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Construction, Expression, and Characterization of Glutathione Synthetase Chimeras: Substitution of a Loop with a Homologous Peptide Region of Dihydrofolate Reductase

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To investigate the functional role of a sequence similarity found between E. coli B glutathione synthetase (GSHase) and mouse dihydrofolate reductase, a chimera GSHase, in which the sequence region showing similarity was replaced with the corresponding region of the mouse enzyme, was constructed. The chimera protein, GDG-FR, was expressed in *E. coli* as inclusion bodies. They were solubilized with 8M urea solution and refolded with step-wise dialysis. Limited proteolysis and PAGE analysis in the presence of urea strongly suggested that the refolded chimera enzyme was in an assembly composed of several subunits, each of which might take a specific conformation similar to that of GSHase. In spite of the conformational similarity to GSHase, the refolded GDG-FR showed no glutathione synthetic activity.

KEY WORDS : Glutathione Synthetase / Dihydrofolate Reductase / Sequence Homology Search / Chimeric Protein / Module /Protein Refolding

Introduction

How enzymes evolved? This problem is one key of protein engineering to construct new enzymes. Shultz and Schirmar proposed that enzymes are constructed from several domains each of which are specific for a substrate or a cofactor (1). Blake argued that, if exons encode structural units as well as functional units, then combinations of such exons would have the advantage of producing stable functional proteins (2). Gô indicated that compact structural units in hemoglobin correspond exons of the gene (3). She called these compact structural units "module". These propositions suggested that, if modules in enzymes are identified, the construction of new enzymes could be achieved.

Glutathione synthetase (γ-L-glutamyl-L-cysteine: glycine ligase (ADP forming), EC 6.2.3.2; GSHase) catalyzes the synthesis of glutathione from γ-Glu-Cys and Gly with the

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Abbreviations: DHFR, dihydrofolate reductase; GSHase, glutathione synthetase; γ-Glu-Cys, γ-L-glutamyl-L-cysteine; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate

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Residue No. of fihydrofolate reductase (Mouse)
                                                    Source              Score
47--+---- ----- +---------+---------+-------89
 P  VIMGR HTWES I G  RPLPGRKNIILSSQPGTDDRVT WV  E.coli K12           -58
 P  VIMGR HTWES I G  RPLPGRKNIILSSQPGTDDRVT WV  E.coli B             -58
 P  VIMGR KTWES LPVK  PLPGRRNIVISRQADYCAAGAETV  N.gonorrheae        -60
 I MVV GR RTYES FP K RPLPERTNVVLTHQEDYQAQGA VV  L.casei             -58
 QNLVIMGK KTWFS IPEKNRPLKGRINLVLSRELKEPPQGAHFL  Human               -66
 QNLVIMGR KTWFS IPEKNRPLKDRINIVLSRELKEPPKGAHFL  Bovine liver        -69
 QNLVIMGR KTWFS IPEKNRPLKDRINIVLSRELKEPPQGAHFL  Pig liver           -70
 QNAVIMGK KTWFS IPEKNRPLKDRINIVLSRELKEAPKGAHYL  Chicken             -65
 QNLVIMGR KTWFS IPEKNRPLKDRINIVLSRELKEPPQGAHFL  Chinese hamster     -70
 QNLVIMGK KTWFS IPEKNRPLKDRINIVLSRELKEPPRGAHFL  Mouse               -71
   :       :::  :   :: :     : :  : ::    :
 RTLNVKQNYEEWFSFVGEQDLPLAD LDVIKMR  KDPPFDTEFI  Glutathione synthetase
55----+---------+--------- +------  ---+----96
Residue No. of glutathione synthetase (E.coli)
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Fig. 1 Amino acid alignment of the *E.coli* GSHase with Mouse DHFR. The score is the measure of homology based on the amino acid mutation data (32). The values shown are the minimum value of this pair of sequences. Underlined residues are the fragment substituted between GSHase and DHFR.

hydrolysis of ATP. Amino acid homology search against a protein sequence database, NBRF, revealed that the amino acid sequence of the *E.coli* GSHase from Arg-55 to Ile-96 was highly similar to that of mouse dihydrofolate reductase (DHFR) from Gln-48 to Leu-90 (Fig. 1) (4). This region of DHFR is a part of the NADPH binding site according to the X-ray crystallographic studies on DHFR. No common substrate to these two enzymes was found except that ATP is a moiety of chemical structure of NADPH. Later, we have shown methotrexate, which is a competitive inhibitor of DHFR to NADPH, is a potent inhibitor of GSHase (4). These results suggested that the sequence region similar between GSHase and DHFR is expected as a part of the adenosine-binding module. In the present paper, we investigated the functional role of the homologous region of GSHase by constructing of a chimera GSHase, GDG-FR, in which the substitution of the peptide from Trp-55 to Arg-75 of GSHase was substituted with the corresponding peptide from Trp-66 to Arg-86 of the mouse DHFR.

Materials and Methods

Bacterial Strains, Phage, and Plasmid

Plasmid pKGS00 was a pKK223-3 derivative containing a 1.2 kilobase pair fragment that is coding the GSHase gene from *E. coli* B (5). Plasmid pMTVdhfr, which carries DHFR gene, was a gift from Dr. P. Berg, Stanford University School of Medicine (6). Bacterial strain *E. coli* BW313 (*dut*, *ung*, *thi*-1, *rel* A, *spo*T1/F'*lys* A) for Kunkel method of site-directed mutagenesis was a gift from Dr. Kagamiyama of the Medical College of Osaka. Bacteriophage M13 mp19 and *E. coli* JM109 were provided by Takara Shuzo Co., Ltd. (Kyoto, Japan).

Restriction Enzymes, DNA Modification Enzymes, and Chemicals

Restriction enzymes were obtained from Toyobo Co., Ltd. (Osaka, Japan) and New En-

gland Biolabs, Inc. (Beverly, Massachusetts, USA). Restriction enzymes, DNA modifying enzymes, and arginyl endopeptidase (7) (Mouse submandibular protease; treated with TLCK and TPCK; 1.045 units/mg) were purchased from Takara Shuzo Co., Ltd. Trypsin (12000 units/mg: treated with TPCK) from Bovine pancreas was purchased from Sigma Chemical Co. (St.Louis, Missouri, USA). Glutathione reductase from yeast were obtained from Oriental Yeast Co., Ltd. (Tokyo, Japan). All the γ-Glu-Cys was a special gift from Kohjin Co., Ltd. (Tokyo, Japan). The Tris-HCl buffer used in the present study contains 5 mM $MgCl_2$, unless noted otherwise. Chemicals used in the present work were the purest grade commercially available.

Site-directed Mutagenesis and Construction of Expression Plasmid for the Chimera

Construction of GSHase-DHFR chimera was carried out as shown in Fig. 2.

For the mutation of GSHase gene, a *Hind* III-*Hinc* II fragment (792 base pairs) encoding the partial GSHase gene was isolated from pGS400 (8) and inserted into bacteriophage M13 mp10 to give ϕHH792 for site-directed mutagenesis. Site-directed mutagenesis was carried out by the method of Kunkel (9) and Taylor (10). Oligonucleotide primers used for the mutations were designed to create *Cla* I on amino terminal side and *Dra* I on carboxylate terminal side of homologous region. Mutants were screened with this created site. Oligonucleotide primers used in the present studies were as follows. (The underlined bases encode the new restriction site.)

G-Cla I 5' AGTGGTTATCGATCGTCGG 3'

G-Dra I 5' CCTGATGTTTAAAGAC 3'

For the mutation of DHFR gene, 4.6 kb of *Hind* III fragment was excised from pMTVdhfr (6) and was self-ligated to yield shorten plasmid pMMdhfr. Site-directed mutagenesis was carried out with gapped-duplex method described by Inoue and Inoue (11). Oligonucleotide primers used for the mutations were designed to create *Cla* I on amino terminal side and *Bgl* II on carboxylate terminal side of homologous region. Mutants were screened with this created site. Oligonucleotide primers used in the present studies were as follows. (The underlined bases encode the new restriction site.)

D-Cla I 5' CCTGGTTATCGATTCCTG 3'

D-Bgl II 5' GTAGAGATCTCAAAG 3'

Resulting plasmid, pMMdhfr-CB, was cleaved with *Bgl* II, and then treated with S1 nuclease. This fragment was digested with *Cla* I to yield 60 base pairs fragment.

After verifying DNA sequence by dideoxy method (12), a short DNA fragment containing the mutation site was excised from ϕHH792 mutant (ϕHH792-CD) with two appropriate restriction enzymes, *Hinc* II and *Hind* III. This short fragment replaced its counterpart of pMCgshII between *Hpa* I site and *Hind* III site. Resulting plasmid, pMCgshII-CD, was digested with the new site, *Cla* I and *Dra* I, to give 5.0 kb fragment. This fragment was ligated with the 60 base pair fragment from pMMdhfr-CB to give pMCgdg'. This pMCgdg' contained a mutation, P67L, on the junction region of GSHase gene. To revert this mutation to the wild-type, a codon of Leu-67 was mutated to a codon for Phe with gapped-duplex method. Oligonucleotide primers used in the present studies were as follows.

L67P 5'-GAATCGAAAACCACTC-3'

Resulting plasmid was pMCgdg which containing the partial GSHase gene from Met-1 to Phe-67, the partial DHFR gene from Ser-57 to Arg-75 and the partial GSHase gene from Lys-87

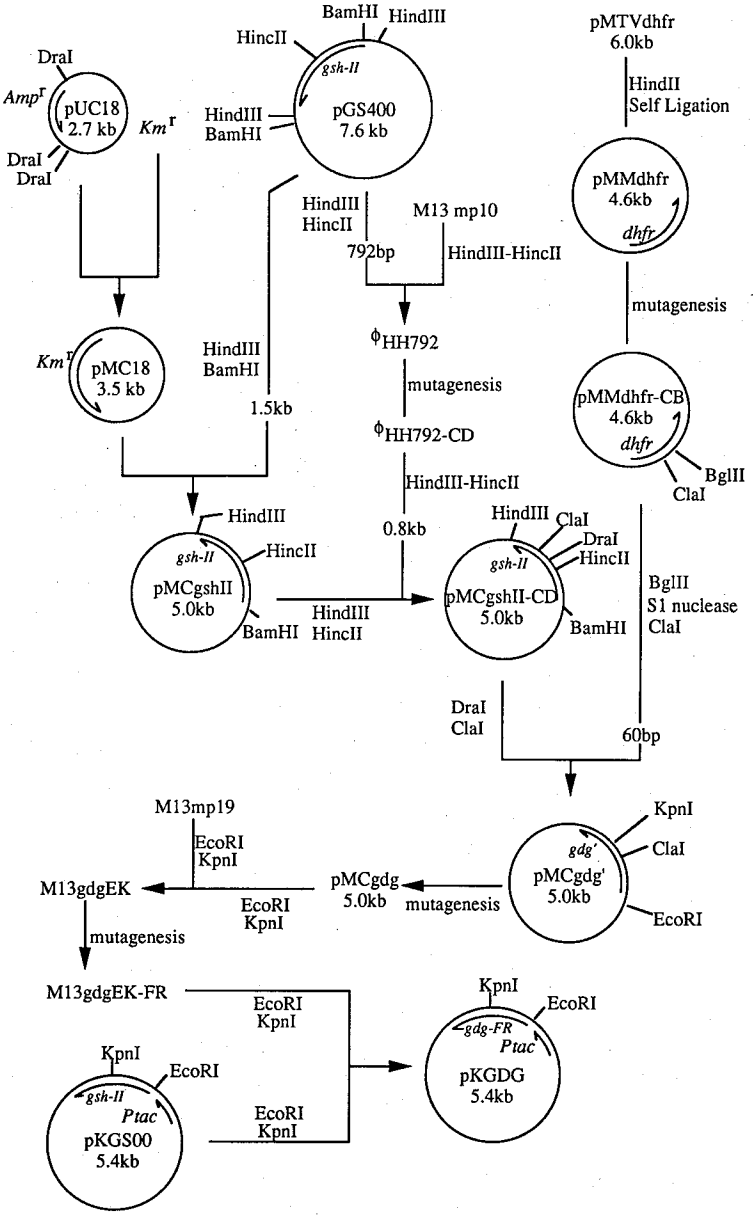

Fig. 2 Construction of chimera GDG-FR gene

to Gln-316. A short DNA fragment containing the chimera fragment was excised from pMCgdg with two appropriate restriction enzymes, *EcoR* I and *Kpn* I. This short fragment replaced its counterpart of pKGS00 (13) between *EcoR* I and *Kpn* I site to yield pKSGDG which carries chimera GDG gene.

To align the hydrophobicity profile between GSHase and chimera GDG (Fig 3), a

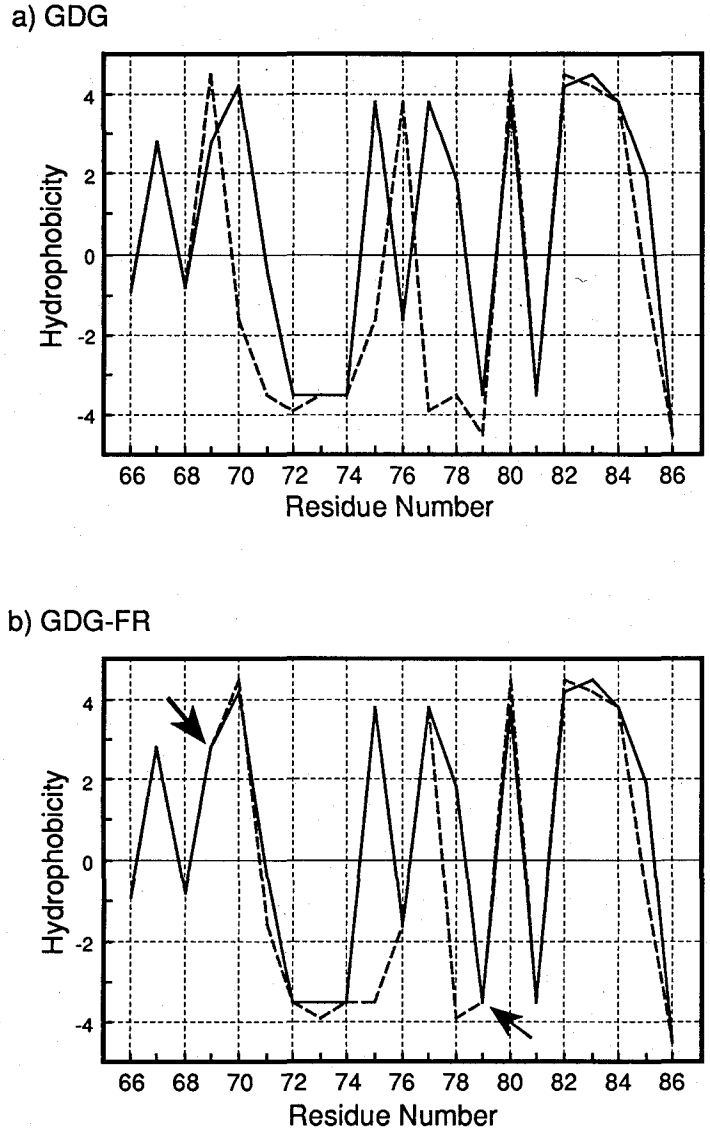

Fig.3 Hydrophobicity profile of chimeras and GSHase. a. Comparison between GDG and GSHase. Hydrophobicity profile of GDG between 70 to 78 is very different from that of GSHase. This is caused by the gap and the insertion in amino acid sequence alignment. b. Comparison between GDG-FR and GSHase. The insertion of Phe after Ser-69 and the deletion of Arg-79 on GDG results the high similarity of the profile between GDG-FR and GSHase.

deletion of Arg-79 and an insertion of Phe between Ser-69 and Ile-70 were introduced into chimera GDG gene with oligonucleotide site-directed mutagenesis as described above. A *EcoR* I-*Kpn* I fragment (0.8 kilobase pairs) encoding the partial chimera GDG gene was isolated from pMCgdg and inserted into bacteriophage M13 mp19 to give M13gdgEK for site-directed mutagenesis. Oligonucleotide primers used in the present studies were as follows. (The underlined bases encode the new restriction site.)

ΔR79 5'-CCTTTAAAGGATATCAATATAGTTC-3'

+70F 5'-GTGGTTTTCGTTCATCCCGGAGAAGAATCG-3'

After verifying DNA sequence by dideoxy method (12), a short DNA fragment containing the chimera fragment was excised from M13gdgEK mutant with two appropriate restriction enzymes, *EcoR* I and *Kpn* I. This short fragment replaced its counterpart of pKGS00 (13) between *EcoR* I and *Kpn* I site to yield pKGDG which carries chimera GDG-FR gene.

Expression and Purification of the Chimera GDG-FR

Expression plasmids, pKGDG transformed *E.coli* JM109 by the method of Hanahan (14). Transformants were grown in 500 mL of LB medium (1 % bacto-tryptone, 0.5 % bacto-yeast extract, 1 % NaCl; pH 7.5) at 37℃ with vigorous shaking. At log phase (OD_{600}=0.6−0.7), isopropyl-β-D-thio-galactopyranoside was added to yield a final concentration of 1 mM. After 10 hours culture, the cells were harvested by centrifugation at 8,000 × *g* for 10 minutes at 0℃ (13). The cells were suspended in 50 mL of Buffer I (50 mM Tris-HCl buffer, pH 7.5, 5 mM $MgCl_2$), and were sonicated. The suspension was centrifuged at 15,000 × *g* for 20 minutes at 0℃. The chimera protein was collected as inclusion bodies.

The inclusion bodies were resuspended in Buffer I containing DNase I (20 μg/mL) and RNase A (25 μg/mL). After standing for 2 hours at 37 ℃, it was centrifuged at 8,500 × *g* for 10 minutes at 0℃. Recovered inclusion bodies were washed with 200 mL of Triton buffer (Buffer I, 2 % Triton X-100) three times. To wash away Triton X-100, the inclusion bodies were washed with Buffer I twice.

Chimera GDG protein was purified from the transformants with pKSGDG as described above.

Solubilization and Refolding of Inclusion Bodies

Five mL of 8 M urea-buffer (8 M urea, 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 30 mM mercaptoethanol) or 6 M GdnHCl-buffer (6 M guanidine hydrochloride, 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 30 mM mercaptoethanol) was added to 50 mg of the inclusion bodies. Without mixing, it was gently shaken over night. Solubilized fraction was recovered with centrifugation at 19,000 ×*g* for 10 min. Protein concentration of this solubilized fraction was determined with coomassie brilliant blue G-250 method.

Refolding of this solubilized chimera GDG-FR was carried out through step-wise dialysis. First, the solubilized GDG-FR solution was diluted into 10, 50 and 100 μg/mL in 4M urea-buffer and in 6M GdnCl-buffer. Then detergent concentration was stepwisely lowered to 2M, 1M, and finally 0M for urea-solubilized GDG-FR and to 3M and finally 0M for GdnCl-solubilized GDG-FR. After removing the detergent until each concentration, the refolded GDG-FR was sampled and concentrated to 2 mg/mL with using Centricon (Amicon, Danvers, Massachusetts, USA). Refolding of GDG was carried out with the same method as GDG-FR.

Measurement of the Activity of Refolded GDG-FR

The glutathione synthetic activity of the refolded GDG-FR at 1M urea was measured with the GR method (15). The refolded GSHase that was refolded from 8M urea-buffer with the same method as GDG-FR was used to reference enzyme.

Limited Proteolysis of the Refolded GDG-FR

Under the native condition, arginyl endopeptidase and trypsin cleave native GSHase only at Arg-33 in spite of that each subunit of GSHase has nineteen Arg residues. The authors showed that this cleavage was dependent on the conformation of GSHase (15). To probe the conformation of refolded GDG-FR, limited proteolysis was carried out.

To 100 μL of 1M urea-buffer containing the protein preparation (2.0 mg/mL), arginyl endopeptidase or trypsin (2 μg/ 5 μL) was added at 30 ℃. Applied samples were the refolded GDG-FR in 1M urea-buffer and the refolded GSHase in 1M urea-buffer. An aliquot of the reaction mixture, 10 μL, was withdrawn at 0, 60, and 120 minutes (0, 5, 10, 30, 60, and 120 min in the case of trypsin) after the beginning of the reaction, and 3 μL of SDS-dye solution (20 mM Tris-HCl pH7.6, 2% SDS, 0.01 % bromophenol blue, 2 % mercaptoethanol, 50 % glycerol) was added immediately to stop the proteolysis. The aliquots of the reaction mixture were analyzed on SDS-PAGE (16).

NH_2-terminal amino acid sequence analysis of the products of proteolysis was carried out to determine the digested site. The GSHase was treated with arginyl endopeptidase or trypsin under the conditions as mentioned above. After 120 minutes reaction, the reaction mixture was separated on SDS-PAGE. Separated peptide fragments were blotted on polyvinylidene difluoride membrane (17, 18) by electroblotting method (19). Edman degradations were performed automatically by using gas-phase peptide sequencer, Applied Biosystems model 477A/120A protein sequencer system. The 3-phenyl-2-thiohydantoin-amino acid derivatives were identified on high performance liquid chromatography.

Analysis on Urea-PAGE

The refolded GDG-FR, the refolded GDG, the refolded GSHase and the native GSHase were applied onto urea-PAGE. Urea-polyacrylamide gel was prepared with the method described by Creighton (20). Separation gel was containing 9 % polyacrylamide (w/v, mono-:bis = 30:0.8), 0.375 M Tris-HCl (pH 8.8) and urea (8M, 4M, 2M, or 1M). Concentration gel was containing 3.125 % polyacrylamide (w/v, mono: bis = 10:2.5), 0.125 M Tris-HCl (pH 6.8), and urea (the same concentration as that in the separation gel). Samples were the refolded GDG-FR until each concentration of urea, the refolded GSHase and native GSHase. Electrophoresis was carried out at 4 ℃ with 15 mA constant current.

Results

Expression and Purification of Chimeric Protein GDG-FR

IPTG-induced transformant cells of pKGDG were harvested and sonicated. Their cell extracts with Buffer I, their cell debris and the whole cells were applied on SDS-PAGE. The electrophoresis showed that the gene of GDG-FR was induced and expressed in large amount. However, the induced GDG-FR was not extracted from ultarasonic-disrupted cells into Buffer I, but remained in the cell debris.

Chimeric GDG-FR protein was purified from the cell debris by washing out contaminated proteins. Recovered GDG-FR was analyzed with SDS-polyacrylamide gel electrophoresis. This purification procedure gave a purified preparation of GDG-FR higher than 90% in purity based on SDS-PAGE. Average yield of mutant protein was about 0.50 g (wet weight) from a 500 mL culture.

Refolding of GDG-FR

The inclusion body of chimera GDG-FR was dissolved in 8M urea-buffer or 6M GdnHCl-buffer at the concentrations of 100, 50 and 10 μg/mL.

After dialysis against 1M urea-buffer, 100 μg/mL solution yielded insolble fraction. Both 50 and 10 μg/mL solution did not yield the insolble fraction after dialysis against 1M urea-buffer, but both solution yielded the insoluble fraction after concentrating into 2 mg/mL.

After dialysis against 3M GdnHCl-buffer, each concentration of the chimera GDG-FR remained in soluble fraction. However, removing of GdnHCl by dialysis resulted in the formation of insolble material at each concentration.

Activity of the Refolded GDG-FR

The activities of the GDG-FR refolded in 1M urea-buffer, the GSHase refolded in 1M or 2M urea-buffer, and the native GSHase was measured with GR method (Table 1).

Table 1. Activities of GSHase and GDG-FR

	Enzyme	Specific Activity (units/mg)	Relative Activity (%)
Refolded	GSHase in 2M urea	<0.01	-
	GSHase in 1M urea	6.6	24
	GDG-FR in 1M urea	<0.01	-
native	GSHase	27.8	100

(1 unit = 1 μmole GSH/min)

It showed that the refolded GDG-FR showed no glutathione synthetic activity while the GSHase refolded in 1M urea-buffer has 24 % activity at 1M urea (6.6 unit/mg) that of the native GSHase in a plain buffer (27.9 unit/mg). Even at 50 mM γ-Glu-Cys, 100 mM ATP and 150 mM Gly, the activity of the refolded GDG-FR was not observed.

Limited Proteolysis of the Refolded GDG-FR

The wild-type GSHase was cleaved by arginyl endopeptidase and trypsin at only Arg-233 on a loop structure under the native conditions, although the heat-denatured GSHase was cleaved at almost all the arginine residues (15). It indicates that limited proteolysis is a good probe of the folding of GSHase.

The refolded GSHase and the refolded chimeric protein, GDG-FR, were applied on the limited proteolysis with arginyl endopeptidase in 1M urea buffer. In both cases, the intact peptide fragment (36kd) was disappeared and two new fragments (26 and 10 kd) were appeared as the reaction time (Fig 4a). Amino terminal sequence analysis of 10 kD fragment from GSHase and GDG-FR gave the same sequence; G-N-L-A-A. This sequence consisted

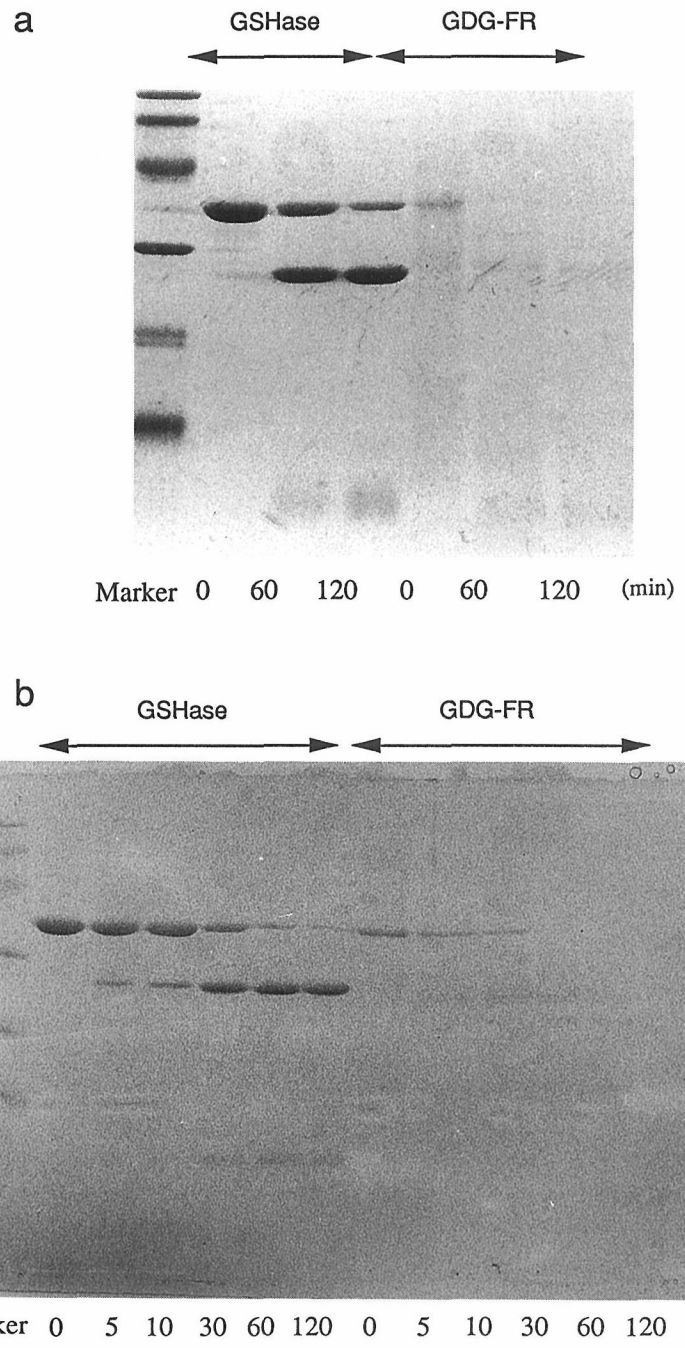

Fig 4. Limited proteolysis of GDG-FR and GSHase with arginyl endopeptidase (panel a) and with trypsin (panel b). In the both case, GSHase was cleaved only at Arg-233. GDG-FR was cleaved only at Arg-233 with arginyl endopeptidase as same as GSHase. However, trypsin digested GDG-FR into many small fragments. This degradation of GDG-FR indicated that GDG-FR had a similar but not the same conformation as GSHase.

the sequence from Gly234 on GSHase and its counterpart on GDG-FR. SDS-PAGE and amino terminal sequence analysis showed that the refolded GSHase was cleaved only at Arg-233 with both arginyl endopeptidase and trypsin. These results indicated that, in 1M urea buffer, the refolded GSHase has the similar conformation to that of the native enzyme. This conclusion was supported by the recovery of the activity of the refolded GSHase.

The tryptic digestion of the GDG-FR refolded in 1M Urea-buffer gave no specific band on SDS-PAGE (Fig 4b). This result was not the same as those of the native and the refolded GSHases, which were cleaved only at Arg-233 by trypsin.

The limited proteolysis indicated that the refolded GDG-FR would take a folding, in which the distribution of arginine residues is similar to the native GSHase but that of lysine residues was different.

Analysis on Urea-PAGE

To analyze the conformation of the proteins in different concentrations of urea, the refolded GDG-FR, the refolded GDG, the refolded GSHase and the native GSHase was applied onto native PAGE containing urea (urea-PAGE). Fig 5 a-d were 8M urea-PAGE, 4M urea-PAGE, 2M urea-PAGE, and 1M urea-PAGE, respectively.

The results of urea-PAGEs were separated into two groups depending on the migrations of the native GSHase. On 8M and 4M urea-PAGE, the native GSHase was separated into several bands, but on 2M and 1M urea-PAGE, it formed the single band. These results indicated that GSHase takes several denatured states through electrophoresis in 8M and 4M urea-polyacrylamide gel. In contrast, the conformation and assemble of the subunits of GSHase would be little affected by the presence of 2M or 1M urea.

On 8M urea-PAGE, GDG-FR migrated slightly larger than GDG, which has additional positive charged arginine residue to GDG-FR. Also, the migration of the denatured GSHase, which has four more negative charge residues than GDG-FR, was larger than that of GDG-FR. The ratio of the migrations of GDG-FR and GDG was one fourth of that of the denatured GSHase and GDG-FR. This indicated that the differences of the migrations among GDG-FR, GDG, and the denatured GSHase were dependent on charges, rather than conformations of the proteins.

On 4M urea-PAGE, GDG-FR was a single band, but still different in migration from that of the refolded GSHase.

On 2M and 1M urea-PAGEs, the refolded GSHase and the native GSHase showed the same migration. This showed that the refolded GSHase and the native GSHase were in the same conformation. GDG-FR was a single band, which has much lower migration than that of the refolded GSHase and the refolded GDG-FR in 4M urea.

Discussion

The chimera GSHase, GDG-FR, was expressed as inclusion bodies. It is difficult to investigate the character of inclusion bodies without refolding. Then we first tried to refold the inclusion bodies of GDG-FR. There are several reports on the mechanism of the formation of inclusion bodies (21, 22, 23, 24). They proposed that the formation of inclusion body is caused by wrong S-S bond formations and by wrong hydrophobic interactions

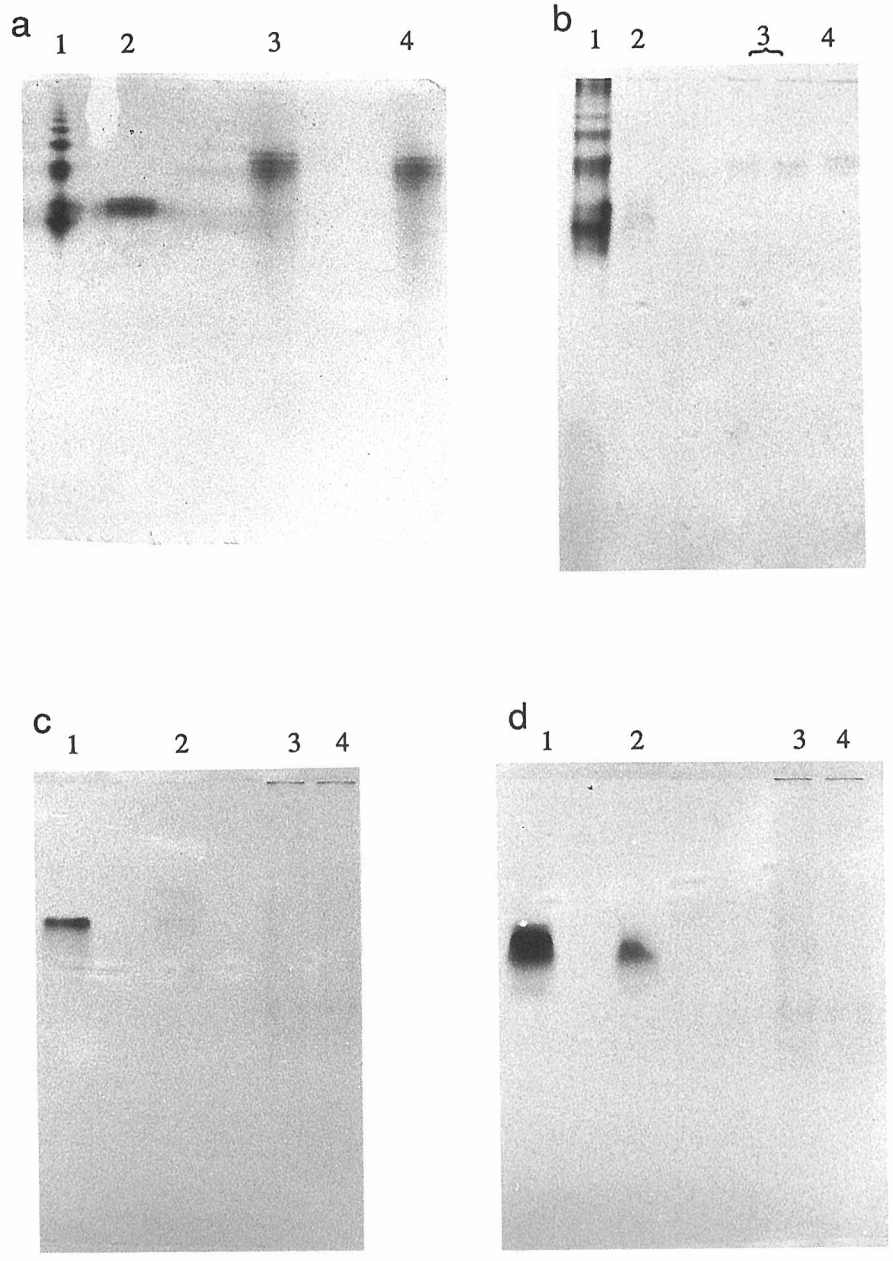

Fig 5. Analysis on Urea-PAGEs
a. 8M urea-PAGE. b. 4M urea-PAGE. c. 2M urea-PAGE. d. 1M urea-PAGE. In all panels, samples except native GSHase are proteins refolded into each concentration of urea.

(25, 26). Some groups succeeded in the refolding of inclusion bodies by solubilization with detergent following gradually dialysis to remove the detergent (23, 26, 27). First we tried the refolding of the inclusion bodies of the chimera GSHase with detergent-dialysis method. GDG-FR was dissolved in a urea or a GdnHCl solution, but became insoluble again with the removal of the detergent.

To investigate refolding state, the refolded GDG-FR was applied onto urea-PAGE. Urea-PAGE has been applied on the analysis of folding state of proteins (20, 28) to detect the transient states from native to denatured on bovine pancreatic ribonuclease A, hen egg lysozyme, bovine serum albumin. Migration on urea-PAGE is dependent on the folding, assembly and charge of the sample as much as that on native-PAGE (20). Since protein unfolded must be larger in volume, its migration on native-PAGE should be smaller than that of protein folded. The analysis of GDG-FR on urea-PAGEs indicated that the assembly of subunit was dependent on the concentration of urea. GDG-FR formed larger conformation on 2M or 1M urea-PAGE than that on 4M urea-PAGE. However, the limited proteolysis of the refolded GDG-FR showed that GDG-FR took a certain conformation similar to that of the refolded GSHase, which was active in the presence of 1M urea.

X-ray analysis of GSHase (29) showed that the peptide region substituted forms a part of the inter-subunit β-sheet, which is in the N-terminal domain. The substitution of the homologous peptide in GDG-FR might disturb the formation of the β-sheet and the N-terminal domain. In multidomain proteins, the formation of each domain is independent with each other (30, 31). This suggests that three domains in GSHase independently take their folds. These folds were partly formed at 4M to 2M urea, and was almost completed in 1M urea. The substitution of the homologous region might disturb the formation of the N-terminal domain but not the other domains. This assumption is consistent with the observations on limited proteolysis and urea-PAGEs analysis of GDG-FR. Limited proteolysis and urea-PAGE strongly suggested that the refolded GDG-FR was in an assembly composed of several subunits, each of which has a specific conformation similar to that of GSHase.

In spite of the conformational similarity to GSHase, GDG-FR showed no glutathione synthetic activity. The disturbance of the formation of the N-terminal domain might result in the distortion of the γ-Glu-Cys binding site, which is between the N-terminal and C-terminal domains. In addition, subunits might be randomly assembled, then the active site cleft will be disordered by other subunits or domains. These give rise to failures of the binding of substrates and of the formation of the catalytic intermediate and products.

The peptide regions substituted are on the surface of the GSHase and DHFR. It is to say, the two peptides are in a similar environment, and both form a similar U-shaped structure (Fig 6). When the structures of the two regions in the wild-type enzymes are superimposed with least square method, r.m.s. deviation of Cα carbons is 5.38 Å. On the comparison, dihedral angles of the main chains at each residue are similar except at Pro-71 on GDG-FR, which is glycine on GSHase. The dihedral angles allowed to Pro is narrow, while those of Gly allowed almost unrestrictly. Then one of the reasons of aggregation of GDG-FR is probably the Pro-Gly substitution.

We first thought that the homologous peptide region was a part of the nucleotide binding module (4). The crystallography of GSHase, however, revealed that the region was a part of the N-terminal domain, not the ATP binding site. GDG and GDG-FR have been express

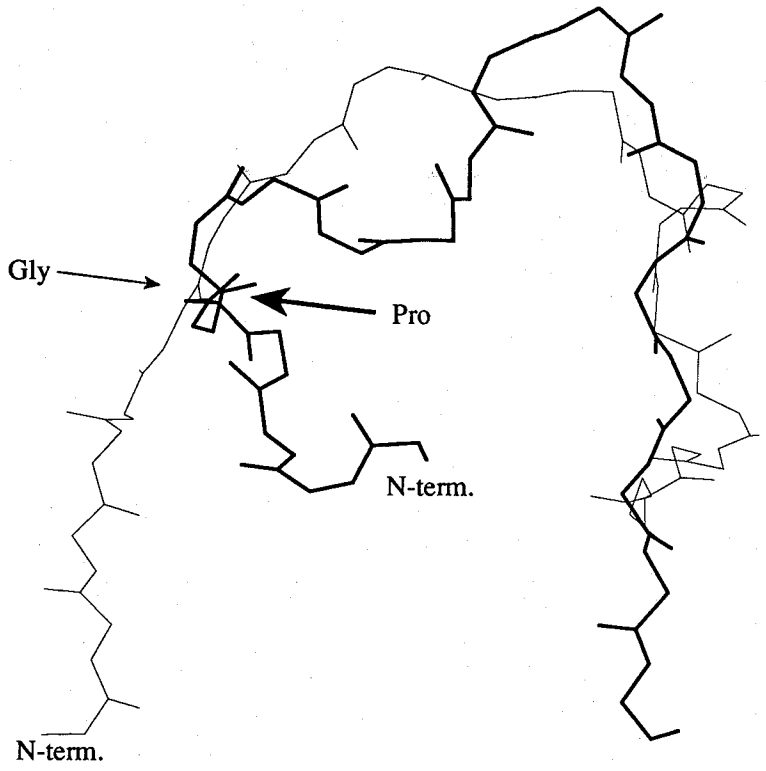

Fig 6. Superimposition of the homologous region of GSHase (thin line) and DHFR (thick line). GSHase peptide from Trp-66 to Arg-86 is superposed onto DHFR peptide from Trp-55 to Arg-75. R.m.s. deviation between both main chains is 5.38 Å DHFR coordinate is taken from Protein Data Bank (entry name is 8dfr.). Arrows indicate Pro-61 of DHFR, which is Pro-72 on GDG-FR, and Gly-72 of GSHase (see in the text).

ed as inclusion bodies and have not folded in the native conditions. But refolding experiments on GSHase and GDG-FR suggested that i) each domain independently folds, ii) domain folding occurs in a transition state from 4M to 2M urea, iii) residue substitutions on narrow region, 11 of 22 residues, between GSHase and GDG-FR failed the holding of the N-terminal domain.

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