme obtained by gel filtration in 20 mM-potassium phosphate, pH 7.2, containing 1 mM-EDTA exhibited an absorption peak at approx. 330 nm in addition to an absorption maximum at 280 nm, indicating the formation of protein-S–TNB mixed disulphide. Assuming $\epsilon_{330} = 7.5 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$ for the molar absorption coefficient of protein-bound TNB [17], the modified enzyme was calculated to contain 1.98 mol of bound TNB/mol of enzyme. Treatment of the enzyme with 20 mM-dithiothreitol resulted in a rapid appear-

ance of 1.89 mol of TNB/mol of enzyme with concomitant recovery of activity. Thus only 1.9–2.0 mol of TNB is fixed to the enzyme in the form of mixed disulphide, despite the formation of approx. 3 mol of TNB anion/mol of enzyme. Since the TNB half of a protein-S–TNB mixed disulphide is a good leaving group, the result suggests that a fraction of mixed disulphide has undergone the attack of a neighbouring thiol group to form a disulphide bond with the displacement of TNB. To examine the point in more detail, C90A was treated with limited graded amounts of DTNB, and the amount of TNB anion released after each addition was determined. When the reaction with 1 equivalent of DTNB was allowed to go to completion, there appeared roughly 2 equivalents of TNB. Thereafter a 1:1 relationship was observed between the amount of DTNB added and that of TNB released (Table 3). The results suggest that one thiol group is extremely reactive towards DTNB, and that the mixed disulphide formed is very susceptible to disulphide interchange with a thiol group that occurs in close proximity. No detectable activity remained after the addition of 2 equivalents of DTNB. The DTNB-modified enzyme was eluted from a gel-filtration column at the same position as the untreated enzyme, indicating that a disulphide bond was formed intramolecularly.

Identification of the cysteine residues modified by the reaction with 1 equivalent of DTNB was carried out as follows. The unchanged free thiol groups were first blocked with *N*-ethylmaleimide at pH 6.8 under denaturing conditions, and the cysteine residues engaged in the disulphide bond were labelled with iodo[14 C]acetate after reduction with dithiothreitol (see the Materials and methods section). Radioactivity corresponding to approx. 1.8 mol of iodo[14 C]acetate was incorporated per mol of modified enzyme. The control sample treated similarly but in the absence of DTNB showed no appreciable radioactivity. The radiolabelled sample was digested with chymotrypsin, and the

chymotryptic-digest peptides were fractionated on a reverse-phase h.p.l.c. column. As shown in Fig. 3, radioactivity was associated with three peptides. The amino acid composition of peptide 3a differed from that of peptide 3b by one tyrosine residue (Table 1). GAMT has two consecutive tyrosine residues at positions 221 and 222, and the amino acid analysis data were consistent with peptide 3a and peptide 3b being the peptides comprising residues 212–221 and 212–222 respectively. Peptide 3c had an amino acid composition compatible with residues 9–19. The sum of radioactivities associated with peptides 3a and 3b is close to the radioactivity of peptide 3c. Thus the results obtained establish that a disulphide bond is formed specifically between Cys-15 and Cys-219.

Properties of C90A and WT modified by treatment with equimolar concentration of DTNB

Treatment of C90A with 1 equivalent of DTNB results in an enzyme preparation having 12% residual activity (under the standard assay conditions) with the formation of 1.88 equivalents of TNB (Table 3). The u.v.-absorption spectrum of the preparation (after dialysis) revealed the presence of < 0.09 mol of bound TNB/mol of enzyme. Thus a population of enzyme greater than 90% appears to have a disulphide bond between Cys-15 and Cys-219. In the previous paper [6] we reported that reaction of WT and an equimolar amount of DTNB leads to an almost quantitative disulphide cross-linking of Cys-15 with Cys-90, but not with Cys-219. Consistent with the previous observation, incubation of WT and 1 equivalent of DTNB resulted in the appearance of 1.95 mol of TNB/mol of enzyme. The enzyme after the reaction contained < 0.08 mol of enzyme-bound TNB/mol of enzyme, and had 10% residual activity.

The fact that approx. 10% of the initial activity is found in each of C90A and WT thus modified raises the possibility that the residual activity is due to the unchanged intact enzyme. It is possible that protein disulphide and TNB mixed disulphide are formed in the same enzyme molecule, leaving a small fraction of enzyme unmodified. If, in a mixture of modified and unmodified

Fig. 3. Fractionation of chymotryptic peptides from C90A modified with equimolar concentration of DTNB and treated with *N*-ethylmaleimide and [14 C]iodoacetate

The chymotryptic peptides were prepared as described in the Materials and methods section. About 16 nmol of peptides was subjected to h.p.l.c. as described in Fig. 1. Stippled bars represent the total radioactivity associated with each peptide.

Fig. 4. Initial-velocity studies with C90A and WT before and after modification with equimolar concentration of DTNB

C90A (22 μ M) (a and b) and WT (22 μ M) (c and d) were each incubated with an equimolar amount of DTNB at pH 8.0 and 30 $^{\circ}$ C, and the reaction was allowed to go to completion. The reaction mixture was dialysed against 50 mM-potassium phosphate buffer, pH 8.0, containing 1 mM-EDTA. Initial-velocity measurements were made at pH 8.0 and 30 $^{\circ}$ C by a coupled assay as described in the Materials and methods section with 2.0 mM-guanidinoacetate (GAA) when the AdoMet concentration was varied (b and d) and with 56 μ M-AdoMet when the guanidinoacetate concentration was varied (a and c). $\circ$, Modified enzymes; $\bullet$, unmodified enzymes. The amounts of enzyme used: modified C90A, 28 μ g; unmodified C90A, 5 μ g; modified WT, 28 μ g; unmodified WT, 4 μ g.

Fig. 5. C.d. spectra of (a) C90A and (b) WT before and after modification with equimolar concentration of DTNB

The DTNB-modified enzyme was prepared as described in Fig. 4 legend. The spectra were taken at 25 °C in 20 mM-potassium phosphate buffer, pH 7.2, containing 1 mM-EDTA. —, Before modification; ·····, after modification.

enzymes, the modified enzyme is totally inactive, initial-velocity analysis should yield K_m values of the untreated enzyme. If, on the other hand, the cross-linked enzyme is partially active and has altered K_m values, the reciprocal velocity is related to the reciprocal substrate concentration as [18]:

$$\frac{1}{v} = \frac{1 + a(1/A) + b(1/A)^2}{d + c(1/A)}$$

where a is the sum of the Michaelis constants of intact and modified enzyme (K_1 and K_2), b is their product, d is the sum of the maximum velocities (V_1 and V_2) and c is ($V_1K_2 + V_2K_1$). The plot of $1/v$ against $1/A$ is non-linear and concave down near the vertical axis, and the values of K_1 and K_2 cannot be determined directly from the curve. Fig. 4 shows double-reciprocal plots for C90A and WT before and after modification with 1 equivalent of DTNB. The plots obtained with the modified enzymes are apparently linear, as with the unmodified enzymes. However, a different horizontal intercept is obtained for modified C90A when AdoMet is varied, and for modified WT when guanidinoacetate is varied. The linearity of the double-reciprocal plots would be consistent with the following possibilities: (1) there exists no intact enzyme, i.e. intramolecular protein disulphide and TNB mixed disulphide are formed in a mutually exclusive manner, and the enzyme having intramolecular disulphide is partially active and the enzyme having mixed disulphide is inactive; (2) the intact enzyme is absent as above, but the cross-linked enzyme is inactive and the enzyme-TNB (which is present in an amount less than 0.1 mol/mol of enzyme) is as active as the native enzyme; (3) the intact enzyme does exist (< 0.1 mol/mol of enzyme), but the partially active cross-linked enzyme has K_m values not greatly different from those of intact enzyme. If we assume that a comparable activity is contributed by the intact enzyme and the cross-linked enzyme, a greater than 5-fold difference in K_m would be required to observe deviation from linearity in the double-reciprocal plots such as those shown

in Fig. 4. In view of the data of Table 3, it is unlikely that the enzyme with TNB mixed disulphide has an activity comparable with the activity of unmodified enzyme [possibility (2)]. Therefore the observed initial-velocity patterns give the qualitative indication that disulphide bond formation between Cys-15 and Cys-90 and between Cys-15 and Cys-219 does not abolish activity and also does not change the K_m values significantly.

Fig. 5 shows the c.d. spectra of C90A and WT and those obtained after modification with 1 equivalent of DTNB. The c.d. spectra of unmodified C90A and WT are very similar over the entire range of wavelength studied. Cross-linking of Cys-15 and Cys-219 in C90A and of Cys-15 and Cys-90 in WT have little effect on the far-u.v. c.d. spectra, but lead to a slight change in the intensities of near-u.v. c.d. bands. The chromophores that give rise to near-u.v. c.d. bands in proteins are aromatic residues and disulphide bonds [19]. Therefore the observed differences in c.d. may be considered to be due to the introduction of a disulphide bond and/or a conformational change associated with cross-linking. In any event, the near-u.v. spectra of the two modified enzymes are almost indistinguishable from each other. The fluorescence spectra of C90A and WT showed no significant change on modification (results not shown).

Reaction of C90A with NTCB

As described above, reaction of C90A with 2 equivalents of DTNB leads to complete loss of enzyme activity (Table 3). In an attempt to test whether the third cysteine residue that is modified in the form of TNB mixed disulphide is essential, we used NTCB as a modification reagent. Protein thiol groups are known to react with NTCB to form *S*-cyano derivatives or TNB mixed disulphides [17,20,21]. Even a single thiol group can react concurrently by two pathways [21]. If a cyano group is introduced, its small size and uncharged character would exert minimal effect on the local conformation, thereby providing a useful check on whether the thiol group is essential or not [20–24].

Incubation of C90A with excess NTCB (20-fold molar excess) at pH 8.0 resulted in a progressive appearance of TNB. The appearance of TNB was complete within 20 min, at which time 2.20 mol of TNB/mol of enzyme were released and approx. 10% of the initial enzyme activity remained. The modified enzyme after gel filtration contained 0.92 mol of free thiol group titratable with DTNB under denaturing conditions. The unmodified free cysteine residue(s) was labelled with *N*-ethyl[14 C]maleimide under denaturing conditions as described in the Materials and methods section, and chymotryptic-digest peptides derived therefrom were analysed under the conditions of Fig. 3. Radioactivity was found in two peptides eluted at 42.5 (peptide 4a) and 43 min (peptide 4b) (chromatogram not shown). Since both peptides had an identical amino acid composition that is consistent with residues 168–173 (Table 1), it appears that one of the peptides arises from deamidation of Asn-169. Thus Cys-168 is the only residue that has escaped modification by NTCB, and the fact that the modified enzyme retains considerable activity indicates that Cys-207 as well as Cys-15 and Cys-219 is not essential. In order to gain insight into the types of modified residues, the enzyme was incubated with [14 C]NTCB under the same conditions, and the resulting enzyme was analysed for [14 C]cyanide incorporated and TNB bound. The reaction yielded 2.10 mol of TNB/mol of enzyme in this case, and the modified enzyme was found to contain 1.78 mol of [14 C]cyanide and 0.61 mol of TNB (determined spectrophotometrically). The disagreement between the amount of TNB anion released and that of [14 C]cyanide incorporated suggests that a fraction of residues is modified in the form of protein disulphide. Cyanide as well as TNB can be displaced from the modified residue by the

Table 4. Reaction of C90A and WT with equimolar concentration of NTCB

C90A (21 μM) and WT (17 μM) were each incubated with an equimolar concentration of [^{14}C]NTCB (600 c.p.m./mol) at pH 8.0 and 30 °C. When the absorbance at 412 nm reached a final value, the reaction mixture was dialysed against 20 mM-potassium phosphate buffer, pH 7.2, containing 1 mM-EDTA, and the modified enzyme was determined for activity, [^{14}C]cyanide incorporated and free thiol groups remaining. The cysteine residues engaged in disulphide linkage were determined separately with the enzymes modified with unlabelled NTCB under the same conditions (see the Materials and methods section).

Enzyme	Activity (%)	TNB formed (mol/mol of enzyme)	Thiol groups disappeared (mol/mol of enzyme)	[^{14}C]Cyanide incorporated (mol/mol of enzyme)	Cysteine residues linked
C90A	28.1	1.06	1.79	0.14	Cys-15–Cys-219
WT	29.4	1.01	1.82	0.14	Cys-15–Cys-90

nucleophilic attack of a protein thiolate, and 1 mol of TNB is produced for each mol of disulphide formed irrespective of whether protein-S–CN or protein-S–TNB is initially formed. Thus 0.64 mol of cysteine residue would be in the form of intramolecular protein disulphide, and a total of 3.03 mol of cysteine residues would be modified by treatment with excess NTCB. This is in agreement with the result that only Cys-168 is refractory to modification by NTCB. Neither AdoMet nor guanidinoacetate exerted appreciable influence on the modification reaction as monitored by the absorbance change at 412 nm.

Table 4 summarizes the results obtained when C90A and WT were treated with an equimolar concentration of [^{14}C]NTCB. There was a loss of cysteine residues close to 2 mol/mol of enzyme and an incorporation of 0.14 mol of [^{14}C]cyanide/mol of enzyme in each case, indicating efficient formation of a protein disulphide. No appreciable formation of TNB mixed disulphide was observed under these conditions. The cysteine residues forming the disulphide bond were determined as described above for the DTNB-modified enzyme, and shown to be Cys-15 and Cys-219 in C90A, and Cys-15 and Cys-90 in WT. The remaining activities after modification are similar for C90A and WT, but are considerably higher than those observed after the reaction with DTNB (Table 3). It is possible that disulphide bond formation is less complete in the present case, and fractions of relevant cysteine residues remain unmodified or exist as S-cyano derivatives.

DISCUSSION

The mutation of Cys-90 to alanine in rat GAMT results in a catalytically active enzyme having kinetic constants similar to those of wild-type recombinant enzyme. This clearly indicates that Cys-90 is not a catalytic residue nor has it any structural role in catalysis, although a large loss of activity occurs when this residue is linked to Cys-15 by a disulphide bond [6]. The spectroscopic properties (u.v. absorption, fluorescence and c.d.) of the mutant are also indistinguishable from those of wild-type. Thus it is evident that no significant alteration in the secondary and tertiary structures accompanies the mutation.

As reported previously [6] and confirmed in the present study, incubation of wild-type GAMT with an equimolar amount of DTNB leads to an almost quantitative disulphide cross-linking between Cys-15 and Cys-90. Unexpectedly, C90A, which lacks Cys-90, undergoes the same type of reaction between Cys-15 and Cys-219. The DTNB-mediated formation of protein disulphides is reported for many proteins and is considered to occur by the nucleophilic attack of a neighbouring thiolate to displace TNB from the initially formed protein-S–TNB mixed disulphide. Since the mutant and the wild-type enzymes appear to have similarly

folded structures as evidenced by their catalytic and spectroscopic properties, the ease with which Cys-15 is linked to Cys-90 and Cys-219 by disulphide bonds suggests that these residues occur in close proximity in the three-dimensional structure. The same residues, Cys-15 and Cys-90 in WT and Cys-15 and Cys-219 in C90A, are also cross-linked upon incubation with 1 equivalent of NTCB, although it is not known in this case whether the initially modified species is protein-S–CN or protein-S–TNB. If Cys-15, Cys-90 and Cys-219 are located close together, a question arises why Cys-15 is linked almost exclusively to Cys-90 in WT. The reason for this could be that Cys-90, compared with Cys-219, is a better nucleophile and/or positioned more favourably for the reaction with TNB-modified Cys-15. It would also be possible that replacement of the charged cysteine residue with an uncharged alanine residue results in slight alteration in the local geometry around these residues, thereby enabling the mutant to form the Cys-15–Cys-219 disulphide bond more readily. The similar catalytic and spectroscopic properties exhibited by the enzymes cross-linked between Cys-15 and Cys-90 and between Cys-15 and Cys-219 are not inconsistent with the idea that the three cysteine residues are vicinal residues.

Besides establishing that Cys-90 has no functional and structural role and providing evidence that Cys-15, Cys-90 and Cys-219 are located in close proximity in the tertiary structure, the use of the mutant C90A enables us to conclude that Cys-15, Cys-219 and Cys-207 are not essential; cross-linking of Cys-15 and Cys-219 does not appear to eliminate activity, and treatment of C90A with excess NTCB, which modifies all cysteine residues except Cys-168, does not abolish activity. Carboxymethylation of Cys-219 has been shown previously to result in complete loss of activity [6], and nothing has been known about Cys-207. Another interesting suggestion that can be made by use of the mutant is on the relative reactivity of Cys-15 and Cys-90 with DTNB. In the DTNB-dependent formation of disulphide in wild-type, it has not been possible to decide which of Cys-15 and Cys-90 reacts with DTNB. Since Cys-219 is far less reactive than Cys-15 and Cys-90 [6], the finding that a disulphide bond is formed between Cys-15 and Cys-219 in C90A strongly suggests that Cys-15 is the site of attack of DTNB (or NTCB).

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