PROTEIN FOLDING AND ITS CATALYSTS IN THE CELL

細胞内における蛋白質の高次構造形成を促進する酵素に関する研究

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(1)

PROTEIN FOLDING AND ITS CATALYSTS IN THE CELL

Structural and Functional Analyses of Peptidyl-Prolyl *cis-trans* Isomerase and Protein Disulfide Isomerase

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CONTENTS

ABBREVIATIONS

Å	: angstrom
Abs	: absorbance
ADH	: alcohol dehydrogenase
AMP	: adenosine-2'-monophosphoric acid
AP-1	: activator protein-1
ARRE-1	: antigen receptor response element-1
ARRE-2	: antigen receptor response element-2
bp	: base pair(s)
CsA	: cyclosporin A
CyP	: cyclophilin
Da	: Dalton
DAG	: diacylglycerol
DNA	: deoxyribonucleic acid
E. coli	: *Escherichia coli*
ER	: endoplasmic reticulum
FKBP	: FK506-binding protein
GAP	: glyceraldehyde 3-phosphate dehydrogenase
GSH	: glutathione, reduced form
GSSG	: glutathione, oxidized form
h	: hour(s)
HEPES	: N-(2-hydroxyethyl)-piperazine-N'-2-ethanesulfonic acid
h-lysozyme	: human lysozyme
h-PDI	: human protein disulfide isomerase
i.d.	: in diameter

IL-1	: interleukin-1
IL-2	: interleukin-2
IP3	: inositol 1,4,5-triphosphate
kb	: kilo base pairs
MCA	: 4-methyl-coumaryl-7-amide
min	: minute(s)
NADPH	: β-nicotinamide adenine dinucleotide phosphate
NFAT-1	: nuclear factor of activated T-cells-1
NFIL-2A	: nuclear factor of IL-2 A
PAGE	: polyacrylamide gel electrophoresis
PCR	: polymerase chain reaction
PDI	: protein disulfide isomerase
PKC	: protein kinase C
PMA	: phorbol myristate acetate
PPIase	: peptidyl-prolyl *cis-trans* isomerase
RNA	: ribonucleic acid
s	: second(s)
SDS	: sodium dodecyl sulfate
SSC	: saline sodium citrate
TPA	: phorbol myristate acetate
TPOR	: thiol:protein disulfide oxidoreductase
TRE	: TPA or PMA response element
y-PDI	: yeast protein disulfide isomerase

One- and three-letter amino acid abbreviations

A=Ala	: alanine
C=Cys	: cysteine

D=Asp : aspartic acid
E=Glu : glutamic acid
F=Phe : phenylalanine
G=Gly : glycine
H=His : histidine
I=Ile : isoleucine
K=Lys : lysine
L=Leu : leucine
M=Met : methionine
N=Asn : asparagine
P=Pro : proline
Q=Gln : glutamine
R=Arg : arginine
S=Ser : serine
T=Thr : threonine
V=Val : valine
W=Trp : tryptophan
Y=Tyr : tyrosine

Nucleotide one-letter abbreviations

A : adenine
C : cytosine
G : guanine
T : thymine

CHAPTER 1

BACKGROUND

Since Anfinsen and his colleague succeeded in demonstrating the spontaneous renaturation of RNase A (1), it has been believed that the amino-acid sequence dictates conformation of the protein, i. e., the three-dimensional structure of the protein is the one in which the Gibbs free energy of the whole system is a minimum with respect to all degrees of freedom and the spontaneous folding of the protein is driven without the help of additional factors (catalysts) or the input of energy. The observations that the folding of proteins *in vivo* is generally faster and more efficient than that *in vitro* have led to our advanced understanding in protein folding. While the principle that the three-dimensional structure of a protein is determined by its amino-acid sequence still holds, a different concept of protein folding is emerging through studies on physical biochemistry and cell biology. Detailed analyses of the refolding pathway *in vitro* by trapping the intermediates, and of protein folding and assembly in the cell in relation to protein targeting and secretion have led to the idea that there is a machinery which facilitates protein folding in the cell. This machinery consists of two factors: (i) "foldase" which accelerates the rate-limiting steps in the protein folding pathway; (ii) "molecular chaperone" (2) which prevents nascent polypeptides from aggregation and incorrect folding.

The kinetic analysis of protein refolding *in vitro* has shown the existence of two rate-limiting steps in the protein folding pathway, i. e., isomerization of disulfide bonds and proline imide bonds. In the process of protein folding, formation of secondary structures such as the α-helix and the β-sheet occurs with half-times in microseconds,

whereas the disulfide isomerization and prolyl isomerization require half-times in the range of minutes to hours. How can the existence of these well-defined slow chemical steps in protein refolding *in vitro* be reconciled with the rate of appearance of functional activity during protein synthesis *in vivo*? Thus the search for enzymic catalysts of these isomerization reactions was challenged, leading to the discovery of foldases, protein disulfide isomerase and peptidyl-prolyl *cis-trans* isomerase. Evidence that molecular chaperones actually promote protein folding in the cell has been available (3), however, biological significance of foldases remains obscure. In order to study the mode of action of foldases during protein folding in the cell, I have focused on the structural and functional analyses of peptidyl-prolyl *cis-trans* isomerase (**CHAPTER 2**) and on the biochemical and genetical analyses of protein disulfide isomerase (**CHAPTER 3**).

CHAPTER 2

Structural and Functional Analyses of Peptidyl-prolyl *cis-trans* isomerase

2.1. Structural Analysis of Pig Kidney Peptidyl-Prolyl *cis-trans* Isomerase: Peptidyl-Prolyl cis-trans Isomerase Is the Cyclosporin A-Binding Protein, Cyclophilin (4)

SUMMARY

Peptidyl-prolyl *cis-trans* isomerase (PPIase) catalyzes the *cis-trans* isomerization of proline imide bonds of oligopeptides and has been shown to accelerate the refolding of several proteins *in vitro* (5-8). Its activity has been detected in yeast, insects and *Escherichia coli* as well as mammals, and it is thought to be essential for protein folding during protein synthesis in the cell. I purified PPIase from pig kidney and found that its amino-acid sequence is identical to that reported for bovine cyclophilin, a protein known to bind the immunosuppressive drug, cyclosporin A (9). To investigate the functional relationship between PPIase and cyclophilin, I examined the effect of cyclosporin A on PPIase activity and found that it was inhibitory. Thus I propose that the peptidyl-prolyl *cis-trans* isomerizing activity of PPIase may be involved in events, such as those occurring early in T-cell activation, that are suppressed by cyclosporin A.

INTRODUCTION

Many proteins exhibit slow and complex refolding kinetics. From the observations that unfolded ribonuclease A (RNase A) consists of heterogeneous molecules, fast- (U_F) and slow- (U_S) species, in a slow equilibrium, and that the rate of refolding is strongly dependent on the number of proline residues in the protein, it was pointed out that the *cis-trans* isomerization of prolyl imide bonds is one of the rate-determining steps in the refolding of some proteins (10,11) (Fig. 1). In the case of RNase A, the fast- and slow-folding molecules, U_F and U_S, differ in the conformational state of prolyl imide bonds (*cis* and *trans*). The *cis-trans* isomerization is slow process with a high activation energy of approximately 20 kcal/mole and it was postulated that only the fraction (U_F) of the unfolded protein with the same set of isomers found in the native conformation can fold directly to the native protein and that the remaining populations (U_S) must first isomerize all of the prolyl imide bonds to the native isomeric forms before folding can begin. In the random-coil state of proteins, proline is the only amino acid that is known to exist in a *cis-trans* isomeric equilibrium mixture, in which the *cis* form amounts to 10-20 % on the average. In folded proteins, by contrast, each bond has a specific conformation and although *trans* conformers are predominant, there are a number of well-defined *cis* peptide bonds for proline residues (amounting to 5 % to 8 % of all peptide bonds to proline in the current database). The content of the *cis* form depends on the kinds of amino acid neighboring to prolines, the length of polypeptides, the environmental conditions (pH, temperature, and ionic strength) and so on.

It is now established that the *cis-trans* isomerization is rate-determining during folding of a variety of proteins (7, 10, 12-21) and is also responsible for the activation of already folded native proteins (22-28) (Table 1). Thus a search for potential enzyme catalysts of this reaction was challenged. Fischer *et al.* (5) first developed a simple assay system for the catalyst of prolyl isomerization using peptides of X-Ala-Pro-Phe-*p*-

nitroanilide as its substrates (the chymotrypsin-coupled enzymic assay). These molecules exist in an equilibrium between the *cis* and *trans* forms. The carboxy-terminal *p*-nitroanilide group is cleaved off by chymotrypsin only when the prolyl imide bond is exclusively in the *trans* form. Release of the chromogenic group is easily monitored by measuring the change in the absorbance with time. In the presence of the catalyst the rate of the *cis-trans* isomerization of the peptides is accelerated and the half-time for release of the chromogenic group is shortened. Using this system, Fischer *et al.* partially purified an enzyme (peptidyl-prolyl *cis-trans* isomerase; PPIase) from pig kidney, which is able to catalyze the prolyl isomerization (5). From the observation that PPIase is shown to be widely distributed among species including yeast, insects and *Escherichia coli* (*E. coli*) as well as mammals, it is thought to play a significant role in protein folding during protein synthesis in the cell.

In this study, I determined the amino-acid sequence of PPIase purified from pig kidney and found its identity with cyclosporin A-binding protein, cyclophilin. I discuss the involvement of PPIase in T-lymphocyte activation on the basis of the result that the PPIase activity was inhibited by cyclosporin A.

EXPERIMENTAL PROCEDURES

Purification of pig kidney PPIase.

The ammonium sulfate extract containing PPIase activity was prepared from 690 g of pig kidney cortex by the method of Fischer *et al.* (5). The extract was dialyzed against 10 mM Tris-HCl buffer, pH 8.0, containing 0.05 % NaN_3 and applied to a DEAE-Sephacel column (2.5 cm i.d. X 35 cm). PPIase activity was measured in a coupled assay with chymotrypsin by a modified method of Fischer *et al.* (5), in which the oligopeptide N-succinyl-Ala-Ala-Pro-Phe-4-methyl-coumaryl-7-amide (MCA) (Peptide Institute Inc.) was used as the substrate instead of N-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide (6). The eluate rich in PPIase activity passed through the column and was obtained as a single peak, which was concentrated by ammonium sulfate fractionation and separated on a Sephacryl S-200 column (2.5 cm i.d. X 90 cm) equilibrated with 0.1 M Tris-HCl buffer, pH 8.0, containing 0.15 M NaCl and 0.05 % NaN_3. A major PPIase activity was found in the last peak and this fraction was rechromatographed on a Sephadex G-75 column (2.5 cm i.d. X 90 cm). Finally, PPIase was purified by reversed-phase chromatography on a TSK-phenyl 5PW-RP (TOSO) column with a linear gradient of acetonitrile (0-70 %) containing 0.1 % trifluoroacetic acid, and eluted as two peaks at about 40 % of acetonitrile concentration. Although the specific activity of PPIase was decreased by one-fifth after the reversed-phase chromatography, it was associated with only the peaks of M_r = 18,000.

Amino-acid sequence analysis of purified pig kidney PPIase.

The amino-acid sequence analysis was carried out by automated Edman degradation using Applied Biosystems 477A protein sequencer equipped with a model 120A on-line PTH analyzer. The native protein (20-100 µg) was subjected to separate digestions with L-1-(tosylamido)-2-phenylethyl chloromethyl ketone-treated trypsin, and cyanogen

bromide. The digest were separated on an Aquapore RP-300 column or on a Spheri-5RP-18 column with the use of 130A separation system (Applied Biosystems). The columns were eluted at a flow rate of 200 µl/min with a linear gradient of acetonitrile (0-100 %) containing 0.1 % heptafluorobutyric acid for 45 min. The amino-terminal cyanogen bromide peptide was further digested with *Staphylococcus aureus* V8 protease. Amino-acid analysis was carried out by Beckman 6300 E amino-acid analyzer after hydrolysis with 6 M HCl for 24 h.

Computer analysis.

The computer-assisted homology analysis was carried out by Bion programs provided by IntelliGenetics, Inc.

Inhibition of PPIase activity by CsA.

Aliquots (2.0 ml) of 35 mM HEPES buffer containing 5 mM 2-mercaptoethanol, pH 7.8, were incubated at 25 °C for 10 min in the spectrophotometer cell (Spectrophotometer U-3210 with temperature controller SPR-7 and cell stirrer, Hitachi) before adding 50 µl of 1.68 mM N-succinyl-Ala-Ala-Pro-Phe-MCA in the same buffer. Samples were equilibrated for 1 min. The PPIase fraction (0.27 µg) was incubated with the substrate for 30 s and the reaction was started by adding 20 µl of 0.76 mM of chymotrypsin (Sigma). The effect of cyclosporin A (CsA) on PPIase activity was examined by mixing CsA with PPIase for 1 min before incubating with the substrate. CsA was kindly provided by Dr. Y. Yamakawa of the National Institute of Health, Japan and was dissolved in saline containing 7 % ethanol and 2.85 % Tween 80 before use (this solvent did not affect the PPIase activity measured in these experiments). The rate of isomerization at a given time was calculated by using a rate assay program provided for Spectrophotometer U-3210.

RESULTS

Purification and amino-acid sequence analysis of pig kidney PPIase.

I detected PPIase activity by the assay described in **EXPERIMENTAL PROCEDURES**, in a crude ammonium sulfate extract prepared from pig kidney (5). A representative profile obtained in the chymotrypsin-coupled assay of the PPIase activity is shown in Fig. 2. PPIase was purified further as described in Table 2, and after a final step of purification by reversed-phase column chromatography, was separated into two forms (*a* and *b*). These isoforms have an apparently identical relative molecular mass (M_r) of 18,000 and very similar amino-acid compositions (data not shown). Although the observed relative molecular mass is somewhat at variance with the value previously reported (M_r = 14,000) (7), I was not able to detect any other major component with the PPIase activity during the purification. Exposure to a high concentration of acetonitrile during the reversed-phase column chromatography decreased but did not eliminate the specific activity detected (Table 2).

The two isoforms *a* and *b* were present in a ratio of 53:47 and their specific PPIase activities were distinguishable. A comparison of their tryptic peptide maps indicated that the structural difference between the two isoforms resides in the amino-terminal peptides (data not shown). The first 30 amino-acid residues of form *a* were unambiguously determined by Edman degradation (Fig. 3) but the amino terminus of form *b* could not be sequenced using this method, possibly due to the modification.

Sequence analysis of form *a* reveals a total of 163 amino-acid residues and predicts a relative molecular mass of 17,735 in good agreement with the estimate by SDS/PAGE. Peptide mapping data indicates the existence of a disulfide bridge between Cys61 and Cys160, although I cannot exclude the possibility that this was formed *in vitro*, as I did not use anti-oxidant during the purification procedure. The remaining two cysteine residues, Cys51 and Cys114 appear to have free sulfhydryl groups.

PPIase is identical with cyclosporin A-binding protein, cyclophilin.

On searching for homologous sequences in the National Biomedical Research Foundation database, I found that the sequence of pig PPIase is identical to that of bovine cyclophilin. Cyclophilin is known to bind specifically to CsA, a cyclic undecapeptide of fungal origin, that is a potent immunosuppressant used to prevent rejection on transplantation of organs such as kidney, heart, bone marrow and liver (9, 29). Cyclophilin is a soluble cytosolic protein and is present in high concentrations (0.1-0.4 % of total cytosolic proteins) in many mammalian tissues (9, 29) and nearly all organisms (9, 29, 30). Two isoforms have been isolated for bovine cyclophilin and are expected to have very similar molecular structures (29).

The amino-acid sequence of pig PPIase (and bovine cyclophilin) is 96 % identical to the published sequences for rat (29) and human (31) cyclophilin, and about 60 % identical to the recently published sequence for *Neurospora crassa* (32), indicating a high degree of conservation across species. PPIase was suggested to be present on ribosomal particles (5) and it is interesting that cyclophilin was found to be localized in both the mitochondria and the cytosol of *Neurospora crassa* (32).

Inhibition of PPIase activity by cyclosporin A.

The CsA-binding activity of cyclophilin is sulfhydryl dependent (9) and the activity of PPIase is also affected by sulfhydryl blocking reagents (5), suggesting that CsA binding may modulate PPIase activity. I therefore examined the effect of CsA and found that it blocked PPIase activity (Fig. 4). The activity was almost completely inhibited in the presence of CsA at 3×10^{-7} M, a concentration that is consistent with the dissociation constant of CsA to cyclophilin (2×10^{-7} M) (9). These results, and the observation that both PPIase activity and the CsA-binding activity of cyclophilin are heat-sensitive at around 50 °C, and abolished by incubation with trypsin but not with chymotrypsin (5, 29), support, on functional grounds, the identity between PPIase and cyclophilin.

DISCUSSION

Despite of the significance of PPIase as a potential catalyst of protein folding in the cell, no structural information on PPIase had been available. Thus I purified pig kidney PPIase to homogeneity and determined its complete amino-acid sequence. Surprisingly, PPIase was found to be identical with cyclosporin A-binding protein, cyclophilin.

CsA is a lipid-soluble, cyclic undecapeptide produced by *Tolypocladium inflatum* (Fig. 5). Since it was first approved for clinical use in 1983, CsA has revolutionized organ transplantation through its widespread use in the prevention of graft rejection. CsA is immunosuppressive because it blocks T-cell activation or proliferation (33, 34) through inhibition of synthesis of interleukin-2 (IL-2), the main growth factor of T-cell. The IL-2 gene is silent in resting T-cells, but is activated upon antigen recognition by the T-cell receptor (TCR) and the subsequent signal transduction. CsA blocks this antigen-induced transcription of the IL-2 gene (Fig. 6) (35). Several trans-acting factors which bind to the enhancers of the IL-2 gene are affected by CsA, including NFIL-2A and NFAT-1, perhaps most dramatically, NFAT-1.

Cyclophilin has been shown to be responsible for the binding activity of the soluble fraction of T-cells to CsA and supposed to be involved in the signal transduction pathway described above, however, its function has remained obscure. Thus, I examined the effect of CsA on the PPIase activity to investigate functional relationships between the enzymic activity of PPIase and the binding activity of cyclophilin to CsA. The result that PPIase activity was completely inhibited by CsA suggests that PPIase is involved in the signal transduction pathway by means of its prolyl isomerizing activity.

Coupled with the knowledge on the signal transduction pathway described above, I propose a mechanism how CsA blocks T-cell activation via its inhibitory action on PPIase (Fig. 7) (35, 36). Based on the fact that NFAT-1 requires *de novo* protein

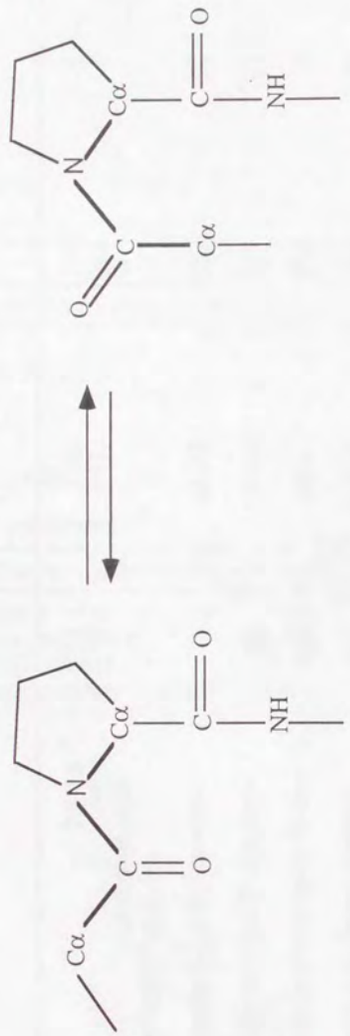
synthesis for its activation and that its binding to the enhancer of the IL-2 gene (ARRE-2) is inhibited by CsA, there are two possibilities to explain the mode of action of PPIase in the signal transduction pathway: (i) the newly synthesized protein after the stimulation is NFAT-1 and its nascent polypeptide chain requires the PPIase activity to fold correctly with the binding activity to ARRE-2 (Fig. 7A); (ii) the newly synthesized protein is PPIase which is specifically expressed in T-cell and it activates NFAT-1 which has already existed in the inactivated form (Fig. 7B). In the latter case, PPIase which is expressed ubiquitously must not catalyze the activation of NFAT-1. T-cell specific PPIase has not been identified so far, however, the fact that multiple cyclophilin-related DNA sequences are present in the mammalian genome (30, 31) supports this idea. I also propose that PPIase may act, not only as an enzyme that facilitates folding during protein synthesis, but as a fundamental modulator in various intracellular signal transduction processes, through *cis-trans* isomerization of the partner molecules. And it is interesting to know whether some endogenous factors with similar structures to that of CsA would be present in the cells and they would play important roles in various aspects by modulating the PPIase activity.

Table 1. Examples of PPIase-Catalyzed Processes (222)

Proteins in which *cis-trans* isomerization is rate-determining during folding	
RNase A	lactoglobulin
RNase A, S-fragment	chymotrypsinogen
Ig light chain	myokinase
cytochrome c	Barstar
RNase T_1	carbonic anhydrase II
collagen, type III	chymotrypsin inhibitor
pepsinogen	dihydrofolate reductase
tryptophan synthetase	
Proteins of which conformations are changed by *cis-trans* isomerization	
concanavalin A	calbindin D_{9K}
prothrombin fragment I	angiotensin II
superoxide dismutase	bradykinin
Staphylococcus aureus nuclease	corticotropin

Table 2. Purification of Pig Kidney PPIase

Purification step	Total protein (mg)	Total activity (arbitrary units)	Specific activity (arbitrary units/mg)	Activity recovery (%)
Ammonium sulfate fraction	7322	183050	25	100
DEAE-Sephacel chromatography	699	90222	129	49
Sephacryl S-200 gel filtration	67	63784	952	35
Sephadex G-75 gel filtration	6.7	37218	5555	20
TSK-phenyl 5PW-RP chromatography				
form *a*	1.4	1417	1012	1
form *b*	1.2	1368	1146	1



cis form

trans form

Fig. 1. *cis-trans* Isomerization of prolyl imide bonds.

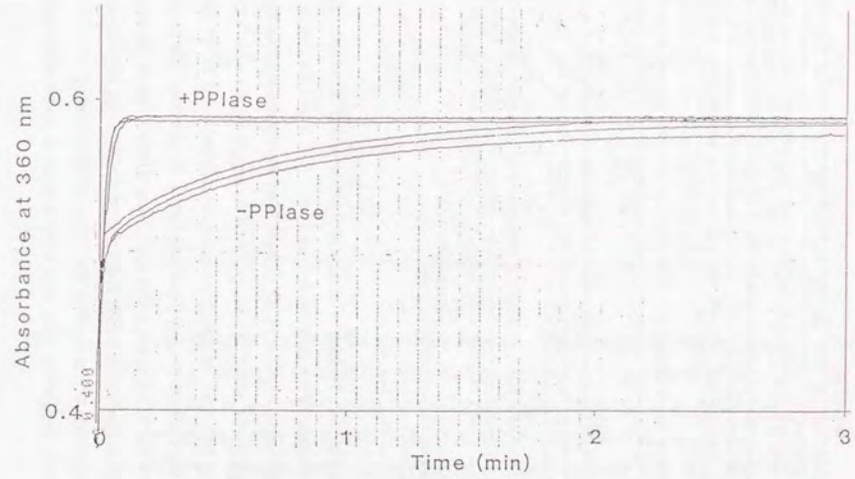


Fig. 2. A representative profile obtained in the chymotrypsin-coupled assay. The detailed assay condition is described in **EXPERIMENTAL PROCEDURES**. 5µl of the crude extract of pig kidney was used for the assay. Three independent results obtained with and without PPIase are shown, respectively.

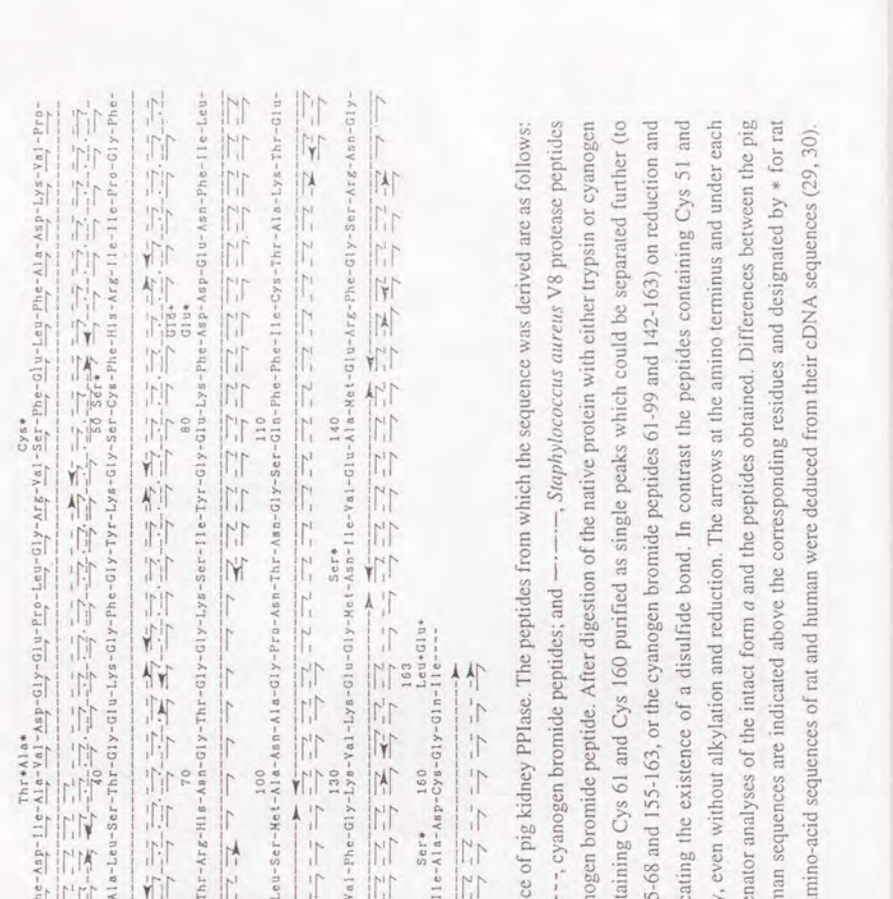


Fig. 3. Amino-acid sequence of pig kidney PPIase. The peptides from which the sequence was derived are as follows: – – –, tryptic peptides; - - -, cyanogen bromide peptides; and —·—·—, *Staphylococcus aureus* V8 protease peptides of the amino-terminal cyanogen bromide peptide. After digestion of the native protein with either trypsin or cyanogen bromide, the peptides containing Cys 61 and Cys 160 purified as single peaks which could be separated further (to give the tryptic peptides 55-68 and 155-163, or the cyanogen bromide peptides 61-99 and 142-163) on reduction and carboxymethylation, indicating the existence of a disulfide bond. In contrast the peptides containing Cys 51 and Cys114 purified separately, even without alkylation and reduction. The arrows at the amino terminus and under each peptide line indicate sequenator analyses of the intact form *a* and the peptides obtained. Differences between the pig and the rat, and/or the human sequences are indicated above the corresponding residues and designated by * for rat and by + for human. The amino-acid sequences of rat and human were deduced from their cDNA sequences (29, 30).

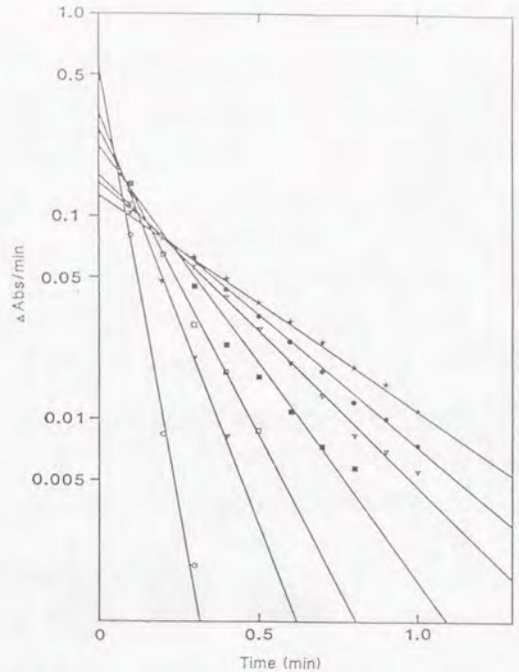
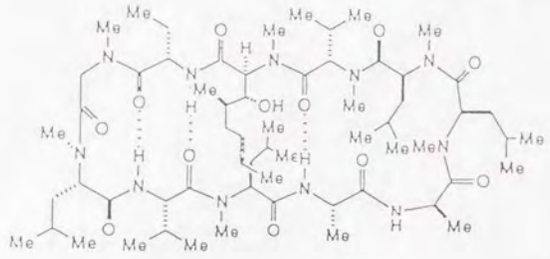
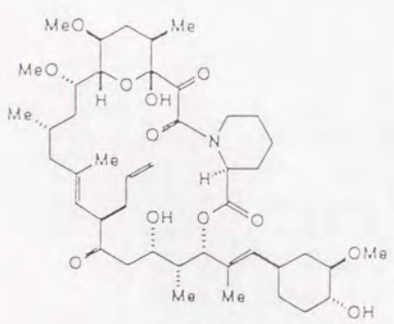


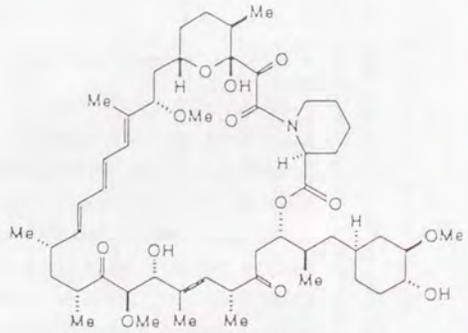
Fig. 4. Inhibition of PPIase activity by CsA. The kinetics of cis-trans isomerization of *N*-succinyl-Ala-Ala-Pro-Phe-MCA was monitored at 360 nm in a coupled assay with chymotryptic cleavage. The MCA moiety can only be cleaved off by chymotrypsin when the preceding Ala-Pro peptide bond is in the *trans* conformation (5). At the equilibrium about 12 % of the peptide has a *cis* Ala-Pro bond. In the presence of a high chymotrypsin concentration, hydrolysis of the trans peptide occurs within a few seconds, but cleavage of the *cis* peptide is limited in rate by the *cis* -> *trans* isomerization of the Ala-Pro bond. The trans peptide was cleaved within the deadtime and the kinetic data of this phase is not shown. The rate of *cis* -> *trans* isomerization of *N*-succinyl-Ala-Ala-Pro-Phe-MCA, represented as the absorbance change at a given time (ΔAbs/min) is plotted against the proceeding times (min) in semi-logarithmic plots. CsA clearly affected the rate of the isomerization catalyzed by PPIase: ○, without CsA; ▾, 0.04 μg; □, 0.08 μg; ■, 0.16 μg; ▿, 0.4 μg; ●, 1 μg and ★, without PPIase.



Cyclosporin A (CsA)



FK506



Rapamycin

Fig. 5. Structures of immunosuppressive drugs, cyclosporin A (CsA), FK506 and rapamycin.

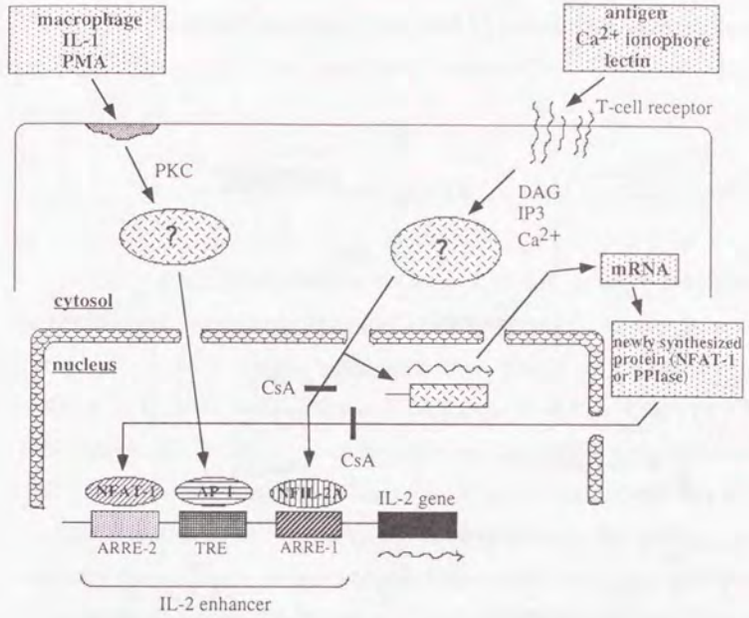


Fig. 6. A model of the pathways involved in the activation of the IL-2 gene in human T-cell. There are two possible independent signal transduction pathways. One is stimulated by macrophage, IL-1, or PMA and mediated by PKC, leading to the activation of AP-1, a nuclear factor, which binds to the TRE region in the IL-2 enhancer. The other is stimulated by antigens, Ca^{2+} ionophores, or lectins and mediated by DAG, IP3 and Ca^{2+}, resulting in the activation of NFAT-1, a transcription factor, which binds to ARRE-2. The activation of NFAT-1 requires *de novo* protein synthesis and is inhibited by CsA. The proposed sites of action of CsA are shown. Based on the results described in this study, the action of PPIase is suggested to be involved in these sites. See text and Fig. 7 for further details. IL-1, interleukin 1; IL-2, interleukin 2; PMA, phorbol myristate acetate; PKC, protein kinase C; DAG, diacylglycerol; IP3, inositol 1,4,5-trisphosphate; NFAT-1, nuclear factor of activated T-cells-1; AP-1, activator protein-1; NFIL-2A, nuclear factor of IL-2 A; ARRE-1, antigen receptor response element-2; ARRE-2, antigen receptor response element-2; TRE, TPA or PMA response element.

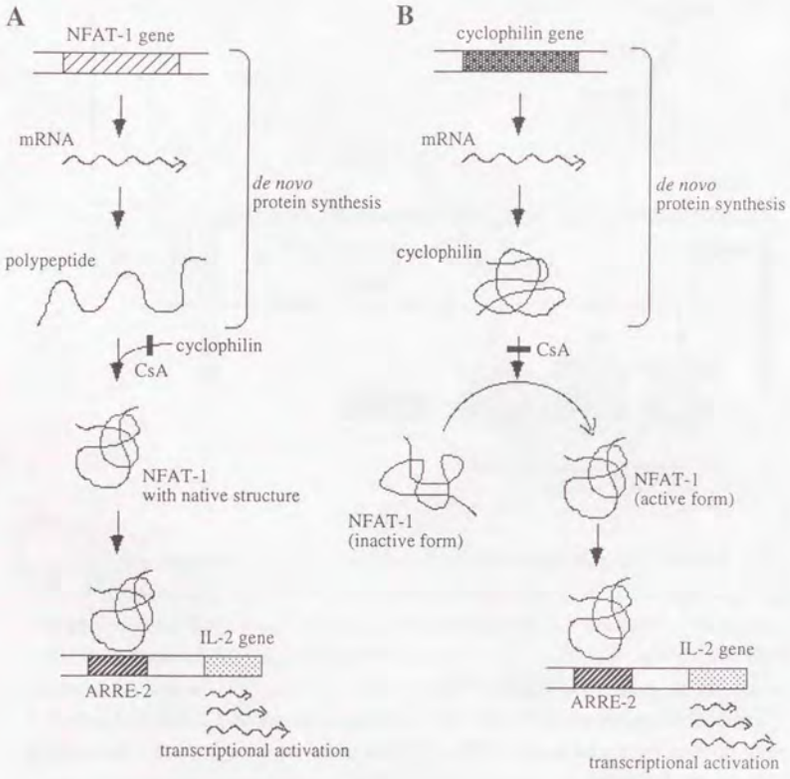


Fig. 7. The possible mode of the action of cyclophilin (PPIase) on the transcriptional activation of the IL-2 gene. Thick bars indicate the inhibition sites of CsA. See text for further details.

2.2. Two Distinct Forms of Peptidyl-Prolyl *cis-trans* Isomerase Are Expressed Separately in Periplasmic and Cytoplasmic Compartments of *Escherichia coli* Cells (37)

SUMMARY

PPIase is thought to be essential for protein folding in the cell. Two forms, *a* and *b*, of PPIase and their corresponding genes were isolated from *E. coli* cells. Despite their insensitivity to CsA, both amino-acid sequences were homologous and related to that of cyclophilin, a protein that has PPIase activity sensitive to CsA (**CHAPTER 2.1**). Although the cysteine residue was suggested to be responsible for PPIase activity, no cysteine residue was found in the sequence of *E. coli* PPIase *a*. In addition the two cysteine mutants of *E. coli* PPIase *b* were shown to retain the full catalytic activity, indicating that the cysteine residues in *E. coli* PPIase *a* and *b* play no essential role in its catalysis. PPIase *a* is found to be identical with the *E. coli* ORF 190 gene product that was sequenced by Kawamukai *et al.* (38) and overexpressed by Liu and Walsh (39). It is translocated into *E. coli* periplasmic space by means of the signal sequence. PPIase *b* lacks a signal sequence or a transmembrane domain and is detected mainly in the bacterial cytoplasm. These findings indicate that proteins with the ability to assist protein folding of various polypeptides are located on both sides of the bacterial inner membrane. Thus, I propose that the folding of some exported proteins may be catalyzed by the periplasmic PPIase and, in turn, that some proteins which have been isomerized may not be translocated efficiently.

INTRODUCTION

PPIase catalyzes the *cis-trans* isomerization of Xaa-Pro peptide bonds in oligopeptides and accelerates the slow, rate-limiting steps in the refolding of several proteins *in vitro* (7, 8, 12); it is thought to be essential for protein folding during protein synthesis in the cell (5). The action of PPIase, however, appears to be restricted to specific proteins *in vitro* (8, 12). As described in **CHAPTER 2.1**, PPIase is identical with cyclophilin, a protein known to bind the immunosuppressive drug CsA, and its activity is inhibited by CsA. These findings lead to the hypothesis that the effect of CsA is mediated through inhibition of the peptidyl-prolyl *cis-trans* isomerizing activity of PPIase in T-cell activation. After my finding, a cellular binding protein for FK506 (Fig. 5 in **CHAPTER 2.1**), an immunosuppressant remarkably similar to CsA in the effect on T-cell activation, was also found to have the same enzymic activity as cyclophilin (40, 41). However, cyclophilin and FK506 binding protein (FKBP) are quite distinct in terms of ligand specificity: cyclophilin binds to and is inhibited by CsA but shows no binding of FK506, whereas the converse holds for FKBP (40, 41). These two PPIases are unrelated to each other in their entire amino-acid sequences, indicating that PPIases are classified into at least two different superfamilies, cyclophilin and FKBP superfamilies (42). On the basis of earlier data, which indicated that CsA and FK506 act on T-lymphocytes in an essentially equivalent fashion (43), these findings indicate that the two drugs act through distinct pathways but that their mode of action converges on PPIase activities (44), and suggest that PPIase activity is essential for T-cell activation.

The CsA-binding and PPIase activities are present in nearly all organs as well as in species (8, 45). In *Neurospora crassa* and *Saccharomyces cerevisiae*, cyclophilin mediates the cytotoxic effect of CsA (46). This finding, coupled with the result that the PPIase activity of cyclophilin is inhibited by CsA, suggests that cytotoxic effects of CsA on many organisms of widely diverging phylogenic origin are also mediated through

inhibition of the PPIase activity. However, it is not yet known whether or not the mechanism of CsA action in these organisms is similar to that proposed for T-cell activation, e.g., that CsA inhibits the appearance of DNA binding and/or transcriptional activities of nuclear proteins in T-cells. In any case, the presence of cyclophilin-like PPIase activity across many species and the widespread effects of CsA, in conjugation with its inhibitory effect on PPIase, suggest that the control of the prolyl isomerization may be a ubiquitous regulatory mechanism in some cellular events, such as those in cell growth and differentiation.

On the basis of the probable participation of a cyclophilin-like protein in the formation of rhodopsin in the photoreceptor cell of *Drosophila*, it was proposed that the PPIase activity is necessary for the posttranslational folding of rhodopsin (47, 48). In this case, however, the existence of a cell-specific isoform was postulated to explain the specific action of cyclophilin-like PPIase on the formation of rhodopsin in the photoreceptor cells. In fact, a number of cyclophilin-related DNA sequences have also been detected in mammalian genomes (30, 31), suggesting the presence of a large family of cyclophilin-like PPIases encoded by multiple genes, and the functional diversity of cyclophilin-like PPIase within a single species. In this chapter, I present the existence of two distinct forms of cyclophilin-like PPIase encoded by two separate genes and their possible function in different cellular compartments of the *E. coli* cell.

EXPERIMENTAL PROCEDURES

Purification of E. coli PPIases a and b.

Two hundred grams of *E. coli* ST249 cells (50) was treated with 2 g of lysozyme in 500 ml of 0.1M Tris-HCl buffer, pH 7.8, containing 5 mM 2-mercaptoethanol for 30 min. The soluble fraction was concentrated with ammonium sulfate fractionation and dialyzed against 10 mM Tris-HCl buffer, pH 7.8, containing NaN_3. The dialyzate was applied on a DEAE-Sepharose CL-6B column (2.5 cm i.d. X 40 cm) and eluted by stepwise increasing NaCl concentrations: 0, 0.05, 0.1, 0.2, 0.3, and 0.5 M. The PPIase activity was detected in the eluents with 0 and 0.1 M NaCl. The two fractions with PPIase activity were collected separately, and each of the collections was applied on a Sephadex G-50 column (2.5 cm i.d. X 90 cm) equilibrated with 10 mM Tris-HCl buffer, pH 8.0, containing 0.15 M NaCl and 0.05 % NaN_3. Form *b* was purified as a single band on SDS/PAGE at this step. Form *a* was further purified on a CM-Sepharose CL-6B column (1.5 cm i.d. X 20 cm) with linear gradient elution (from 0 to 0.25 M NaCl/10 mM sodium acetate buffer, pH 6.0). Electrofocusing was done with Ampholine, PAGPLATE, pH 3.5-9.5, by a Multiphor II electrophoresis apparatus (Pharmacia LKB Biotechnology). SDS/PAGE was done by a method modified from that of Laemmli with a gradient gel from 12 % to 30 % (51, 52).

Assay of PPIase activity and effects of CsA and FK506 on the activity.

The *cis-trans* isomerization of the Ala-Pro peptide bond in the synthetic peptide N-succinyl-Ala-Ala-Pro-Phe-MCA was measured in a coupled assay with chymotrypsin based on the ability of this peptide only when the Ala-Pro is in *trans* configuration. The synthetic peptide (50 µl of a 1.68 mM solution) was preincubated with the appropriate concentrations of PPIase and CsA in 2 ml of 35 mM HEPES buffer, pH 7.8, containing 5 mM 2-mercaptoethanol and the assay was started by mechanical mixing with 20 µl of 0.76 mM chymotrypsin (Sigma) in a spectrophotometer cell (Spectrophotometer U-3210

with a temperature controller SPR-7 and a stirrer, Hitachi) at 25 °C. The *trans* peptide was cleaved by the deadline; hydrolysis of the MCA in the *cis* peptide is limited in rate by *cis-trans* isomerization at Ala-Pro and was monitored by the increase in absorbance at 360 nm. The inhibitory effects of CsA and FK506 on PPIase activity were measured by determining the residual activity of PPIase preincubated in the presence of various concentrations of CsA and FK506, respectively.

Protein sequencing analysis.

The amino-acid sequence analysis was carried out by automated Edman degradation using an Applied Biosystems 477A protein sequencer equipped with a Model 120A on-line PTH analyzer. The native protein (50-200 µg) was subjected to separate digestions with L-1-(tosylamido)-2-phenylethyl chloromethyl ketone-treated trypsin and cyanogen bromide. The digests were separated on an Aquapore RP-300 column or on a Spheri-5RP-18 column using a 130-A separation system (Applied Biosystems). The peptides were eluted from the columns at a flow rate of 200 µl/min with a linear gradient of acetonitrile (0-100%) containing 0.1 % trifluoroacetic acid or 0.1 % heptafluorobutyric acid for 45 min. The amino-acid analysis was carried out with a JOEL JLC-300 amino-acid analyzer (Nihon Denshi) after hydrolysis with 6 M HCl for 24 h.

Construction and screening of the genomic library.

To detect genomic DNA fragment carrying the *E. coli* PPIase *b* gene, the high molecular weight DNA isolated from *E. coli* HB101 (53) was digested with various restriction endonucleases and was subjected to Southern blot analysis (54) with the radiolabeled mixed oligonucleotide probes 5'-ATGAA(A/G)CA(A/G)AA(A/G)GCNACCAAAGAA-CC-3', corresponding to the amino-acid sequence Met-Lys-Gln-Lys-Ala-Thr-Lys-Glu-Pro which was obtained from protein sequencing of PPIase *b*. A genomic DNA fragment of about 1 kb generated by *Hin*dIII/*Bgl*II digestion was hybridized to the probes. The

*Hin*dIII/*Bgl*II fragments, of which sizes were roughly 1 kb, were fractionated by 0.8 % agarose gel electrophoresis and subcloned into the *Hin*dIII/*Bam*HI site of pUC118 (TAKARA SHUZO). *E. coli* JM109 cells were transformed with the plasmid library, and the transformants were screened for the *E. coli* PPIase *b* gene with the probes described above. The hybridization was performed overnight at 37 °C in 50 mM Tris-HCl buffer, pH 7.5, containing 1 M NaCl, 10 mM EDTA, 0.1 % sodium dodecyl sarcosinate, 0.1 % polyvinylpyrrolidone, 0.1 % Ficoll 400, 0.1 % bovine serum albumin, and 20 μg/ml sheared salmon sperm DNA. The filters were washed with 3 X SSC (1 X SSC: 0.15 M NaCl/0.015 M sodium citrate, pH 7.0) and 0.1 % sodium dodecyl sarcosinate at 37 °C for 30 min. Autoradiography was carried out at -70 °C with an intensifying screen. One out of approximately 1500 recombinant clones, designated as pEPPIb, gave a positive hybridization signal. To isolate a genomic DNA fragment coding for the PPIase *a* gene, a genomic library for *E. coli* strain HB101 (53) was constructed as follows. The high molecular weight DNA isolated from *E. coli* HB101 cells was partially digested with *Sau*3AI, followed by fractionation through a sucrose density gradient (55). The 10-20 kb genomic DNA fragments were recovered and ligated to EMBL4 *Bam*HI arms (STRATAGENE). After *in vitro* packaging *E. coli* strain LE392 was transfected with the EMBL4 library, and the transfectants were screened for the PPIase *a* gene inserts with radiolabeled mixed oligonucleotide probes 5'-GAACA(A/G)ATGCA(A/G)CA(A/G)-AA(A/G)AAACC-3', corresponding to the amino-acid sequence Glu-Gln-Met-Gln-Gln-Lys-Lys-Pro, obtained from protein sequencing of PPIase *a*. The hybridization was performed as in the case of the screening for the PPIase *b* gene described above. Of approximately 10^4 phages screened, five clones gave positive signals after subtraction using the PPIase *b* gene as a probe. The restriction endonuclease analysis showed that these five clones overlapped with each other; one of them, designated as λEPPIa, was analyzed further. A fragment of approximately 2.5 kb size was generated by *Ava*I digestion of λEPPIa DNA which hybridized to the oligonucleotide probes used for the

screening described above. It was subcloned into the *Sma*I site of pUC119 after filling its protruding ends with Klenow fragment. The obtained plasmid was designated as pEPPIa.

DNA sequencing analysis.

The entire insert DNA for pEPPIb and the restricted DNA insert containing the PPIase *a* gene for pEPPIa were sequenced after subcloning into M13mp18, M13mp19, pUC118, or pUC119 (TAKARA SHUZO). Single-strand templates were prepared according to standard procedures, and dideoxy sequencing analyses (56) were performed with the 7-deaza sequencing kit (TAKARA SHUZO or TOYOBO), using the universal primer except for the oligonucleotide 5'-ATAAAGATACGGATCGCGCCCTTCGCCGAA-3' used for the sequencing of a part of the PPIase *a* gene.

Computer analysis.

The computer-assisted homology analysis compared the *E. coli* PPIase genes and proteins to the data in the NBRF, GenBank, and EMBL databases accessed through DDBJ (DNA Data Bank of Japan, National Institute of Genetics Information Analysis, Mishima, Shizuoka, Japan). SEQFP and SEQHP programs for the homology search (57) and the HPLOT program for hydropathy calculation (58) were provided by DDJB.

Southern blot analysis for E. coli total DNA.

High molecular weight DNA (2 μg) isolated from *E. coli* W3110 cells (53) was digested with *Eco*RV, *Pst*I, or *Pvu*II (TAKARA SHUZO or NIPPON GENE). The digests were subjected to 0.8 % agarose gel electrophoresis and transferred to nylon membranes (Hybond N, Amersham) as described (54). The filters were probed with the *Pvu*I/*Eco*RI DNA fragment of pEPPIb or the *Ava*II DNA fragment containing the *E. coli* PPIase *a* gene, which were radiolabeled with the random primer DNA labeling kit (TAKARA SHUZO). The hybridization was carried out overnight at 68 °C in 6 X SSC containing 0.5

% SDS, 0.2 % polyvinylpyrrolidone, 0.2 % Ficoll 400, 0.2 % bovine serum albumin, and 20 μg/ml of sheared salmon sperm DNA. Following hybridization, the filters were washed with 0.1 X SSC containing 0.1 % SDS at 68 °C for 30 min. Autoradiography was performed at -70 °C using an intensifying screen for the appropriate times.

Separation of periplasmic and cytoplasmic proteins.

Ten grams of *E. coli* cells (wet weight) was suspended in 80 ml of 20 % sucrose/10 mM Tris-HCl buffer, pH 7.5, and incubated with the addition of 5.4 ml of 0.25 M EDTA solution, pH 7.5, at 4 °C for 15 min. *E. coli* cells were harvested by centrifugation, and the periplasmic fraction was obtained by the osmotic shock method, in which the cells were resuspended in 120 ml of cold water by vortexing. After centrifugation, the precipitate was suspended in 50 ml of 0.1 M Tris-HCl buffer, pH 7.5, containing 5 mM 2-mercaptoethanol and treated with 11.8 mg of lysozyme at 30 °C for 1 h. The supernatant contained the cytoplasmic fraction. Protein determination was done by using the Bio-Rad protein assay kit. β-Galactosidase activity was measured by the method described by Maniatis *et al.* (55).

The periplasmic and cytoplasmic fractions were separated on a TSK-gel DEAE-5PW column (7.5 mm i.d. X 7.5 cm) (TOSO) equilibrated with 10 mM Tris-HCl buffer, pH 8.0, containing NaN_3. Proteins were eluted at a flow rate of 1 ml/min with a liner gradient of NaCl (0-0.25M) during 60 min after the elution of the equilibration buffer for 10 min, and the eluent was collected at an interval of 1 min. Some of the major peaks obtained were purified further by reverse-phase chromatography on an Aquapore RP-300 column (2 mm i.d. X 3 cm) (Applied Biosystems) to identify the proteins eluted and to compare their levels in the cytoplasmic and periplasmic fractions. The column was eluted at a flow rate of 200 μl/min with a linear gradient of acetonitrile (0-100 %) containing 0.1 % trifluoroacetic acid for 45 min. Identification of the purified proteins was done by computer search on the databases described above after determination of their amino-

terminal sequences. The protein content was compared by calculating the total absorbance of the corresponding protein peaks originating from the cytoplasmic and periplasmic fractions.

Construction of the expression plasmid of E. coli PPIase b.

The isolation of the *Hin*dII/*Bgl*II genomic DNA fragment containing the entire gene coding for cytoplasmic PPIase was described above. The plasmid pEPPIb was digested with *Pvu*I which cleaves at the site of 78 bases upstream from the initiation codon of the cytoplasmic PPIase gene, and the generated protruding end was planed with mung bean nuclease (TAKARA SHUZO). After *Eco*RI digestion of the DNA, the resulting fragment was subcloned into the *Eco*RV/*Eco*RI site of Bluescript II SK+ (TOYOBO). This plasmid DNA, designated as pBLEPPIb, was digested with *Sal*I, filled up the protruding end with thio-dNTP and Klenow fragment for the protection of the flanking *Xho*I site from the following exonuclease treatment, and was digested with *Hin*dIII. After limited exonuclease III digestion and the following mung bean nuclease treatment, the linealized DNA was self-ligated. The extent of deletion was examined by the DNA sequencing analysis (56). One of the clones, designated as pBLΔEPPIb, in which the nucleotides upstream the initiation site were properly deleted, was selected for further construction. The plasmid pBLΔEPPIb was digested with *Xho*I, followed by Klenow fragment treatment, and the DNA fragment containing the PPIase *b* coding sequence was excised from the vector by *Bam*HI digestion and cloned into the *Cla*I/*Bam*HI site, in which the former protruding end was filled up with Klenow fragment, just downstream the *trpE* promoter in the plasmid pATtrp. The obtained plasmid, designated as pATtrpEPPLb, was used for expression of PPIase *b*.

Expression and purification of recombinant E. coli PPIase b.

E. coli strain HB101 was transformed with the expression plasmid pATtrpEPPIb. Four liters of M9CA medium (0.05 % NaCl, 0.6 % Na_2HPO_4, 0.3 % KH_2PO_4, 0.1 % NH_4Cl, 0.2 % casamino acid, 0.2 % glucose, 2 mM $MgSO_4$, 0.1 mM $CaCl_2$, pH 7.4) containing 50 μg/ml of ampicillin, was inoculated with 40 ml of overnight culture of the plasmid-harboring cells. During cultivation, 3-indolyl acrylic acid was added to a final concentration of 50 mg/ml when the absorbance of the culture at 600 nm reached approximately 0.3, followed by further incubation at 37 °C for 24 h. The cells were harvested by centrifugation and the bacterial pellet (11.8 g by wet weight) was suspended in 50 ml of 0.1 M Tris-HCl buffer, pH 7.5, containing 5 mM 2-mercaptoethanol and 11.8 mg of lysozyme. After incubation at 30 °C for 1 h, followed by sonication, the soluble fraction was obtained by centrifugation (18,000 rpm, 30 min). The supernatant was dialyzed against 10 mM Tris-HCl buffer (pH 8.0) containing 0.05 % NaN_3. The dialyzate was loaded onto a DEAE-Sepharose CL-6B column (2.5 cm i.d. X 40 cm), and was eluted at a flow rate of 30 ml/h with an increasing NaCl concentration in a manner of stepwise elution; 0, 0.05, 0.1, 0.2, 0.3, and 0.5M. The PPIase activity was measured in a coupled assay with chymotrypsin as described above. The fractions rich in PPIase activity were collected, concentrated by ammonium sulfate fractionation, and purified on a Sephadex G-75 column (2.5 cm i.d. X 90 cm) equilibrated with 0.15 M NaCl/10 mM Tris-HCl buffer (pH 8.0) containing 0.05 % NaN_3. The column was eluted at a flow rate of 10 ml/h. Protein concentration was determined by the protein assay kit (Bio-Rad).

Construction of the expression plasmids for mutant PPIase b proteins.

The plasmid pBLΔEPPIb was digested with *Xho*I and *Bam*HI. The DNA insert containing the PPIase *b* gene was subcloned into the *Sal*I/*Bam*HI site of pUC119. Starting with this plasmid, the expression plasmids for two PPIase *b* mutant proteins were constructed by the site-directed mutagenesis technique described by Kramer et al. (59), using the site-

directed mutagenesis kit Mutan G (TAKARA SHUZO). The oligonucleotides used for the mutagenesis were as follows: 5'-ACCTTCGCGGGAGTAGTCCAG-3' for substitution of Cys31 to Ser and 5'-AGCAAACACGAAGTAGCCCCA-3' for Cys121 to Phe. These amino-acid substitutions were designed with minimal changes in the predicted secondary structures, referred to Chou and Fasman algorism (60). The mutations were checked by DNA sequencing analyses. The resulting plasmids were digested with *Pst*I, treated with mung bean nuclease, and followed by *Bam*HI digestion. Each excised DNA fragment coding for the PPIase *b* gene with a single amino-acid change was subcloned into the *Cla*I/*Bam*HI site, in which the former protruding end was filled up with Klenow fragment, of the plasmid, pATtrp. *E. coli strain* HB101 was transformed with these expression plasmids and transformants obtained were subjected to expression of the mutant PPIase *b* proteins. The mutant proteins were purified in the same manner as described for purification of the wild-type PPIase *b* protein.

RESULTS

Two distinct forms of PPIase are present in E. coli cells.

Two peaks of PPIase activity were detected in the chromatogram of the extract of *E. coli* ST249 cells on a DEAE-Sepharose CL-6B column; one passed through the column, and the other was eluted with 0.1 M NaCl. They were separately purified further as described under **EXPERIMENTAL PROCEDURES**. Each of the two peaks contained a single component of PPIase. The two PPIases obtained were designated as *a* and *b* which have apparently different isoelectric points of 9.7 and 5.0, and molecular weights of 21,000 and 20,000, respectively (Fig. 8A and B). PPIases *a* and *b* were present in a ratio of about 1:9, and their specific PPIase activities were almost equal when the synthetic peptide was used for the substrate in the enzyme assay (*a*, 56,600 units/mg; *b*, 51,500 units/mg). These values were very close to that of pig kidney PPIase (57,700 units/mg) (**CHAPTER 2.1**). A comparison of their tryptic peptide maps indicated that they are apparently different molecular species (data not shown). This is in contrast to the case of the isoforms of pig PPIase reported in **CHAPTER 2.1**; the structural difference between the pig isoforms is probably the result of incomplete posttranslational modification at the amino terminus of the PPIase molecule. The amino-terminal sequence analyses of the two PPIases gave entirely different sequences for the first 18 amino-acid residues, i.e., Ala-Lys-Gly-Asp-Pro-His-Val-Leu-Leu-Thr-Thr-Ser-Ala-Gly-Asn-Ile-Glu-Leu for *a* and Met-Val-Thr-Phe-His-Thr-Asn-His-Gly-Asp-Ile-Val-Ile-Lys-Thr-Phe-Asp-Asp for *b*. These results, in combination with those of the peptide mapping, indicate that at least two distinct PPIases are present in *E. coli* cells.

E. coli PPIases are insensitive to both CsA and FK506.

The sensitivity of two forms of PPIase to immunosuppressants, CsA and FK506, was examined to investigate their relationships to known type of PPIases, CsA- and FK506-

sensitive PPIases. However, the activity of neither of the PPIases was inhibited by CsA in the same concentration range as required for the inhibition of pig PPIase (**CHAPTER 2.1**, 49), nor was either sensitive to FK506 (Fig. 9). The result for CsA is consistent with my observation that the growth of *E. coli* cells was not affected by the presence of CsA in the culture medium, and with the fact that no CsA binding activity was detected in *E. coli* extracts despite its ubiquitous presence in all eukaryotes so far analyzed (5, 45).

Cloning of the two PPIase genes.

Genomic libraries were constructed from the high molecular weight DNA isolated from *E. coli* HB101 cells. The libraries were screened with the synthetic oligonucleotide probes designed on the basis of the amino-acid sequences obtained by direct protein sequencing of the purified PPIases. Five positive clones for the PPIase *a* gene and one positive clone, designated as pEPPIb, for the *b* gene were isolated. The five clones for the *a* gene overlapped each other, and one of them, designated as λEPPIa, was further analyzed. Southern blot and restriction mapping analyses of the λEPPIa resulted in the finding of an approximately 2.5-kb *Ava*I DNA fragment containing the PPIase *a* gene. This fragment was then subcloned into pUC119 (pEPPIa) and sequenced. The insert of pEPPIa contained an entire open reading frame encoding a polypeptide of 190 amino acids with a molecular mass of 20,430 (Fig. 10A). On the other hand, sequencing of the insert of pEPPIb revealed that it contained an open reading frame encoding a polypeptide of 163 amino acids with a molecular weight of 18,184 (Fig. 10B).

The amino-acid sequences deduced from the nucleotide sequences indicated the presence of a single Met, an amino terminal to the amino-terminal sequence determined directly for purified PPIase *b*, and of a sequence of 24-residue amino terminal to that determined for purified PPIase *a*. The amino-acid sequences of the cyanogen bromide and tryptic peptides obtained from the purified PPIases gave no discrepancies in the amino-acid sequences deduced from the cloned DNA sequences for both PPIases except

two amino-acid residues in the sequence of form *a*, in which Ala and Asp were identified by direct peptide sequencing instead of Thr and Ser at positions 124 and 125, respectively (Fig. 10A). Furthermore, the amino-acid compositions and the molecular weight values calculated from the deduced amino-acid sequences were in good agreement with those determined by amino-acid analysis and SDS/PAGE for both PPIases isolated. These results indicate that the isolated DNA sequences represent the genes coding for *E. coli* PPIases *a* and *b*, respectively. Thus, the two *E. coli* PPIases are encoded separately by two different genes in the *E. coli* genome.

To investigate the genomic complexity of *E. coli* PPIase genes, Southern blot analysis was performed. *E. coli* genomic DNA was separately digested with three different restriction endonucleases and hybridized with the fragments of the *a* and *b* gene inserts as probes under highly stringent conditions (Fig. 11). Only one band for any of the restriction fragments was found to be hybridized with each of the two PPIase inserts, suggesting that only one copy of each of the two PPIase genes is present in *E. coli* genome.

Northern blot analysis showed that both *a* and *b* genes are transcribed in approximately 600-base RNA species (data not shown). Judging from the sizes of their coding regions, it can be concluded that both of the *E. coli* PPIase genes are transcribed in a monocistronic manner.

Two E. coli PPIases are related to cyclophilin in their amino-acid sequences.

Despite the insensitivity of *E. coli* PPIases to CsA, the amino-acid sequences of the known cyclophilns and *E. coli* PPIases are similar to one another. To search for the possible occurrences of other protein sequences homologous to the *E. coli* PPIases, computer-assisted homology analysis was done by comparing the *E. coli* PPIase genes and proteins to several databases. The amino-acid sequences of *E. coli* PPIases were confirmed to have homology with that of bovine cyclophilin as expected, and were

shown to have no sequence similarity to any other proteins and peptide segments in these databases used for the homology search. Despite the fact that the amino-acid and nucleotide sequences of cyclophilin from many species other than bovine have been reported, these data had not yet been included in the databases updated as of the end of 1989. During a search of the literature, *E. coli* PPIase *a* was found to be identical with the ORF190 gene that was sequenced by Kawamukai *et al.* (38). A comparison of the amino-acid sequences of *E. coli* PPIases and those of the other species indicated that the former, which are about 50 % identical with each other, have about 25 % homology with those of mammalian (**CHAPTER 2.1**, 29-31), fly (47), fungi (32), and yeast PPIases (61, 62), suggesting some conservation in the structures of PPIases from prokaryote to eukaryote (Fig. 12). Although the homology among all molecules is much lower than that observed among eukaryotic cyclophilin/PPIases, two highly homologous regions were found in the middle of the sequences across several species including *E. coli*; one is located around residues 40-70 and the other around residues 90-120 of the PPIase sequences (i.e., the *E. coli* PPIases). On the other hand, the amino-terminal and carboxy-terminal regions of *E. coli* PPIases differ entirely from those of other species. On the basis of these structural features, the two regions with high homology seem to be of great significance for the PPIase activity. In contrast to the clear similarity of *E. coli* PPIases with known cyclophilins, no significant homology to FKBP was found.

Cysteine residues are not essential for the activity of E. coli PPIase.

Earlier studies on the enzymic mechanism of PPIase suggested that it involves the nucleophilic addition of an active site thiol to the carbonyl carbon of Xaa-Pro, to form a tetrahedral, hemithioorthamide intermediate (49). However, no cysteine residue was found in the amino-acid sequence of *E. coli* PPIase *a* (Fig. 10A) and no conserved cysteine residue was found in that of *E. coli* PPIase *b*, comparing with those of PPIases of other species (Fig. 12). To investigate the involvement of the cysteine residue in the

PPIase activity, I measured the activities of *E. coli* PPIase *b* mutants in which Cys31 and Cys121 was replaced with serine and phenylalanine, respectively, by the site-directed mutagenesis technique. I began with the construction of the expression system of wild-type PPIase *b* in *E. coli* cells, in which the recombinant protein was produced under control of anthranilate synthetase (*trpE*) promoter (Fig. 13). The expressed protein was purified by the method used for the purification of endogenous *E. coli* PPIase *b*. In this system, almost all the recombinant protein was detected in soluble fractions and the amount of the protein was estimated as at least 50-fold larger than that present in the control cells. The purified wild-type recombinant PPIase *b* protein was virtually identical with the native protein in terms of apparent molecular mass, isoelectric point, amino terminal sequence, amino-acid composition, reversed-phase chromatographic behavior, and specific activity (data not shown).

Two cysteine mutants of PPIase *b* were also successfully produced in this system and were subjected to kinetic analysis of their enzymic activity. Their kinetic constants were compared with that of the wild-type PPIase *b* (Fig. 14). Based on the observed slope of each line, k_{cat}/K_m of the wild-type protein was estimated to be 1.6×10^{-7} $M^{-1}s^{-1}$ at 25 °C and those of the Cys31 -> Ser and the Cys121 -> Phe mutants were 1.9×10^{-7} $M^{-1}s^{-1}$ and 1.8×10^{-7} $M^{-1}s^{-1}$, respectively. Thus I could not detect any significant difference in the value of k_{cat}/K_m among the wild type and the mutant proteins. This result indicates that the cysteine residues in *E. coli* PPIase *b* play no essential role in catalysis of peptidyl-prolyl *cis-trans* isomerization.

PPIase a has a hydrophobic signal sequence.

To investigate the structural features of *E. coli* PPIases, their hydropathic patterns were calculated by the HPLOT program (58) (Fig. 15). The hydropathy plots along the amino-acid sequences corresponding to those of isolated *E. coli* PPIases indicated that their overall patterns are very similar, as expected from the nucleotide sequence homology.

However, the first 24 residues in the amino-acid sequence deduced from the nucleotide sequence of the *a* gene, which is not present in the isolated protein, showed a striking hydrophobic character. This 24-residue sequence has the following characteristics of a signal peptide: (i) there is a basic amino-acid residue very close to the amino terminus (lysine at the third position from the initiator methionine); (ii) there is a hydrophobic amino-acid stretch in the middle part; and (iii) an amino-acid residue having a small side chain (alanine) occupies the carboxy-terminal site. On the basis of these criteria, it is strongly suggested that the amino-terminal hydrophobic stretch serves as the signal peptide to transport PPIase *a* into the periplasmic space of the *E. coli* cell. On the other hand, PPIase *b* is not thought to have a signal sequence for the following reasons: (i) no hydrophobic sequence was present around its amino-terminal region; (ii) the stop codon appears in the frame upstream of the codon for a putative initiator methionine in its gene sequence (Fig. 10B); and (iii) the amino-acid sequence of the purified PPIase *b* is completely identical with that starting at the putative initiator methionine deduced from the gene sequence.

PPIases a and b are present in periplasm and cytoplasm, respectively.

Because purified PPIase *a* lacked the amino-terminal hydrophobic stretch of 24 residues which is predicted from the nucleotide sequence of the gene and since it has structural features characteristic of signal peptides, I examined whether form *a* is secreted into the periplasmic space of the cell. The periplasmic fraction was prepared by osmotic shock treatment of *E. coli* cells. Table 3 indicates that the PPIase activity was present in the periplasmic fraction as well as the cytosolic fraction at almost equal activity per milligram of total protein. I measured simultaneously the enzymic activity of β-galactosidase as a marker enzyme, which is naturally present in cytoplasm. This measurement allowed me to estimate that the contamination of the cytoplasmic components in the periplasmic fraction was less than 10 %. This level of contamination in

the periplasmic fraction was also confirmed by other cytosolic proteins, such as superoxide dismutase, and is comparable to that reported previously for many cytosolic proteins in osmotically shocked *E. coli* cells (63). Furthermore, the chromatographic patterns of the two fractions on a DEAE-5PW column were quite different from each other (Fig. 16). The major proteins purified from the periplasmic fraction were identified by the combination of protein sequencing and database search, and this fraction was found to contain galactose binding protein, asparaginase, and periplasmic oligopeptide binding protein (data not shown). All of the identified proteins were known to be related to be released from the periplasm by osmotic shock treatment (63). In contrast, only traces of these proteins were detected in the cytoplasmic fraction. To investigate the presence of forms *a* and *b* in each fraction, the PPIase activity in the eluent of the DEAE column was measured (Fig. 16A, B). By this method, the two purified forms *a* and *b* were completely separated and eluted at the retention times of 2 and 39 min, respectively. Thus, the contents of the two forms in each fraction could be estimated by measuring the enzymic activity in the eluent from the column. As shown in Fig. 16, the cytoplasmic fraction contained mainly form *b* (*a*:*b* = 1:20), whereas the ratio of form *a* over form *b* in the periplasmic fraction increased about 20-fold (*a*:*b* = 1:1). On the basis of these results and the measurement of the total PPIase activity in the periplasmic fraction (Table 3), the specific activity of form *a* in the periplasmic fraction was estimated to be 294 units/mg and that in cytoplasm to be 16 unit/mg. This result thus indicates that form *a* is exclusively translocated into the periplasmic space of the *E. coli* cell. The chromatographic analysis and enzyme assay revealed that PPIase *b* can be recovered in the periplasmic fraction upon osmotic shock treatment in levels far beyond those observed for other cytosolic proteins. However, the specific activity of form *b* in the cytosolic fraction was estimated to be about 1.5 times higher than that in the periplasmic fraction. Since form *b* is not expected to have a signal sequence, it is probably leaked from the cytoplasm by osmotic shock treatment; it is known that some of the

nonperiplasmic proteins, such as elongation factor Tu and uridine phosphorylase, are released during osmotic shock treatment (63). These observations are thus consistent with the idea that processed form *a* is exclusively translocated into the periplasmic space whereas form *b* is present in the cytoplasm. After this result was obtained, I found that Liu and Walsh reported the overexpression and isolation of an ORF190 gene product, which is identical with *E. coli* PPIase *a*, from the periplasmic fraction of *E. coli* (39). Their result confirms that *E. coli* PPIase *a* is translocated into the periplasmic space of the *E. coli* cell.

DISCUSSION

PPIase is thought to be essential for protein folding during protein synthesis in the cell based on its refolding accelerating effect on several denatured proteins *in vitro* (5, 7). Despite its presumed significance in protein folding, its biological function is totally unknown. A conviction that PPIase plays a critical role in certain biological events came from the findings that cyclophilin and FKBP, which were thought to mediate the effects of the immunosuppressants in T-cells, possess PPIase activity and that each PPIase is inhibited by its respective ligand (**CHAPTER 2.1**, 40, 41, 49). This conviction was strengthened further by the finding that cyclophilin mediated the cytotoxic CsA effect in *Neurospora crassa* and yeast (46). Thus, on the assumption that the effect of CsA is mediated through inhibition of PPIase activity, PPIases are suggested not to be restricted to immunoresponses but to be involved ubiquitously across several species in many cellular events.

In order to determine the biological functions of PPIase, I looked for a much simpler cellular system and focused on analyzing *E. coli*, since its cellular events are the best studied. Then, I examined PPIase activity in *E. coli* cells and isolated the enzyme from them guided by the activity. This approach led me to the finding that *E. coli* PPIase exists as at least two distinct forms. Because two kinds of PPIase, cyclophilin and FKBP, were found in mammalian cells (**CHAPTER 2.1**, 40, 41), their relationships to *E. coli* PPIases were examined in terms of sensitivity to the immunosuppressants, CsA and FK506. However, neither *E. coli* PPIase activity (*a* or *b*) was affected by these two drugs, indicating that there is the third type of PPIase with regard to drug sensitivity. The structural basis of the insensitivity of *E. coli* PPIases to the immunosuppressants was studied by sequencing the purified enzymes and the corresponding genes isolated. Such sequence analyses indicated that both *E. coli* PPIases had structures homologous to those of cyclophilins from many other species. In this regard, both *E. coli* PPIases are classified

into the cyclophilin superfamily. I categorized them as non-CsA-sensitive type, emphasizing the relationship to cyclophilin in their amino-acid sequences, but indicating their insensitivity to CsA. Table 4 summarizes the physicochemical features of the three known PPIase types, FK506-, CsA-, and non-CsA-sensitive PPIases. Currently, the non-CsA-sensitive PPIase is found only in *E. coli*, however, this does not exclude the possibility that non-CsA-sensitive PPIases may also be present in eukaryotic cells.

Although the involvement of a single sulfhydryl group in the activity of pig PPIase was suggested (49), no conserved cysteine residues were found in the aligned PPIase sequences (Fig. 12). In addition, *E. coli* PPIase *a* does not contain any cysteine residue in its amino-acid sequence, indicating the sulfhydryl group is not necessarily essential for PPIase to exhibit the enzymatic activity. This was confirmed by my results that a sulfhydryl-modifying reagent, *p*-(hydroxymercuri)benzoate, which effectively inhibited the activity of pig PPIase, did not affect that of *E. coli* PPIase *a* and only slightly affected that of PPIase *b* (data not shown) and that both PPIases *b* with mutations in Cys31 and Cys121, respectively, revealed the isomerase activity equivalent to that of the wild-type protein. Further studies will elucidate the mechanism of peptidyl-prolyl *cis-trans* isomerization catalyzed by PPIase.

The probable existence of two cyclophilin-like proteins in *Drosophila* was postulated based upon the results of Northern blot analysis with the *ninaA* gene, which is a known visual transduction gene (47, 48). However, the existence of the two distinct isoforms was still in question judging from the finding that in *Neurospora crassa* two mRNA species for the cytosolic and the mitochondrial forms of cyclophilin were transcribed from a single gene and eventually produced an identical molecule by posttranslational processing of the transit signal sequence from the precursor of the mitochondria targeted protein (32). In contrast, my current results definitively show that *E. coli* has two different genes for PPIase and that the two distinct forms are expressed in a single cell. Thus, the finding that multiple cyclophilin-related DNA sequences are

present in the mammalian genome (30, 31, 42) suggests that there may be many forms of cyclophilin-like PPIase within a single species or even in the cell, each of which probably has its specific role in the cell. The number of gene copies for PPIases found in the human genome was estimated as over 20 for cyclophilin-like PPIase and 1-2 for FKBP-like PPIase by Southern blot analysis (Table 4), whereas in phylogenically lower organisms such as *Neurospora* and yeast, it is estimated as 1-2 for cyclophilin-like PPIase. Although yeast genome was found to contain one copy of DNA sequence which hybridized with a human FKBP cDNA, I was unable to detect any FKBP-like PPIase in the *E. coli* genome (data not shown). However, this does not exclude the existence of FKBP-like PPIase in *E. coli*. These findings suggest that the cyclophilin gene has diverged extensively with the progressing complexity of cellular events during evolution. My determination that one of the two forms of *E. coli* is present in cytoplasm whereas the other form is transported into the periplasmic space of the *E. coli* cell may give a clue to how cyclophilin-like PPIases have functionally diverged in the cell. Namely, it indicates that proteins with the ability to assist folding of various polypeptides are located on both sides of the inner membrane. Many proteins are transported through this inner membrane, and during translocation, the polypeptides are thought to be in the unfolded state (64), so that some mechanism is needed to modulate folding and unfolding on both sides of the membrane. I propose that two PPIases may be involved in such a mechanism. There are several other proteins such as DnaK, DnaJ, GrpE, SecB, and GroE which have been known to assist protein folding in the *E. coli* cell, however, these factors are all present only in the cytoplasm. Thus, the presence of proteins with the ability to assist polypeptide folding in both the periplasm and the cytoplasm suggests that the folding of some exported proteins may be catalyzed by periplasmic PPIase, which, in turn, implies that the isomerization of prolines in some proteins destined for export may be prevented in the cytoplasm. Of course, in cases where the exported protein is translocated through the inner membrane during translation, the prevention of the isomerization in the cytoplasm

may be necessary. However, for the case that translocation is not coupled with translation, some mechanism must be postulated by which the isomerization is prevented in the cytoplasm, for the translocation of proteins through the inner membrane. One possible concept for such a mechanism is that proteins destined for export cannot be substrate for the cytoplasmic PPIase, but only for the periplasmic PPIase. Another mechanism might involve chaperonins which interact with nascent polypeptides and prevent the action of cytoplasmic PPIase on exported proteins within the cytoplasm. Thus my finding that the two distinct forms of PPIase are localized separately in the cytoplasmic and periplasmic compartments of *E. coli* cells raises many important questions on the understanding of the functional roles of various forms of PPIase on protein translocation, and indicates that *E. coli* offers a much simpler cellular system for studying these multiple functions of PPIases on protein folding in the cell.

Table 3. Presence of PPIase Activity in Periplasmic and Cytoplasmic Fractions

	Cytoplasmic fraction	Periplasmic fraction
Total protein (mg)	620	34
β-Galactosidase activity (units/mg)	16	1.2
PPIase activity (units/mg)	467	567
Total PPIase activity (units)	289540	19278

The values were obtained as an average of the experiments repeated 3 times.

Table 4. Summary of the Features of Known PPIases

PPIase (superfamily)	Type	Sensitivity to CsA	Sensitivity to FK506	Relative specific activity	Mol. wt.	Species found	Content (%)	No. of genes Mammalian	No. of genes Others	Cellular localization
FKBP	FK506	-	+	1/20	~11000	human, bovine	0.1-0.4	1-2	~1 (yeast)	cytoplasm
Cyclophilin	CsA	+	-	1	~18000	human, pig, rat, bovine, yeast, fungi	0.1-0.4	>20	1-2 (yeast, fungi)	cytoplasm, mitochondria (fungi)
	non-CsA	-	-	1	~21000	*E. coli*	0.04-0.4	unknown	2 (*E. coli*)	cytoplasm periplasm

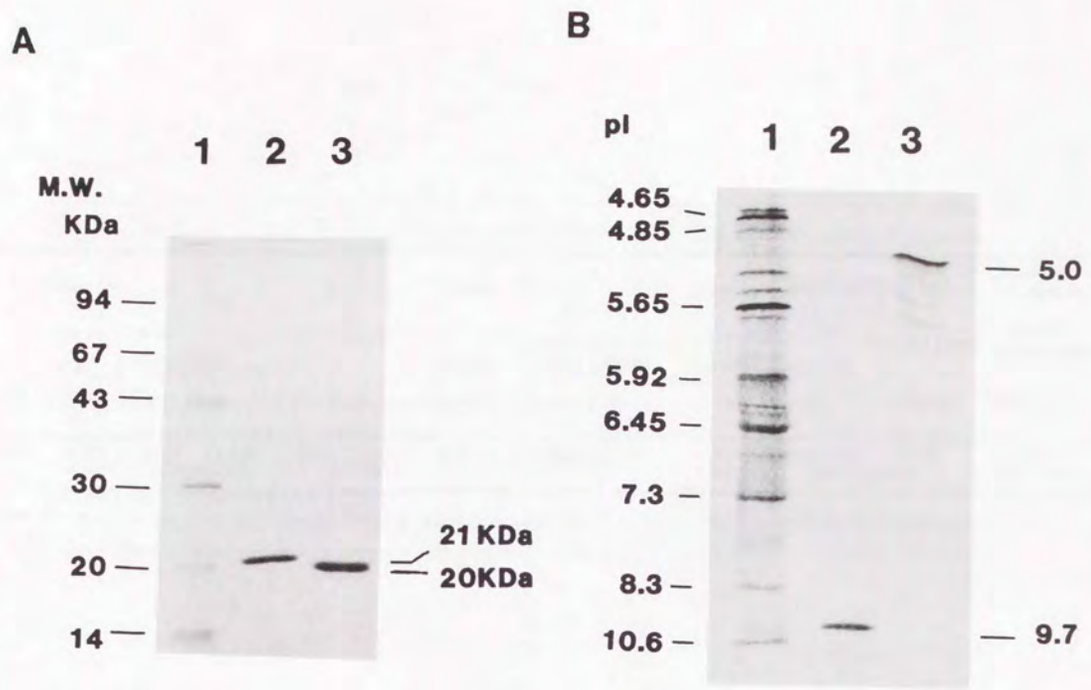


Fig. 8. Two forms of *E. coli* PPIase. A. SDS/PAGE and B. electrofocusing of the purified *E. coli* PPIases *a* and *b*. Lane 1, standard; lane 2, *E. coli* PPIase *a*; and lane 3, *E.coli* PPIase *b*.

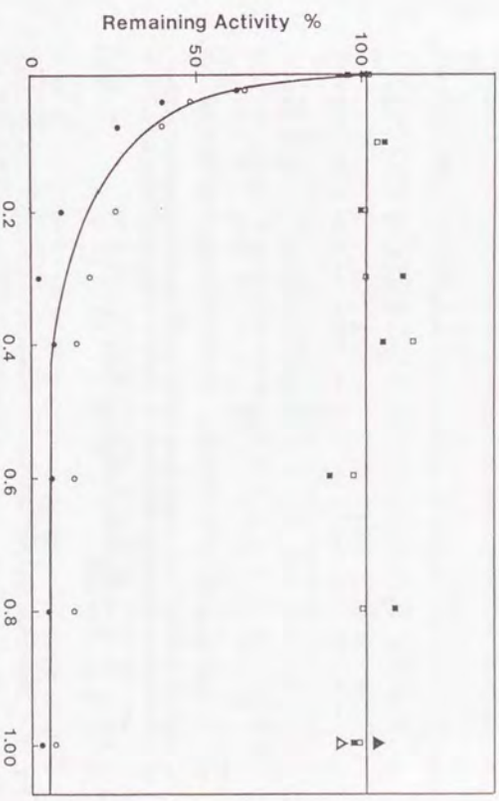


Fig. 9. Effects of CsA and FK506 on the activities of *E. coli* PPIases. The activity of PPIases was determined by measuring the catalysis of the *cis-trans* isomerization of *N*-succinyl-Ala-Ala-Pro-Phe-MCA. Residual PPIase activity is plotted against increasing amounts of CsA and FK506. PPIase (27 ng/2 ml) was preincubated in the presence of varying concentrations of CsA or FK506, respectively, and the remaining PPIase activity was determined. For CsA: □, *E. coli* PPIase *a*; ■, *E. coli* PPIase *b*; ●, yeast PPIase; ○, pig PPIase. Only the remaining activity at the highest concentration is plotted for FK506: ▲, *E. coli* PPIase *a*; △, *E. coli* PPIase *b*.

A

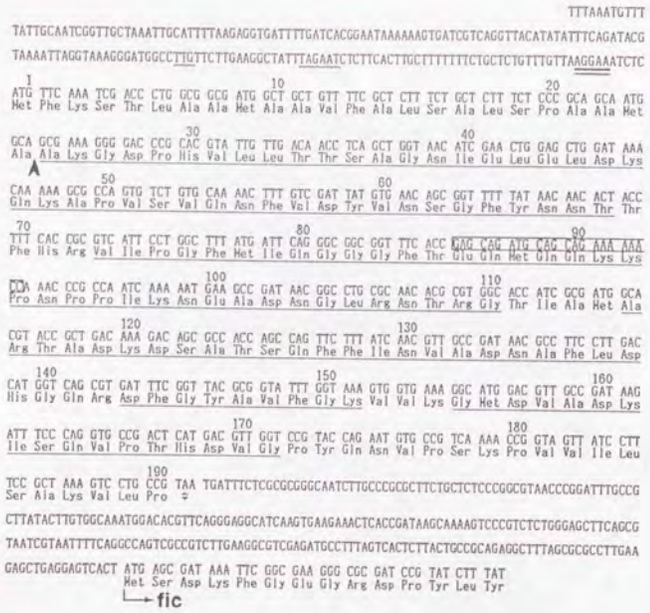


Fig. 10. A. Nucleotide sequence and deduced amino-acid sequence of *E. coli* PPIases *a*. The nucleotide sequence of a portion of the subcloned *Ava*I fragment is shown. The deduced amino-acid sequence is shown under the corresponding nucleotide sequence, and found to be identical with that of the hypothetical protein encoded by the ORF 190 sequence adjacent to the *fic* gene which is involved in the cell filamentation induced by cyclic AMP in *E. coli* (38). The initiation codon of the *fic* gene starts 262 bp downstream of the termination codon of the PPIase *a* gene. The first 14 amino acid sequence of *fic* is indicated under the corresponding nucleotide sequence. The signal peptide processing site is indicated by an arrow head. Amino-acid sequence determined by the protein sequencing of tryptic and cyanogen bromide peptides of the purified PPIases *a* is underlined. The oligonucleotides used as a probe are indicated by a box. The termination codon is indicated by an asterisk. The predicted ribosome binding site is shown by a double line. Putative -35 and -10 (Pribnow box) regions are indicated by a wavy line.

B

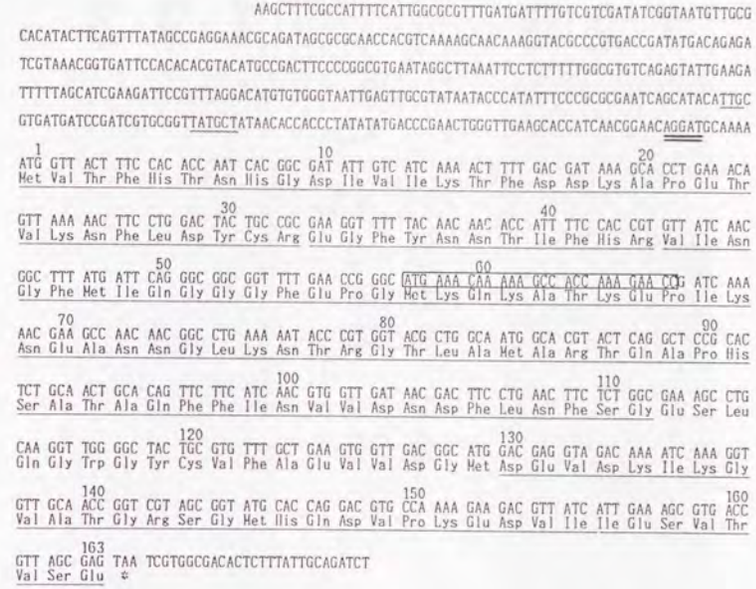


Fig. 10. B. Nucleotide sequence and deduced amino-acid sequence of *E. coli* PPIase *b*. The nucleotide sequence of the *Hin*dIII/*Bgl*II region of the isolated genomic fragment is shown for PPIase *b*. The deduced amino-acid sequence is shown under the corresponding nucleotide sequence. Amino-acid sequence determined by the protein sequencing of tryptic and cyanogen bromide peptides of the purified PPIases *b* is underlined. The oligonucleotides used as a probe is indicated by a box. The termination codon is indicated by an asterisk. The predicted ribosome binding site is shown by a double line. Putative -35 and -10 (Pribnow box) regions are indicated by a wavy line.

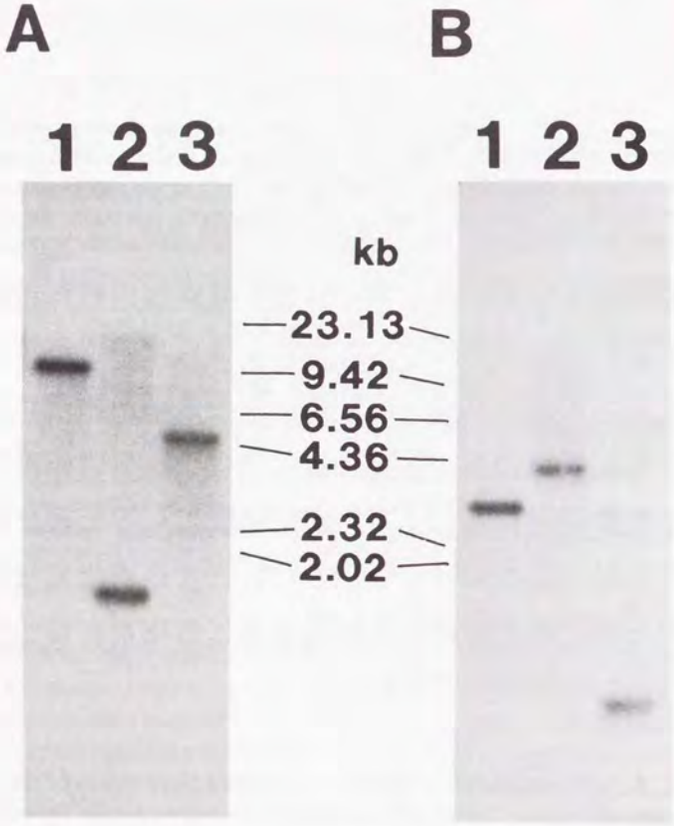


Fig. 11. Genomic Southern blot analyses. *E. coli* strain W3110 DNA (2 μg) was digested with *Eco*RV (lane 1), *Pst*I (lane 2), and *Pvu*II (lane 3), respectively, subjected to 0.8 % agarose gel electrophoresis, transferred to a nylon membrane, and probed with ^{32}P-labeled *Ava*II DNA fragment for PPIase *a* (A) and *Pvu*I/*Eco*RI fragment for PPIase *b* (B). Marker sizes of the electrophoresis are indicated between each autoradiogram.

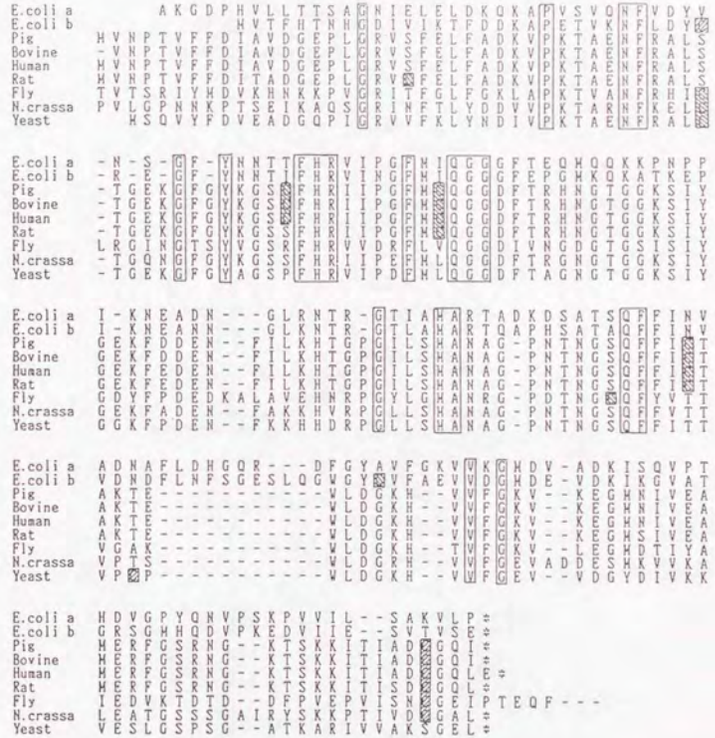


Fig. 12. Comparison of the amino acid sequences of PPIases from mammal, fly, fungi, yeast, and *E. coli*. The amino acid residues identical throughout all species are boxed. Gaps are shown by dashes and are inserted in the sequences to maximize the sequence homology. The amino-terminal sequences of *N. crassa* and fly and the carboxy-terminal sequence of fly are not shown. Cysteine residues are shaded.

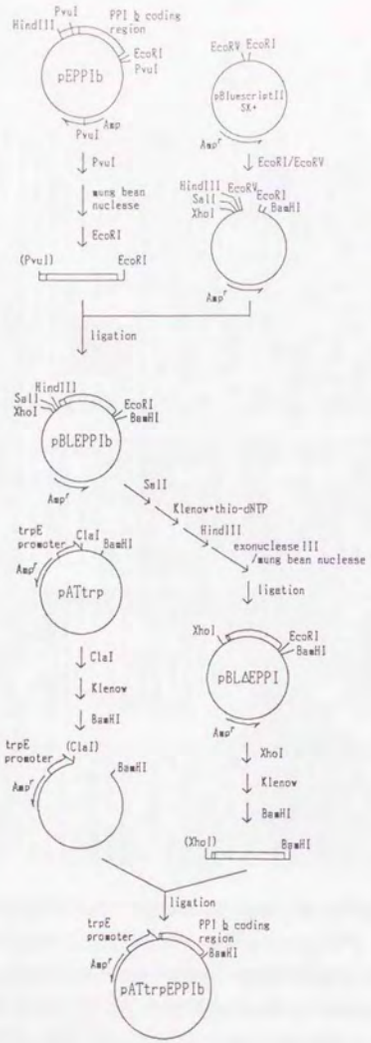


Fig. 13. Construction of the expression vector of *E. coli* PPIase *b*. Details of the procedure are described in **EXPERIMENTAL PROCEDURES**.

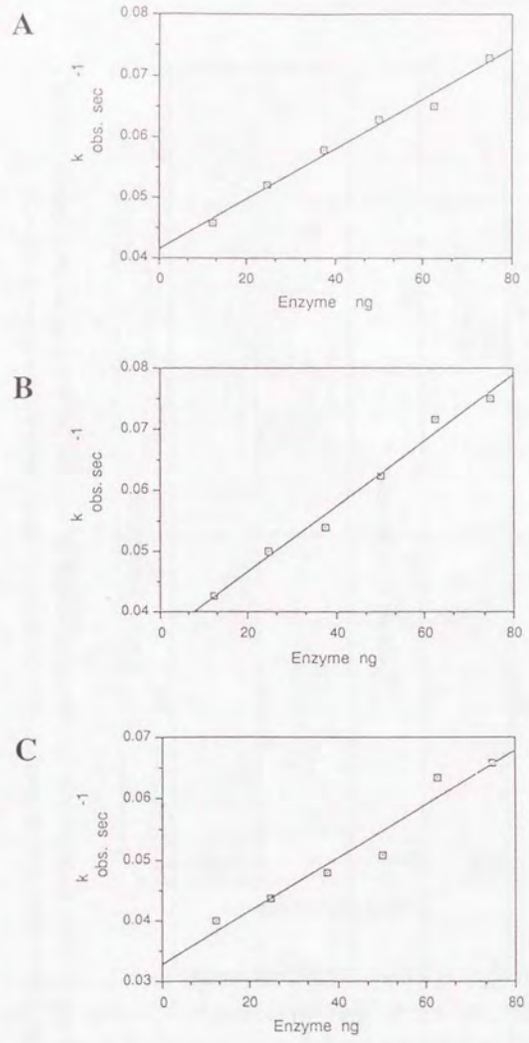


Fig. 14. Catalysis of prolyl *cis-trans* isomerization by wild-type and mutant PPIases. Observed initial velocity, k_{obs}, obtained in the chymotrypsin-coupled assay is plotted against the concentration of the enzyme. A, wild-type; B, Cys31 -> Ser mutant; C, Cys121 -> Phe mutant.

Fig. 15. Hydropathy profiles purified for *E. coli* PPIases calculated by using the HPLOT program. Hydropathic index is plotted along the deduced amino acid sequence. A. PPIase *a*; the processing site is shown by an arrow. B. PPIase *b*.

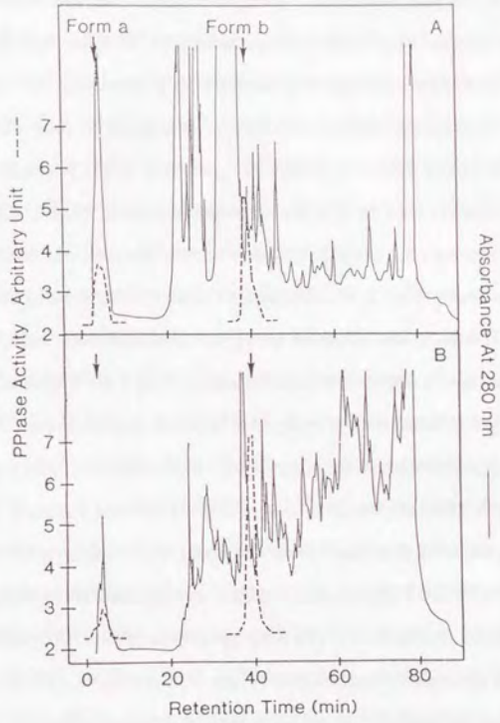


Fig. 16. Comparison of the contents of PPIases *a* and *b* in the periplasmic (A) and cytoplasmic (B) fractions. Each fraction (2 mg of protein) was applied to a DEAE column. The elution positions of the two forms are indicated by arrows in each chromatogram.

2.3. PERSPECTIVE

Since I found that PPIase is identical with cyclophilin (4), much efforts have been made to investigate the mechanism of signal transduction mediated by cyclophilin because of the medical significance of cyclophilin as the immunosuppressant-binding protein. These studies have resulted in a number of new interesting findings about PPIase. Here I discuss current understandings and unanswered questions about PPIase.

From the earlier studies on PPIase as cyclophilin, it was found that there exist up to twenty genes coding for PPIase in the mammalian genome (30, 31, 42). And a variety of cyclophilin isozymes has been identified in both prokaryotic and eukaryotic cells and classified as the cyclophilin (CyP) superfamily (Table 5) (70). It includes CyP-A, -B, -C, -D, -S and -*nina*A-like, and all share a highly conserved core sequence flanked by divergent amino- and carboxy-terminal sequences. The CyP-A species are classically identified as cyclophilins with a molecular mass of approximately 18 kDa and its corresponding molecules are widely distributed in all organisms including yeast (CyP1) and *E. coli*. *E. coli* PPIase *b*, which is categorized as CyP-A in a sense of its contents and localization in the cell, is distinct from the other CyP-A species in regard to CsA-sensitivity (**CHAPTER 2.2**). In principle, these cyclophilins are cytoplasmic enzymes, however, one of the species is secreted from lipopolysaccharide-stimulated macrophages (65). The other cyclophilin species, which are classified into CyP-B, -C, -D, -S and -*nina*A-like, have hydrophobic signal sequences in their amino-termini. CyP-B species reveal high sequence similarity with the CyP-A species in their central parts and have the ER retention signals in their carboxy-termini, while others have the hydrophobic regions there which may serve as anchors to membranes. In this context, *E. coli* PPIase *a* described in **CHAPTER 2.2** and yeast CyP2 are classified into CyP-B. All these species are thought to be involved in the trafficking of proteins in the cell. CyP-C shows a restricted distribution in kidney tissue and binds to 77 kDa glycoprotein, of which

function is unknown (66). From the observation that the inhibition of the activity of ER-resident PPIase by CsA causes apparent retardation of the assembly of the triple helices of collagen (67) and the secretion of transferrin (68), it is suggested that ER-specific PPIase catalyzes the folding of the secretory proteins. Function of CyP-*nina*A-like is the best established. It is expressed specifically in photoreceptor cells and is required for the transport of the folded isoforms of rhodopsin (Rh1 and Rh2) from the ER to the surface (47, 48, 69). CyP-D is an ER-resident protein and is also thought to be involved in the folding pathway of secretory proteins, however its precise function remains obscure. CyP-S is found in the secretory pathway and is shown to be associated with ER-resident proteins such as calreticulin, a soluble Ca^{2+}-binding protein.

FKBP was first identified as a binding protein to FK506, an immunosuppressant of which effect on T-lymphocytes is similar to CsA, and also has the PPIase activity (40, 41). The FKBP superfamily also consists of a diverse spectrum of species (designated as FKBP-12, FKBP-13, FKBP-25, and FKBP-59, in which the numbers are referred to their molecular mass) (Table 5) (70). Yeast FKBPs, which correspond to mammalian FKBP-12 and FKBP-13, are designated as FKB1 and FKB2, respectively. No FKBP species have been identified in *E. coli* so far. The FKBP-12 species are predominant FK506-binding proteins in cytoplasm and are believed to be involved in the pathway of protein folding in the cell. Homologous sequences to FKBP-12 are found in the pilin of *Neisseria meningiditis* (71, 72) and the FKBP-12-like proteins are identified in *Streptomyces hydroscopicus* (244). FKBP-13 has a signal sequence and is localized within the ER, and it appears to be involved in the folding pathway of secretory proteins. It has a substrate specificity similar to that of FKBP-12 (73). FKBP-25 binds to rapamycin, a immunosuppressant with similar structure to that of FK506 (Fig. 5 in **CHAPTER 2.1**), with much higher affinity than FKBP-12 does. It has a nuclear localization signal (NSL) (74-76), suggesting that it may translocate into the nucleus through unknown signal. FKBP-59 is a heat-shock protein and a component of the unstimulated glucocorticoid

receptor complex, which contains HSP90 (77,78). Its molecule consists of the FK506-binding, the calmodulin-binding, and the ATP-binding domains (79, 80). Binding of glucocorticoid to the complex induces the dissociation of the glucocorticoid receptor, resulting in the translocation of the receptor into the nucleus. The role of the PPIase activity in this system remains unknown but FKBP-59 may induce some conformational change of HSP90 or the glucocorticoid receptor by its activity, leading to the dissociation.

The role of CyP-*ninaA*-like in folding of rhodopsin has been proven by detailed biochemical and genetical studies (69). Coupled with the fact that PPIase is found in all cellular compartments in which protein folding occurs, this suggests that PPIase is a catalyst of protein folding in the cell and then it appears to be essential to cell growth. However, it was demonstrated that disruption of CYP1, CYP2, CYP3, FKB1, and FKB2 genes in the yeast genome revealed no apparent anomalies of growth rate, mating efficiency or sensitivity to heat shock (81-85), suggesting the presence of factors which compensate for the missing PPIases. How multiple PPIases within a single cell with different substrate specificities divide their roles is a crucial problem to be solved.

It has been suggested that PPIase acts as a modulator of protein function in a variety of aspects as well as a catalyst of protein folding, however, in most cases presented above further studies are needed to prove the proposals about its modulator functions. In this context, recent reports on the involvement of FKBP-12 in modulation of the calcium release channel are attractive (86, 87). FKBP-12 has been shown to be tightly associated with the ryanodine receptor, a calcium release channel of the sarcoplasmic reticulum and endoplasmic reticulum, and to modulate its gating. It is noteworthy that proline residues are frequently found in junctions, which are exposed to the cytoplasm, between membrane spanning domains of the channel proteins. Further investigation would elucidate the general function of PPIase as a gating-modulator of channels such as acetylcholine receptor and glutamate receptor.

The crystal and solution structures have been solved for cyclophilin A and FKBP-12, both without and with their ligands and proline-containing oligopeptide substrates. CyP-A has a β-barrel structure, consisting of eight antiparallel β-strands wrapping around the barrel surface and two helices sitting on the top and the bottom closing the barrel. Inside the barrel, seven aromatic and other hydrophobic residues form a compact hydrophobic core (Fig. 17). The substrate-binding site is coincident with the CsA-binding site (88-93). FKBP-12 has an antiparallel b-sheet topology that results in a novel loop crossing and produces a large cavity lined by a conserved array of aromatic residues (Fig. 17). This cavity serves as its isomerase active site and the drug-binding pocket (94-96). The possible involvement of cysteine residues in catalysis of PPIase suggested by earlier work on cyclophilin, in which the covalent tetrahedral bond is formed on the carbonyl carbon of the amide bond (49, 97) was excluded by the site-directed mutagenesis experiments as described in **CHAPTER 2.2** (98). The studies on three-dimensional structures of PPIases, coupled with detailed kinetic analyses of the PPIase-catalyzing reactions, has led to the elucidation of the mechanism of the catalysis, i. e., a distortion mechanism in which an intermediate state with a twisted C-N bond is stabilized by interactions with the enzyme (99-101). These investigations will be helpful for further understandings about substrate specificities of PPIases.

My finding that CsA is a potent inhibitor of the PPIase activity of cyclophilin, offered the first model for the molecular mechanism of the action of the immunosuppressant (Fig. 7 in **CHAPTER 2.1**); the PPIase activity of cyclophilin is required for the folding (Fig. 7A) or the conformational activation (Fig. 7B) of NFAT-1 which is involved in the signal transduction pathway, leading to transcriptional activation of the IL-2 gene. This model was supported by the findings that FKBP also has the PPIase activity and that its activity is inhibited by another immunosuppressive drug FK506, which suppresses the activation of the IL-2 gene in the apparently same manner as CsA (40, 41). Recently this simple picture for the mechanism of the

immunosuppressants was corrected by the following observations: (i) both cyclophilin (10-30 μM) and FKBP (5-30 μM) are found intracellularly at concentrations far above the IC_{50} values for CsA (5-200 nM) and FK506 (0.5 nM), indicating that full inhibition of the IL-2 gene expression occurs when most of cyclophilin and FKBP is uninhibited for the PPIase activity; (ii) CsA and FK506 cause a lethal cell cycle arrest in *Saccharomyces cerevisiae* and *Neurospora crassa*, and their isolated mutants, which are able to grow in media containing these drugs, have mutations in these PPIases, suggesting that the PPIase activity is rather not essential for viability and that the cyclophilin-CsA and FKBP-FK506 complexes cause the cytotoxic effects (46, 102); (iii) some of the analogs of CsA or FK506 bind to and inhibit the PPIase activity of their respective PPIases but fail to induce immunosuppression (103, 104). Thus further insights into the mechanism of action of CsA and FK506 were obtained by searching for ligands which specifically associate with the complexes of cyclophilin-CsA and FKBP-FK506 and both the complexes are found to bind to calcineurin. Calcineurin, a heterodimeric complex composed of a catalytic A subunit and a regulatory B subunit, is a Ca^{2+}/calmodulin-dependent protein phosphatase (105). Calcineurin binds CyP-CsA and FKBP-FK506 complexes in a Ca^{2+}-dependent manner and no binding is observed with either ligands or PPIases alone. Association of any complexes with calcineurin leads to inhibition of its phosphatase activity (106, 107). All available data support the notion that calcineurin is the biologically relevant target of CsA and FK506 (108). Further NFAT-1, a transcription factor of the IL-2 gene (Fig. 6 and 7 in **CHAPTER 2.1**), has been shown to be the substrate of calcineurin in T-lymphocytes. The advanced picture of the mechanism of CsA- and FK506-mediated immunosuppression in T-lymphocytes is presented in Fig. 18 (109-113). NFAT-1 is a heterodimeric transcription factor and one of the subunits is a cytoplasmic protein which requires translocation to the nucleus to form an active complex with another subunit (Jun-Fos) present in the nucleus. The trafficking of the cytoplasmic subunit of NFAT-1 is controlled by its calcineurin-mediated

dephosphorylation. Thus the inhibition of the protein phosphatase activity of calcineurin by association with the PPIases and their ligands results in the prevention of transcriptional activation of the IL-2 gene, leading to the immunosuppression.

It has also been demonstrated that calcineurin is involved in other signal transduction pathways of hippocampal long-term depression (114) and recovery from a-factor arrest in *Saccharomyces cerevisiae* (115), suggesting that calcineurin may generally play important roles in the signal transduction cascade. This proposal allows me to suppose the existence of some potential endogenous factors, which act as modulators in the signal transduction pathways by inhibiting and regulating the phosphatase activity of calcineurin in the same manner as CsA and FK506. And it looks mysterious that cyclophilin and FKBP with quite different tertiary structures to each other have the same enzymic activities and that they associate with the same target protein (calcineurin) and inhibit its activity when they bind to their respective ligands with quite distinct structures. How cyclophilin and FKBP have evolved convergently as modulators of the signal transduction and as catalysts of prolyl isomerization are interesting problems to be solved.

In spite of the progress in studies on PPIase as the modulator described above, less efforts have been made in order to understand the mechanism of protein folding catalyzed by PPIase. In **CHAPTER 2.2**, I presented the structural and functional analyses of *E. coli* PPIases. *E. coli* is, of course, the most appropriate system for the genetical and biochemical investigation of the protein function because of its simplicity. Together with the best understanding about chaperonins and the translocators of proteins through the membrane, further studies on *E. coli* PPIases will allow us to know the mechanism of protein folding in the cell.

Table 5. The Families of Cyclophilin and FKBP

Species	Protein	Function	Cellular localization	References
Mammalian	CyP-A	protein folding inhibition of phosphatase activity of calcineurin as a complex with CsA	cytoplasm	4, 29, 30, 31, 227, 228
	CyP-B	protein trafficking	ER, secretory pathway	229, 230, 231, 232, 233
	CyP-C	association with 77K protein	secretory pathway	106
	CyP-D	unknown	ER	234
	CyP-S	association with calreticulin	secretory pathway	235, 236, 237
	CyP-*ninaA*-like (*Drosophila*)	folding of rhodopsin	ER membrane	47, 48, 69
	FKBP-12	protein folding inhibition of phosphatase activity of calcineurin as a complex with FK506 gating of ryanodine receptor inhibition of p70 S6 kinase as a complex with rapamycin	cytoplasm	72, 238, 239
	FKBP-13	folding of secretory proteins?	ER	75
	FKBP-25	DNA-binding macrophage infectivity potentiator (*Legionella pneumophilia*)	nucleus plasma membrane (bacteria)	240, 241
	FKBP-59	involved in a Hsp90/glucocorticoid receptor complex	cytoplasm nucleus	242, 243
Yeast	CyP1	protein folding	cytoplasm	46, 82, 84, 102
	CyP2	folding of secretory proteins?	ER, secretory pathway	82, 84
	CyP3	folding of mitochondrial proteins?	mitochondria	85
	CyP4 (SSC3)	folding of secretory proteins?	ER, secretory pathway	223
	FKB1	protein folding	cytoplasm	82, 83, 102, 224
	FKB2	folding of secretory proteins?	ER, secretory pathway	73, 225
	FKB3 (NPR1)	unknown	nucleus vesicle-like bodies	226
E. coli	PPIase *a*	protein folding	periplasm	37, 39
	PPIase *b*	protein folding	cytoplasm	37

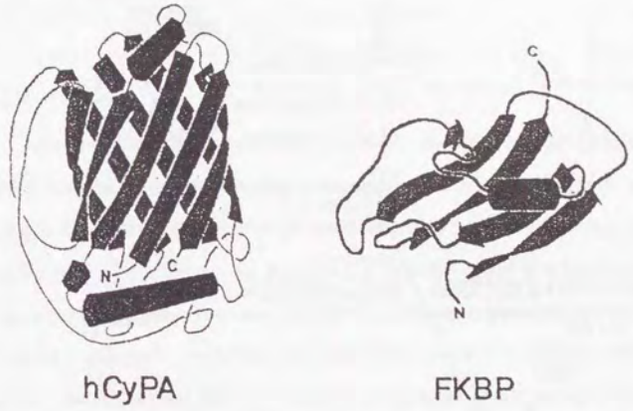


Fig. 17. The structure of human cyclophilin A (CyPA) and FK506-binding protein (FKBP).

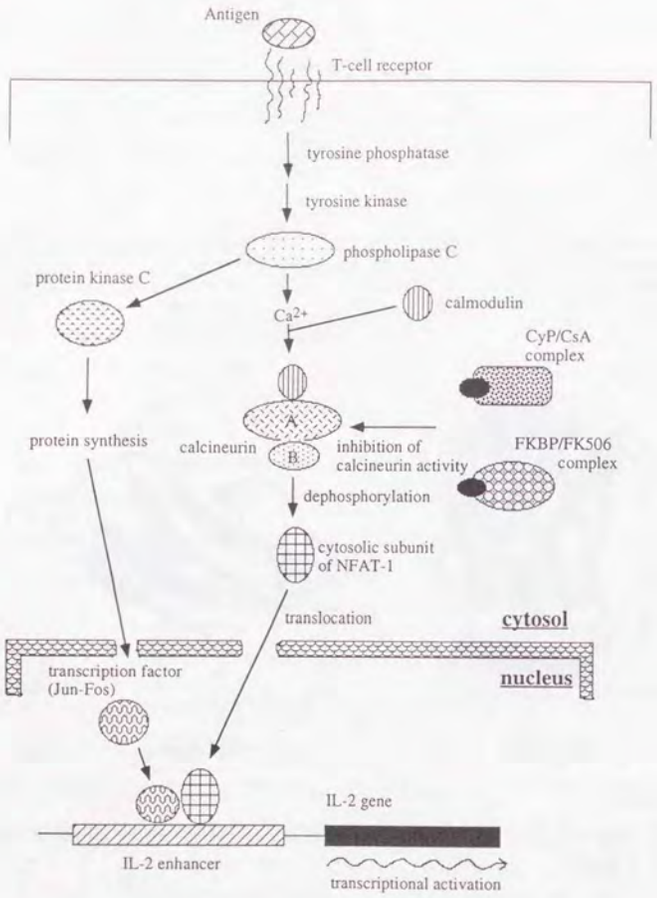


Fig. 18. A current model of the mechanism of CsA- and FK506-mediated immunosuppression in T-cell. Antigen recognition by T-cell receptor results in the activation of tyrosine phosphatase and tyrosine kinase. This is followed by the activation of phospholipase C, which initiates the protein kinase C- and calcineurin-mediated cascades. The former is independent from the action of CsA and FK506. In the latter cascade, CsA and FK506 bind to cyclophilin and FKBP, respectively, and each complex blocks the phosphatase activity of calcineurin. This step blocks dephosphorylation of the cytosolic subunit of NFAT-1 and prevent it from translocation to the neucleus, which results in the activation of the IL-2 gene.

CHAPTER 3

Functional Analysis of Protein Disulfide Isomerase

3.1. A Glutathionylated Protein Which Mimics a Folding Intermediate of Human Lysozyme Is a Substrate of Protein Disulfide Isomerase (116)

SUMMARY

A mutant human lysozyme, designated as C77A-a, in which glutathione is bound to Cys95, has been suggested to mimic an intermediate in the formation of disulfide bond during folding of human lysozyme (h-lysozyme) (117). Protein disulfide isomerase, which is believed to catalyze the formation of disulfide bond and associated protein folding in the endoplasmic reticulum, attacked the glutathionylated h-lysozyme C77A-a to dissociate a glutathione molecule. Results obtained by structural analyses showed that the protein is folded and that the structure around the disulfide bond, buried in a hydrophobic core, between the protein and the bound glutathione is fairly rigid. Thioredoxin, which has more powerful reducing activity of protein disulfides than PDI, catalyzed the reduction with lower efficiency. These results strongly suggest that PDI can catalyze the disulfide formation in intermediates with compact like the native states in the late step of *in vivo* protein folding.

INTRODUCTION

There are many interactions among side-chains of amino acids in proteins, which maintain three-dimensional structures of proteins. Among them, only disulfide linkage is a covalent interaction and is shown to play important roles in stabilization of structures of secretory proteins, assembly of proteins consisting of multi subunits, and facilitation of the folding process of nascent polypeptides.

The isomerization of disulfide bonds is shown to be one of the rate-limiting steps during protein folding *in vitro*. Rates of refolding are affected by the presence of thiol and disulfide compounds and are favored by high pH through ionization of thiol (-SH) to thiolate ($-S^-$), which promotes thiol disulfide interchange. In the best cases of small and simple proteins, such as ribonuclease A, with relatively few disulfides, optimal *in vitro* refolding often occurs with half-times in the range of ten to thirty minutes and is slower at physiological pH; for larger proteins, *in vitro* refolding may take a longer time. Thus a catalyst of this rate-limiting reaction was supposed to exist in the cells.

Protein disulfide isomerase (PDI), an abundant component of the lumen of the endoplasmic reticulum (ER), is an enzyme which catalyzes reduction, oxidation and thiol-disulfide interchanging process in oligopeptides and protein substrates. It was discovered from microsome fractions of rat liver in the early 1960's as a catalyst of *in vitro* oxidative refolding of denatured and reduced ribonuclease A (118,119), first purified soon after (120), and identified in a variety of organs (121). PDI exhibits a broad substrate specificity and acts to catalyze native disulfide bond formation *in vitro* in many proteins, including multi-domain and multi-subunit proteins, starting from either the reduced or incorrectly disulfide-bonded molecules (Table 6). In view of the following findings there has been general acceptance of the proposal, first made by Anfinsen and colleagues (122), that PDI catalyzes the rate-limiting steps associated with native disulfide formation in protein biosynthesis: (i) PDI is an abundant protein mainly existing

in the ER, in which disulfide bond formation occurs; (ii) the PDI activity correlates with the rate of synthesis of disulfide-bonded proteins in various tissues and at different stages of the cell (123); (iii) PDI catalyzes the formation of the interchain disulfide bonds required for monomer IgM assembly and it is chemically cross-linked to IgM *in vivo* (124); (iv) the presence of PDI within the luminal space of the microsome is essential for efficient disulfide formation in γ-gliadin in a cell-free protein synthesis system coupled with an *in vitro* translocation system (125). In the 1980's, PDI was applied for *in vitro* refolding of proteins with incorrect disulfide bonds, which were recovered from the expression system of *E. coli* as inclusion bodies, however, the effect of PDI was found to be modest as unexpected. Thus further studies on its catalytic properties had been needed for application.

The amino-acid sequence of PDI was first determined by cloning of cDNA coding for rat PDI (126) (Fig. 19). The structural characteristics for understanding the thiol-disulfide interchange activity of PDI is the presence of two regions (*a* and *a'* domains, 47 % identical), that reveal high similarity to *E. coli* thioredoxin. Each domain of PDI contains the sequence -Cys-Gly-His-Cys-. Thioredoxin acts in a variety of oxidoreduction process, and its catalytic center is a short disulfide loop consisting of the sequence -Cys-Gly-Pro-Cys-. In X-ray crystallographic structure of *E. coli* thioredoxin in the disulfide state (127), this tetrapeptide sequence forms the first turn of a long α-helix, and the S atom of the more amino-terminal Cys residue is located at the positive pole of the helix dipole, which accounts for the unusually low pK and high reactivity of this Cys residue in the dithiol state of the protein. The high level of homology between the *a* and *a'* domains in PDI and thioredoxin, coupled with preliminary modeling studies (128), suggests that the conformations of these domains are similar to that of thioredoxin. Mutational analysis shows that the sequences (-Cys-Gly-His-Cys-) are responsible for the PDI activity (129). PDI has another set of homologous domains (*b* and *b'*) and a domain (*e*), which reveals homology to the estrogen receptor, however, their function remains unknown.

Biochemical analyses shows that PDI is a soluble enzyme located within the luminal space of the ER (130). This location within the reticuloplasm (131) has been confirmed by immunocytochemical techniques at both the electron microscope and fluorescence microscope level (132, 133). PDI and other soluble luminal content proteins can readily released from isolated microsomal vesicles (134, 135). The localization of PDI within the ER is also confirmed by its structural characteristics (Fig. 19). PDI has a hydrophobic amino-acid stretch, which serves as a signal sequence. The C-terminal sequence of mammalian PDI is -Lys-Asp-Glu-Leu (-KDEL) and the subsequent discovery of this sequence in the carboxy-termini of other ER-resident proteins led to the proposal that this sequence functions as a signal for ER retention or recycling of soluble resident proteins, blocking their secretion outside the cell through bulk flow (135,136). Upstream the -KDEL sequence, there is a region rich in acidic amino acid (Asp and Glu), which is found in other ER-resident proteins such as a glucose-regulated protein 78 (GRP78) and GRP94. It is not clear whether this acidic amino-acid stretch helps the retention of PDI within the ER.

After determination of primary structure of PDI, it was revealed that some proteins with separate functions are identical to PDI. The first case is prolyl-4-hydroxylase, an $\alpha_2\beta_2$ tetrameric enzyme of which the β subunit is identical to PDI (137). This enzyme catalyzes the hydroxylation of proline residues in collagen, which is one of the posttranslational modifications essential to formation of triple helix of collagen. More recently protein structural evidence has appeared to establish that PDI is a component of the heterodimeric microsomal triglyceride transfer protein complex, which facilitates incorporation of triglyceride into nascent very low density lipoprotein (VLDL) particles within the ER (138). Persuasive photoaffinity labeling data at one time identified a luminal protein as an essential component of the N-glycosyltransferase system of the ER, which is responsible for the specific interaction with -Asn-X-Ser/Thr- sequences in nascent proteins (139); this glycosylation-site binding protein (GSBP) appears to closely

relate to PDI, but its role in N-glycosylation has since been disproven (140, 141). PDI is also shown to be a thyroid hormone (T_3) binding protein and suggested to mediate signal transductions in cell growth, development, and tissue differentiation (142, 143). However the mode of function of PDI in this process remains obscure. Characteristics of PDI and its structurally related proteins are summarized in Table 7. While most of these PDI-related proteins have shown to possess the protein-reducing and oxidizing activities, none of them has shown to have the disulfide-isomerizing activity. Further analysis will be needed to know which structural feature of PDI is responsible for the activity.

While there seems little doubt that PDI acts *in vivo* to catalyze disulfide interchange reactions and hence to facilitate the initial folding of disulfide-containing extracellular proteins, less efforts have been made to prove its actual catalysis in the cell because of the absence of proper approaches to investigate the process of protein folding *in vivo*. There are a number of obvious questions that need to be answered before I can provide a crude picture of the action of PDI as a catalyst of protein folding in the cell. In this chapter, I focus on the analysis of mutants as a model, which mimic folding intermediates of human lysozyme (h-lysozyme) with four disulfide bonds: Cys6-Cys128, Cys30-Cys116, Cys65-Cys81 and Cys77-Cys95. One of the mutants, designated as C77A, in which Cys77 is replaced by alanine, was secreted from yeast as two distinct forms, C77A-a and C77A-b. The former has the Cys95 modified with glutathione and the latter has a free thiol at Cys95 (117). The oxidation step in disulfide bond formation in C77A was suggested to involve mixed disulfide with glutathione. Together with the recent finding that glutathione disulfide is the source of the oxidizing equivalent in the ER (144), C77A-a drew my attention because this modification was suggested to occur during *in vivo* folding and the glutathionylated protein mimics the actual intermediate to be a substrate of PDI.

Here I present the new finding that PDI attacks a glutathionylated substrate, which represents the first example for the *in vivo* glutathionylation of the protein. The

characteristics of PDI as an *in vivo* "foldase" is discussed on the basis of the structural analyses of the substrate.

EXPERIMENTAL PROCEDURES

Yeast strain, plasmid, and growth media.

Yeast *Saccharomyces cerevisiae* AH22R$^-$ (*MAT*a *leu2 his4 can1 pho80*) (145) was utilized as a host strain. The expression plasmid of mutant h-lysozyme (C77A), in which Cys77 is replaced to Ala, has been previously described (117). Modified Burkholder minimal medium (146), supplemented with 8 % sucrose was used for growth of yeast.

Purification of mutant h-lysozyme.

Secreted mutant h-lysozyme (C77A) was purified as separate forms, C77A-a and C77A-b, with a CM-Toyopearl 650C column (TOSO) and an Asahipak ES-502C cation exchange column (Asahikasei) as described (117).

Conversion of C77A-a to C77A-b by bovine PDI or E. coli thioredoxin.

Bovine PDI was purified to homogeneity according to the published method (147). *E. coli* thioredoxin was purchased from TAKARA SHUZO. PDI- and thioredoxin-mediated conversion of C77A-a to C77A-b were analyzed as follows. Purified C77A-a was incubated at 37 °C for appropriate time with two-folds amount of purified bovine PDI or that of *E. coli* thioredoxin in the oxidative reaction buffer containing 100 mM sodium phosphate, pH 7.5, 2 mM GSH and 0.2 mM GSSG or in the reductive reaction buffer containing 100 mM sodium phosphate, pH 7.5, 0.1 mM dithiothreitol. The products of these reactions were analyzed by reversed-phase HPLC on a TSK-gel ODS-120T column (4.6 mm i.d. x 25 cm) (TOSO) equilibrated with 15 % acetonitrile and eluted at flow rate of 1 ml/min with a linear gradient of acetonitrile (15-55 %) containing 0.1 % trifluoroacetic acid for 45 min.

Amino-acid analyses.

Amino-acid compositions of the products of PDI- or thioredoxin-mediated conversion were determined with a Beckman Model 6300E amino-acid analyzer. Analyses were performed on protein samples hydrolyzed for 22 h, *in vacuo*, at 110 °C in 6 N HCl.

Crystallographic analyses.

Rod shaped crystals of C77A-a were obtained from a buffer solution which contained 20 mg/ml protein, 30 mM sodium phosphate, pH 6.0, and 2.5 M NaCl using a modification of the published method (148) and seeding technique. The space group was $P2_12_12_1$ and its cell dimensions were a=57.2 Å, b=61.0 Å, c=33.2 Å. The variation of cell dimension was so slight that the C77A-a crystal was considered isomorphous to the wild type h-lysozyme crystal. Diffraction data were collected with an automated oscillation camera system equipped with Imaging Plate (MAC Science, DIP-100) on a rotating anode generator with a 0.3 x 3.0 mm focus. Indexing, evaluation of diffraction intensities, scaling and merging were done using the program system ELMS (149) and PROTEIN (150). The diffraction data were 84 % complete to 1.8 Å resolution and the final merging R-value was 6.67 %. The structure of C77A-a was determined by a molecular replacement method with atomic coordinates of wild type h-lysozyme (151) as a starting model using programs PROTEIN and TRAREF (152). After several cycles of the rigid group refinements, 2Fo-Fc and Fo-Fc maps were calculated. These maps showed clearly the electron densities of the bound glutathione molecule. The initial model of the glutathione molecule was constructed from the atomic model of the crystal structure of γ-L-glutamyl-L-cysteinyl-L-glycine at 120 K (153). The glutathione model was fitted to the respective electron densities on an Evans & Sutherland PS-300 graphics system using the program FRODO (154). Structural refinements of C77A-a were carried out using the stereochemically restrained least squares refinement method as implemented in the program package PROTIN/PROLSQ (155) at 1.8 Å resolution. The refined model finally

consisted of the protein, the glutathione and 113 water molecules and the crystallographic R factor was 0.146 for 8982 independent reflections between 5.0 and 1.8 Å. The r.m.s. bond deviation from target value is 0.017 Å, and 2.6 degree from ideal bond angles. The r.m.s. difference in atomic B factors for covalently bonded pairs was 1.4 $Å^2$. The coordinate error was estimated to be about 0.12 Å based on the statistics of Luzzati (156)

RESULTS

Dissociation of glutathione bound to Cys 95 in C77A-a by PDI.

To elucidate the possible involvement of PDI in the folding pathway of h-lysozyme, I investigated the effect of PDI on C77A-a and C77A-b. As shown in Fig. 20A, PDI was found to convert C77A-a to C77A-b by dissociating the glutathione molecule bound to Cys95 in C77A-a in the presence of glutathione and glutathione disulfide. The conversion of C77A-a to C77A-b was also confirmed by analyzing the amino-acid composition of the product obtained from C77A-a after incubation with PDI (Table 8). Nevertheless, the reverse reaction was not observed at all (data not shown). In the time course of the reaction, any protein molecule except C77A-a and C77A-b was never found, indicating that C77A-a was converted to C77A-b without any shuffling of other three disulfide bonds. Figs. 20B and C show the effect of PDI and thioredoxin on the time course of the conversion under oxidative and reductive conditions, respectively. PDI catalyzed the dissociation of the glutathione with great efficiency under oxidative condition as equivalent in the ER, whereas thioredoxin had almost no effect on the reaction (Fig. 20B). The redox potentials of thioredoxin and PDI are -270mV and -190mV, respectively (157, 158). Although thioredoxin has been shown to have much more powerful protein disulfide reductase activity in the presence of dithiothreitol than PDI (157-160), PDI catalyzed the reduction of the disulfide bond more efficiently than thioredoxin as shown in Fig. 20C. The results indicate that there should lie unknown mechanism in the PDI-mediated reduction. In my understanding, it is the first finding that such a *in vivo* glutathionylated protein has been proven to be a substrate for PDI. My observations suggest that PDI acts on its substrates as an actual foldase in the cell, because C77A-a and C77A-b may mimic a folding intermediate and a wild-type protein, respectively.

Crystallographic analyses of C77A-a.

Until now, there has been no information on the three-dimensional structure of the glutathionylated protein, especially the *in vivo* folding intermediate. Thus, three-dimensional structure of C77A-a was determined and refined by X-ray crystallography at 1.8 Å resolution. The overall structure of C77A-a was essentially identical to those of C77A-b (161) and the wild type, because the root-mean-square deviation of main-chain atoms was 1.0 Å. These results indicate that the binding of the glutathione molecule to h-lysozyme does not affect on the overall main chain folding. The atomic structure of the glutathione molecule and structural environment around the glutathione in C77A-a are shown in Fig 21. The cysteine residue in the glutathione has almost the same geometry of the disulfide bond compared with those of Cys77 in wild type protein which has disulfide bond with Cys95. The glutathione molecule makes a close interaction with the h-lysozyme molecule by many hydrogen-bondings and hydrophobic interactions (Fig. 21). The glutathione molecule had no interaction with neighboring protein molecules in the crystal. However, the cysteine residue, except the Cα atom, was not exposed to the solvent accessible surface of C77A-a (Table 9). Especially, the side-chain atoms of the cysteine and those of Cys95 were buried completely in the molecule. Atomic thermal factors of the glutathione molecule are also shown in Table 9. Although Glu and Gly residues exposed to the solvent region, have high thermal factors, the factor of the sulfur atom (15.8 $Å^2$) was lowest in the glutathione. Further, the disulfide bond between Cys95 and the glutathione was surrounded by many hydrophobic residues: Phe57, Ile59, Trp64, Leu79, Leu84, Val99 and Trp109 (data not shown). The average thermal factor of the side-chain atoms in hydrophobic core was 13.6 $Å^2$, indicating that the structure around the disulfide bond is fairly rigid.

DISCUSSION

There are several examples that glutathione binds free cysteine residues in proteins *in vitro*, but the glutathionylation of Cys95 in C77A-a is believed to occur in the ER (162). It has been suggested that the disulfide bond between Cys77 and Cys95 may be formed in the last step of folding process of h-lysozyme (163, 164). The C77A-a possibly mimics the intermediate just prior to the formation of the Cys77-Cys95 bond which contributes to stabilization of the folded h-lysozyme (165). Together with the recent finding that an oxidative environment in the ER is maintained by glutathione disulfide, glutathione is supposed to be involved in the oxidative formation of disulfide bonds during protein folding step. The participation of PDI in this step is also supported by the observation that the bound glutathione to Cys95 is dissociated by PDI. Thus the PDI-mediated reduction would lead to the formation of the disulfide bond between Cys77 and Cys95 in the wild type h-lysozyme. Further, Cys95 in C77A-b was not glutathionylated by PDI, allowing me to suspect that the cysteine residues in the folding intermediates are glutathionylated spontaneously under the oxidative condition in the ER rather than by catalysis of PDI.

Results obtained from the crystallographic analyses showed that the disulfide bond between Cys95 and the glutathione in C77A-a was surrounded by hydrophobic residues and that the structure around the disulfide bond was fairly rigid. Thus it is unlikely that the disulfide bond is exposed to the accessible surface even by its thermal vibration, indicating that PDI may induce structural change of its substrate prior to the reduction of the disulfide bond. Two possibilities are available to explain how PDI recognizes its substrates. The first possibility is that PDI recognizes some specific amino-acid sequences. No sequence specificity has been found for the binding of peptides or proteins to PDI (140). However, cysteine-containing peptides are found to bind 4 - 8-fold tighter than no-cysteine-containing peptides, suggesting that the cysteine residue

contributes to the interaction with PDI (166). Based on my structural analyses, side chain atoms of the cysteine residues in question are buried in a hydrophobic core. In consequence PDI cannot attack the sulfur atoms of the disulfide bond directly. This observation indicates that the cysteine is not responsible for the first step of recognition of the substrate by PDI. However, PDI may recognize structural features of bound glutathione exhibited by glutamic acid and/or glycine, because they are exposed to the surface of the protein. The second possibility is that PDI can recognize the energetic state of the substrates, which is suggested from the study of S protein (167). The S protein lacks twenty amino-acid residues of its amino-terminal peptide (S peptide) of ribonuclease A. Disulfide bonds of S protein are found to be shuffled by PDI, while those of native ribonuclease A are not, suggesting that PDI does not distinguish substrates by their specific sequences but by their energetics (168). According to this model, the observed conversion might be explained by the energy state of the C77A-a protein, which is high enough to be susceptible to structural change induced by PDI. In any case, proteins are known to be so flexible that the disulfide bond and the sulfur atoms in question could become accessible to PDI in solution. Further study on the structure of C77A-a in solution, such as NMR analysis, will give us more information.

In the *in vitro* experiments, PDI acts on pre-folded or completely denatured proteins, or short polypeptides (123), which correspond to molecular species in the early step during protein folding. In the present study the dissociation of the glutathione bound to Cys95 in C77A-a with folded structure was found to be catalyzed by PDI more efficiently than by thioredoxin. Disulfides in various proteins are shown to be good substrates of thioredoxin, indicating that thioredoxin has no substrate specificity, whereas structurally buried disulfides are not (195). My finding described here is the first example that PDI seems to be able to catalyze the disulfide formation in intermediates, which have become compact like the native states in the late step of *in vivo* protein folding, while most disulfide bonded intermediates already studied are open in conformation (196). By

this feature PDI can be regarded as a real catalyst during *in vivo* protein folding, while thioredoxin can not. I believe that these observations are helpful to understand the actual mechanism of the PDI-mediated disulfide bond formation *in vivo*.

Table. 6 Examples of PDI-Catalyzed Processes (222)

Net protein disulfide formation to form native disulfide bonds	ribonuclease lysozyme albumin bovine pancreatic trypsin inhibitor types I and II collagen IgG IgM
Net protein disulfide reduction	insulin oxytocin vasopressin ricin
Protein disulfide isomerization	ribonuclease lysozyme soya bean trypsin inhibitor proinsulin urokinase
Protein disulfide formation in the *in vitro* cell-free protein synthesis system	prolactin γ-gliadin

Table 7. Summary of the Features of PDI and PDI-Related Proteins

Proteins	Structural features	Function	Cellular localization
PDI	-	reduction, oxidation and isomerization of disulfide bonds	ER
Prolyl-4-hydroxylase (β-subunit)	identical with PDI	hydroxylation of proline in collagen	ER
Glycosylation-site binding protein (GSBP)	identical with PDI	involved in the N-glycosylation system?	ER
T3-binding protein	identical with PDI	binding to thyroid hormone, T3 involved in signal transduction?	ER, plasma membrane, cytoplasm, mitochondria, nuclear membrane
PI-specific phospholipase C (ER60)	two "CGHC" sequences	hydroxylation of phosphatidylinositol 4, 5-bis-phosphate protease activity	plasma membrane ER
ERp72	three "CGHC" sequences	unknown (protein oxidoreductase activity)	ER
P5	two "CGHC" sequences	unknown (protein oxidoreductase activity)	ER
PDI (secreted form)	unknown	unknown	ER, secretory granule, plasma membrane

Table 8. Amino-acid Composition of the Product of PDI-Mediated Reduction of C77A-a

Amino acid	Theoretical value	Wild type	C77A-a	C77A-b	The product
Asx	18	17.0	17.1	16.8	16.5
Thr	5	4.3	4.3	4.3	4.3
Ser	6	4.5	4.5	4.5	4.5
Glx	9	8.7	9.7	8.7	9.1
Gly	11	10.3	11.2	10.3	10.2
Ala	14	13.5	14.5	14.3	13.8
Val	9	7.7	7.7	7.6	7.5
Cys	8	5.7	5.6	4.7	4.2
Met	2	1.9	1.9	1.9	1.9
Ile	5	4.4	4.4	4.4	4.3
Leu	8	8.0	8.0	8.0	8.0
Tyr	6	5.6	5.4	5.6	5.4
Phe	2	1.8	1.8	1.8	1.9
Lys	5	4.7	4.9	4.9	4.9
His	1	1.1	1.1	1.0	1.0
Arg	14	14.2	14.1	13.6	14.0
Pro	2	1.9	1.9	1.9	2.1

Table 9. Thermal Factors and Solvent Accessible Surface Area of Atoms in the Glutathione

Atom name	Thermal factor (Å^2)		Solvent accessible surface area (Å^2)	
Glu N	30.82		0.	
Cα	30.61		1.57	
C	32.05		0.	
O1	33.64		23.22	
O2	32.04		22.17	
Cβ	27.28		0.	
Cγ	23.79		0.	
Cδ	22.70		0.	
Cε	23.10		9.57	
		Cys77 in wild-type		Cys77 in wild-type
Cys N	21.49	12.58	0.	0.
Cα	19.58	12.51	1.58	4.73
C	21.37	12.13	0.	0.
O	19.68	12.30	0.	12.67
Cβ	16.81	11.66	0.	0.
Sγ	15.84	9.73	0.	0.
Gly N	24.84		10.33	
Cα	29.39		7.89	
C	32.86		3.45	
O1	34.27		29.56	
O2	35.47		38.00	

Solvent accessible surface area was calculated by the program ASA (H. Mizuno and N. Go, unpublished) using a solvent probe of 1.4 Å radius.

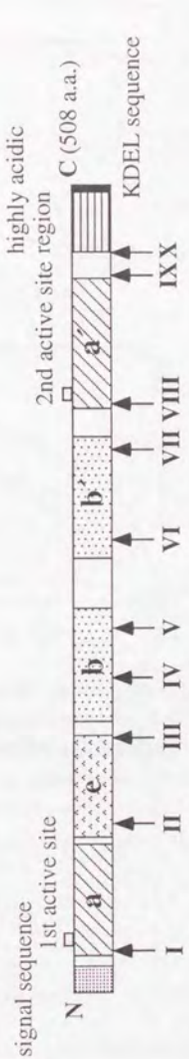


Fig. 19. Structural features of human PDI. The PDI molecule, consisting of 508 amino acid residues, has a signal sequence at the amino terminal and an ER retention signal (-KDEL-) at the carboxy terminal. The a- and a'-domains are thioredoxin-like domains. The e-region reveals similarity to estrogen receptors. Functions of the e-region, the b- and b'-domains, and the highly acidic region remain unknown. The location of the introns in its gene is indicated by arrows.

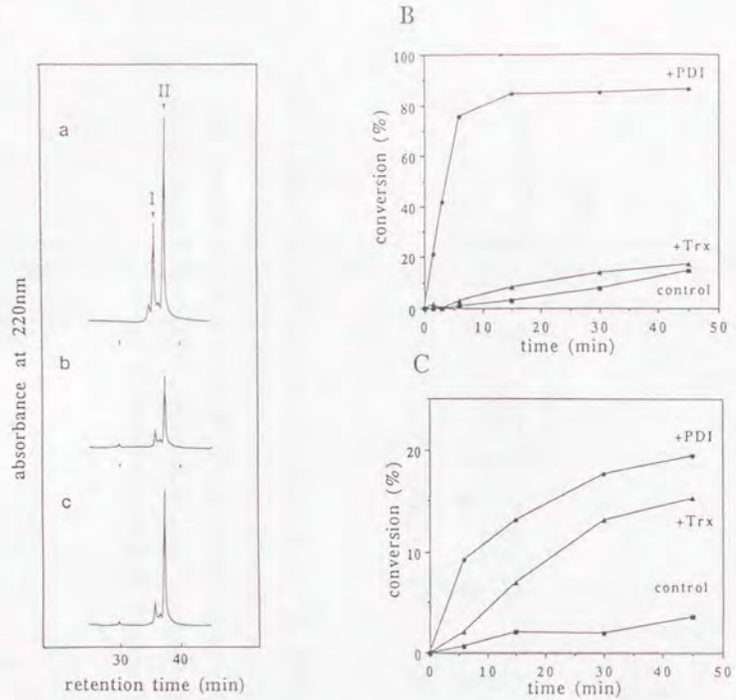


Fig. 20. Dissociation of glutathione bound to Cys95 in C77A-a by PDI or thioredoxin. A, a, 2.1μM of C77-a and 4.2μM of C77A-b were separated by reverse-phase HPLC on a TSK-gel ODS-120T column as described in **EXPERIMENTAL PROCEDURES**. b, 2.1μM of C77A-a was incubated in the oxidative reaction buffer as described in **EXPERIMENTAL PROCEDURES** containing 4.2μM of bovine PDI at 37°C for 45 min, followed by separation as above. c, the reaction product obtained as described in b was separated as above soon after mixing with 2.1μM of C77A-b. Peaks I and II correspond to C77A-a and C77A-b, respectively. Parts of chromatograms are shown here. B, 1.4μM of C77A-a was incubated at 37°C in the oxidative reaction buffer with 2.8μM of bovine PDI (●), with 2.8 μM of *E.coli* thioredoxin (▲), and without the enzymes (■) for 0, 1.5, 3, 6, 15, 30, and 45 min, respectively, followed by reverse-phase HPLC to estimate the rate of conversion. C, 1.4μM of C77A-a was incubated at 37°C in the reductive reaction buffer as described in **EXPERIMENTAL PROCEDURES** with 2.8μM of bovine PDI (●), with 2.8 μM of *E.coli* thioredoxin (▲), and without the enzymes (■) for 0, 1.5, 3, 6, 15, 30, and 45 min, respectively, followed by reverse-phase HPLC.

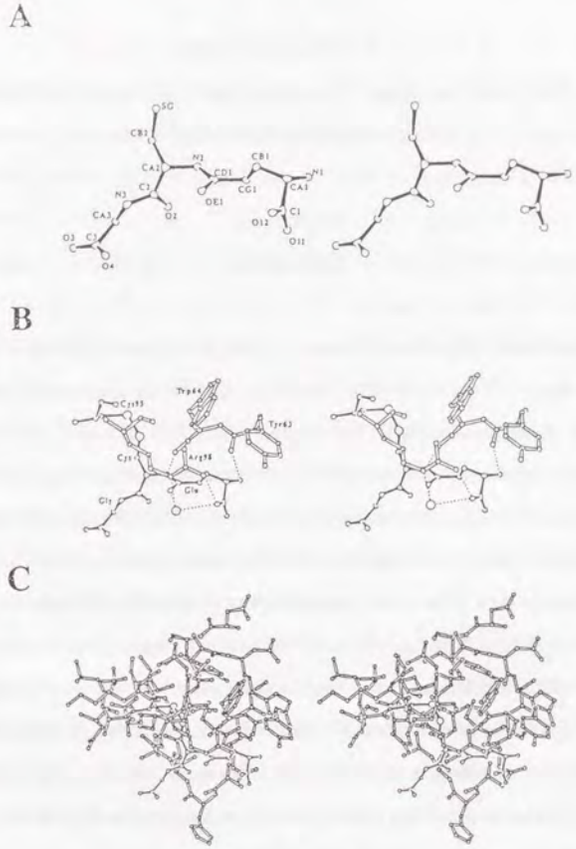


Fig. 21. Stereo drawings of the atomic structure of the glutathione molecule in C77A-a. A, a ball and stick model of the glutathione molecule bound to C77A-a is shown. Each atoms are named and labeled as shown. B, interactions between the glutathione molecule and surrounding residues, Tyr63, Trp64, Cys95 and Arg98 are shown. Models of the glutathione and Cys95 are indicated by closed bonds and other residues are indicated by open bonds. Six hydrogen bonds, including water mediated, are indicated by broken lines. Two sulfur atoms and the water molecule are indicated by large circles. C, an atomic model of the glutathione and the surrounding residues within 12Å from the Sγ atom of the cysteine residue of the glutathione is shown. The glutathione and Cys95 are also indicated as shown in B.

3.2. A Potential Function of Protein Disulfide Isomerase Involved in Protein Folding in the Cell (169)

SUMMARY

I investigated the effect of human and yeast PDIs on *in vivo* protein folding in a specially constructed yeast coexpression system, using human lysozyme (h-lysozyme) as a substrate. Coexpression with human and yeast PDIs increased the amounts of intracellular h-lysozyme with the native conformation, leading to an increase in h-lysozyme secretion. The coexpression of PDI did not affect the transcription of the h-lysozyme gene. These results indicated that PDI actually assists protein folding in the cell. The secretion of h-lysozyme increased even when both active sites of PDI were disrupted, suggesting that the effect of PDI resulted from a function other than the formation of disulfide bonds. This is the first finding that PDI without isomerase activity accelerates protein folding *in vivo*. Coupled with the result that purified recombinant human PDI revealed chaperone-like activity in an *in vitro* assay using rhodanese as a substrate, I present a possibility that PDI would be involved in the pathway of protein folding in the cell by means of its chaperone-like function.

INTRODUCTION

After the genetic engineering technique had been established in early 1980s', much effort has been made to produce proteins in heterologous systems such as *E. coli* and yeast. However, in most cases overexpression of foreign genes was unsuccessful due to degradation or accumulation of incorrectly processed and misfolded proteins in these cells. And the effect of PDI on the refolding of the proteins produced in these systems with incorrect disulfide bonds was vigorously examined *in vitro*, but fruitful results were not obtained, possibly due to the difference of conditions, in which PDI catalyzes protein folding, between *in vitro* and *in vivo*. As earlier works clearly showed that PDI promotes the *in vitro* folding of various proteins (Table 6 in **CHAPTER 3.1**), the mechanism of the *in vivo* protein folding catalyzed by PDI can be used to improve expression systems for foreign genes. Thus in this study, I constructed a coexpression system of PDI and human lysozyme (h-lysozyme) as the substrate in a single yeast cell and I investigated the effect of PDI on the *in vivo* folding of a disulfide-bonded protein. It was found that PDI facilitates protein folding *in vivo*, suggesting that PDI is a real catalyst of protein folding in the cell. The effect of PDI was modest and less than expected but I think this is the first step to understand the mode of action of PDI in the process of protein folding in the cell.

Surprisingly, the effect of the mutant PDI, in which both the active sites were disrupted, on protein folding was also evident. It has been shown that PDI can catalyze disulfide bond formation in the late step of protein folding (**CHAPTER 3.1**, 220) and that PDI has some properties of molecular chaperones, allowing me to investigate the new aspects of PDI as a foldase. In this light of view, I describe the potential function of PDI other than the formation of disulfide bonds during the *in vivo* protein folding and the use of PDI in developing more efficient expression systems for foreign genes.

EXPERIMENTAL PROCEDURES

Strains and vectors.

The *Saccharomyces cerevisiae* haploid strain YPH499 (*MAT***a**, *ura3-52*, *lys801*^{amber}, *ade-101*^{ochre}, *trp1-Δ63*, *his3–Δ200*, *leu2-Δ1*) (170) was used as the host for integrating expression units of h-lysozyme and PDI genes. Plasmids pRS303, pRS304, pRS305, and pRS306 (170) (kindly provided by Dr. A. Nakano of The University of Tokyo) were used to construct integration vectors.

Materials.

The h-lysozyme gene was from Takeda Chemical Industries Ltd. The yeast glyceraldehyde 3-phosphate dehydrogenase (GAP) promoter and the yeast alcohol dehydrogenase (ADH) terminator were from Tonen K.K.

General methods.

Yeast cells were grown in YPD medium or SD medium with auxotrophic requirements (171). Yeast transformations were carried out as described (172). Routine recombinant DNA manipulations were performed as described (173).

Amplification and cloning of genes.

High-molecular-weight yeast DNA was prepared from YPH499 cells as described (174). Polymerase chain reaction (PCR) (175) was performed, using a Perkin-Elmer Cetus DNA Thermal Cycler, in 10 mM Tris-HCl, pH 8.3 containing 1.5 mM $MgCl_2$, 50 mM KCl, 0.01 % gelatin, 200 μM dNTPs, 2.5 units of AmpliTaq DNA polymerase, 2 μmol each of sense and antisense primers, and 200 ng of yeast high-molecular weight DNA as a template. Amplification was performed for thirty cycles of 1 min at 95 °C, 2 min at 37 °C, and 3 min at 72 °C, followed by an incubation for 7 min at 72 °C. Primers for

amplification of the yeast PDI (y-PDI) gene (176-179) were: 5'-AAAACTCGAGTTAT-GAAGTTTTCTGCT-3' (sense) and 5'-TTTTTGGATCCGTTCGTGCAGTGTGA-3' (anti-sense). Primers for the yeast actin gene (174) were: 5'-TTTTTTCTAGAGGTTGC-TGCTTTG-3' (sense) and 5'-TTTTTCTGCAGAGATTAGAAACAC-3' (anti-sense). Restriction sites and some base pair extensions were added to the end of the primers to facilitate further cloning steps. Full-length human PDI (h-PDI) cDNA was cloned by screening a human liver λgt11cDNA library (Clontech) using the oligonucleotide, 5'-TGGCGTCCACCTTGGCCAACCTGATCTCGGAACCTTCTGC-3' (137), as a probe.

Site-directed mutagenesis.

Site-directed mutagenesis, as described by Kunkel (180) was used to introduce a mutation at the junction of the signal sequence and the N-terminus of mature h-PDI to generate a *Nae*I restriction site. The mutagenic primer was: 5'-CGGGGGCGCCGGCGCGC-3'. Mutations in the active sites of h-PDI and y-PDI were introduced by the method described above. The mutagenic primers used for generating mutations in the first and the second active sites of h-PDI were: 5'-CTTGCAGTGGCCAGACCAAGGGGCATA-3' and 5'-AGTGACCAGACCATGGG-3', respectively, and those for mutations in the first and the second active sites of y-PDI were: 5'-AGTGGCCAGACCATGGA-3' and 5'-ACAGTGACCAGACCATGGGGC-3', respectively.

DNA sequencing analyses.

PCR-amplified, cloned, and mutagenized DNAs were sequenced by the dideoxy chain termination method (56) using the 7-deaza or the BcaBEST dideoxy sequencing kits (TAKARA SHUZO).

Construction of integration vectors for expression of h-lysozyme, h-PDI, and y-PDI.

The *Hin*dIII/*Xho*I fragment encoding GAP promoter and the *Bam*HI/*Kpn*I fragment encoding ADH terminator were introduced to the multi-cloning sites of the integration vectors: pRS303, pRS304, pRS305, and pRS306 (170). The resulting plasmids were designated as pRS303E, pRS304E, pRS305E, and pRS306E, respectively. Two integration vectors for the expression of h-lysozyme, designated as pRS-GHLZM-303 and pRS-GHLZM-304, were constructed by introducing an *Xho*I/*Bam*HI fragment encoding h-lysozyme between the GAP promoter and the ADH terminator in pRS303E and pRS304E, respectively. Expression vectors for y-PDI and mutant y-PDI, designated as pRS-GYPDI-305 and pRS-GYPDIM-305, were constructed by introducing the *Xho*I/*Bam*HI fragments encoding y-PDI and mutant y-PDI into the *Xho*I/*Bam*HI site in pRS305E, respectively.

Integration vectors for expression of h-PDI were constructed as follows. A DNA fragment encoding the signal sequence of human serum albumin (HSA) was generated by the annealing and subsequent ligation of the phosphorylated oligonucleotides (5'-TCGAGAATTCATGAAGTGGGTTACCTTCATCTCTTTGTTGTT-3', 5'-AACAAGA-ACAACAAAGAGATGAAGGTAACCCACTTCATGAATTC-3', 5'-CTTGTTCTCTTC-TGCTTACTCTAGAGGTGTTTTCAGAAGGCCTG-3', and 5'-GATCCAGGCCTTCT-GAAAACACCTCTAGAGTAAGCAGAAGAG-3'), and subcloned into the *Xho*I site of pUC119X, which was produced by introducing a *Xho*I linker into the *Eco*RI site of pUC119. The resulting plasmid was designated as pUC119sig. The *Eco*RI/*Bam*HI fragment encoding h-PDI was subcloned into the *Eco*RI/*Bam*HI site of pUC119 and a *Nae*I restriction site was generated at the junction between the signal sequence and the N-terminus of mature h-PDI by site-directed mutagenesis as described above. The resulting plasmid was digested with *Nae*I and *Bam*HI, then the mature h-PDI coding sequence was excised and cloned into the *Stu*I/*Bam*HI site of pUC119sig. The resulting plasmid, designated as pHPDI, was digested with *Eco*RI, followed by fill-in with Klenow

fragment, and the DNA fragment containing h-PDI with the signal sequence of HSA was excised from the vector by *Bam*HI digestion and cloned into the *Xho*I/*Bam*HI site, in which the former protruding end had been filled-in with Klenow fragment, just downstream of the GAP promoter in pRS305E and pRS306E, respectively. The resulting integration vectors were designated as pRS-GHPDI-305 and pRS-GHPDI-306, respectively. An expression vector of mutant h-PDI (pRS-GHPDIM-305) was constructed in the same manner as described for wild type h-PDI. The control integration vectors, designated as pRSD305 and pRSD306, were produced by *Xho*I/*Bam*HI digestion, filled-in with Klenow fragment, followed by the self-ligation of pRS305E and pRS306E, respectively. All of the vectors were linearized at unique restriction sites in selective marker genes (*HIS4*, *TRP1*, *LEU2*, and *URA3*) before transformation.

Northern blotting.

Total yeast RNA was isolated as described (171). RNA (10 µg) was subjected to agarose gel electrophoresis and transferred to a nylon membrane (Hybond-N, Amersham) (173), which was probed with the h-lysozyme or the yeast actin gene (174) fragment radiolabeled using the random primer DNA labeling kit (TAKARA SHUZO). The hybridization proceeded overnight at 65 °C in 6 X SSC (1 X SSC: 3 M NaCl, 0.3 M sodium citrate, pH 7.0) containing 50 % deionized folmamide, 0.5 % SDS, 0.2 % polyvinylpyrrolidone, 0.2 % Ficol 400, 0.2 % bovine serum albumin, and 20 µg/ml of sheared salmon testis DNA. Following hybridization, the membrane was washed with 0.1 X SSC containing 0.1 % SDS at 65 °C for 30 min. Autoradiography was performed at -70 °C using an intensifying screen.

Purification of recombinant h-PDI.

An yeast strain expressing recombinant wild-type h-PDI was grown in selective SD medium at 30 °C for 48 h and further grown in 12 liters of fresh YPD medium, of which

the pH was adjusted to 7.0 by 50 mM sodium phosphate, at 30 °C for 48 h. After centrifugation, the supernatant was applied directly on a DEAE-Sephacel column (5.0 cm i.d. X 30 cm) equilibrated with 20 mM sodium phosphate buffer, pH 6.3, and eluted at a flow rate of 25 ml/h with a linear gradient of NaCl (0-1.0 M). Recombinant PDI was detected in the eluate with about 0.6 M NaCl. The fractions containing PDI were collected, concentrated with a Centricon 10 (AMICON), applied to a Con A Sepharose column (1.5 cm i.d. X 15 cm) equilibrated with 0.5 M NaCl/20 mM Tris-HCl buffer, pH 7.4, and eluted with the same buffer. The flow through fractions were pooled, applied to a Sephacryl S-200 column (1.5 cm i.d. X 170 cm) equilibrated with 0.5 M NaCl/100 mM sodium phosphate buffer, pH 7.0, and eluted at a flow rate of 0.1 ml/min with the same buffer. PDI was eluted at around 120 ml. Mutant h-PDI was purified in the same manner as described above except that the yeast strain secreting the mutant protein was used. Protein concentrations were measured using the protein assay kit (BIO-RAD) with bovine γ-globulin as standard.

Preparation of crude yeast extracts.

Yeast crude cell extracts were prepared basically as described (181). Stationary cultures of yeast were inoculated into 250 ml of fresh YPD medium and grown at 30 °C to mid-logarithmic phase. The cells were harvested, suspended in 50 ml of buffer A (50 mM sodium phosphate, 20 mM EDTA, 50 mM NaCl, pH 7.5), and ruptured at 9,000 psi for five times with a French pressure cell press (SLM INSTRUMENTS, INC.). The resulting suspension was adjusted to pH 7.0 with 1 M Tris, then phenylmethylsulfonyl fluoride was added (2mM). After centrifugation at 15,000 X *g* for 30 min, the supernatant was concentrated with ammonium sulfate (30 %-85 % saturation) and dialyzed against buffer B (50 mM sodium phosphate, 10 mM EDTA, 50 mM NaCl, pH 7.0). The dialyzate was used for assay of intracellular PDI activity.

Assay of enzyme activity.

Protein disulfide isomerase activity was assayed by measuring the rate of reactivation of scrambled RNase A (182). Thiol:protein disulfide oxidoreductase (TPOR) activity was assayed by the method, in which the enzyme-catalyzed reduction of disulfide bonds of insulin by GSH is linked to the reduction of GSSG to GSH, by NADPH and glutathione reductase (183). The lytic activity of h-lysozyme was measured using *Micrococcus lysodeikticus* cells as a substrate (184).

Assay of chaperone activity.

The effect of purified recombinant h-PDI on the reactivation of unfolded dihydrofolate reductase (DHFR) and the aggregation of unfolded rhodanese was investigated as described (185).

RESULTS

To elucidate the possible involvement of PDI in the step of protein folding in the cell, the coexpression system of PDI and human lysozyme (h-lysozyme) as a substrate was constructed using a set of yeast integrating plasmid vectors (YIp) with multi-cloning sites, in which expression units of foreign genes can be irreversibly inserted into yeast chromosomes. In this study, h-lysozyme was selected as the substrate for PDI because it is a small and simple protein, consisting of 130 amino-acid residues, with four disulfide bonds and because the formation of its disulfide bonds are shown to be crucial for *in vivo* folding (186, 187). Furthermore, lysozyme folding has been shown to be catalyzed by PDI *in vitro* (**CHAPTER 3.1**, 188). In the integration vectors, h-lysozyme, human PDI (h-PDI), and yeast PDI (y-PDI) were constitutively expressed under the control of the yeast glyceraldehyde 3-phosphate dehydrogenase (GAP) promoter with transcriptional termination by the yeast alcohol dehydrogenase (ADH) terminator (Fig. 22). The expression of h-lysozyme under the control of the GAP promoter in yeast episomal plasmid vectors (YEp) has been reported (189). In h-PDI expression, it should be noted that the signal sequence of h-PDI was substituted with that of human serum albumin because h-PDI was degraded in yeast, when expressed with its authentic signal sequence, possibly due to a failure in its processing. This construction actually allowed me to express functional h-PDI, while the amino terminal residue (Asp) of native mature h-PDI was changed to Gly (not shown). The expression units of h-lysozyme, h-PDI, and y-PDI in the vectors were integrated by homologous recombination to the sites of the selective marker genes (*ura3*, *trp1*, *his3*, and *leu2*) on yeast chromosomes of the host strain (YPH499), which contain non-revertible auxotrophic mutations. The strains constructed and used in this study are described in Table 10.

As shown in Figs. 23A and B, h-PDI facilitated the secretion of h-lysozyme to an extent of 128 and 122 % of the control after 24 and 48 h in culture, respectively. The

effect of y-PDI on h-lysozyme secretion was greater than that of h-PDI (148 and 157 % of the control at 24 and 48 h of culture, respectively). A dose effect of h-PDI on the h-lysozyme secretion was also evident (Fig. 24), in which the increase was about 43 and 53 % of the control at 24 and 48 h of culture, respectively. These results indicated that the effect was closely related to the amount of intracellular h-PDI. In these experiments, strains (HL10 and HL30), in which control vectors lacking only the coding sequences of h-PDI or y-PDI were integrated, were used as controls, showing that the effect resulted from the coexpression of PDI and not from the rescue of auxotrophic mutations by the integration. There were no apparent differences in the growth rates among strains (data not shown), indicating that the increase in the secretion did not result from the effect of PDI on the cell growth. Thus, functionally expressed h- and y-PDIs facilitated the secretion of the substrate *in vivo*.

The substitution of both catalytic site sequences of PDI with -Ser-Gly-His-Cys- leads to the complete loss of isomerase activity *in vitro* (129). Furthermore, the mutation exhibits a delay in the disulfide bond formation of carboxypeptidase Y *in vivo* (190). Thus, it is believed that these catalytic sites play an important role in the oxidoreduction of disulfide bonds during protein folding in the cell. To determine whether the increase of intracellular isomerase activity was responsible for facilitating h-lysozyme secretion, the effect of mutant h-PDI on h-lysozyme secretion was examined. Western blot analysis of intracellular PDI in the strains HLP11 and HLP12 showed that the expression level of the mutant h-PDI was almost the same as that of the wild type (not shown). Unexpectedly, the mutant h-PDI was found to accelerate the secretion of h-lysozyme to almost the same extent as wild type h-PDI (Fig. 25). Whereas PDI is an ER-resident protein, recombinant PDI was secreted into the culture medium in small amounts in my expression system, probably due to overflow. Thus the wild type and the mutant h-PDIs secreted from yeast strains HP1 and HP2 respectively, were purified to homogeneity by DEAE-Sephacel, Con A Sepharose, and Sephacryl S-200 chromatographies (Fig 26). Con A Sepharose

chromatography completely removed authentic y-PDI with a sugar modification, which would be also secreted into the medium. In both the wild type and the mutant, about 1 mg of the protein was recovered from 6 liters of 24 h-culture. Purified proteins were assayed for the isomerase activity using scrambled ribonuclease A and the thiol:protein disulfide oxidoreductase activity using insulin as a substrate. I confirmed that the mutant h-PDI lost its activity completely (data not shown). Thus, the *in vivo* effect of h-PDI described above was caused by a function other than that of disulfide bond formation catalyzed by isomerase activity.

The amounts of mRNA of h-lysozyme were compared among the strains by Northern blotting to exclude the possibility that the effect of PDI on h-lysozyme secretion was derived from the stimulation of h-lysozyme gene transcription. As shown in Fig. 27, the coexpression of either h-PDI or y-PDI did not affect the transcriptional level of the h-lysozyme gene. This was also confirmed by the densitometric analysis (data not shown), indicating that the effect of PDI is post-transcriptional.

In the experiments described above, the effect of PDI was investigated by comparing the amounts of secreted h-lysozyme. Next, I focused on the intracellular changes induced by the coexpression of PDI. Crude cell extracts were prepared from the yeast strains (HL10, HLP11, HLP12, and HLP21) and the amounts of intracellular h-lysozyme with the native conformation were estimated by measuring the lytic activity. As shown in Table 11, the increase of intracellular, folded h-lysozyme paralleled that of secreted h-lysozyme, indicating that the effect of PDI is exerted in the pre-secretory pathway. The effect of PDI on the increase of intracellular h-lysozyme was more remarkable than on that of secreted h-lysozyme, possibly due to some limitations in the secretory steps, such as a deficiency of the secretory machinery. The intracellular activities of PDI were also measured by scrambled ribonuclease and TPOR assays (Table 11). In the former, the activity was increased 121 % in the extracts of HLP11 and 243 % in those of HLP21, compared with that of the control (HL10), respectively. In the latter,

those were 112 % for HLP11 and 248 % for HLP21, respectively. Supposing that the specific activities of mammalian PDI and y-PDI are similar (191), the intracellular amount of y-PDI was estimated to be 7 - 12 fold greater than that of h-PDI. Together with the findings that the amount of folded h-lysozyme from HLP11 and HLP21 increased 250 and 550 %, the effect of h-PDI on intracellular h-lysozyme folding is greater than that of y-PDI. In this experiment, the complete loss of PDI activities by the mutation and the effect of the mutant h-PDI, namely an increase in the secreted h-lysozyme level, were also confirmed (Table 11).

Recently it was reported that PDI possesses properties of molecular chaperones since it non-specifically binds to various peptides (192) and denatured proteins bound to PDI and dissociates from it in the presence of ATP (193). Furthermore, PDI actually exhibits chaperone activity in the *in vitro* oxidative refolding of lysozyme (194). A molecular chaperone such as GroEL prevents denatured DHFR from reactivation by binding to its refolding intermediate and releases it rapidly after the addition of ATP, leading to refolding of denatured DHFR (185). In contrast, guanidium-denatured and reduced rhodanese aggregates rapidly upon the dilution of the denaturant and it cannot be correctly refolded spontaneously. GroEL prevents the aggregation by associating with unfolded rhodanese and assists its correct folding by dissociation from the unfolded polypeptide, hydrolyzing ATP (185). Thus, chaperone activity of purified recombinant h-PDI was tentatively investigated. In the DHFR assay, no significant chaperone activity of h-PDI was evident even when PDI was present at a large molar excess (10-fold against DHFR at a molar ratio) (data not shown). On the other hand, the chaperone-like activity of recombinant h-PDI was observed in the rhodanese assay (Fig. 28A). Unlike GroEL, addition of Mg-ATP did not lead to the dissociation of h-PDI from the denatured polypeptide (Fig. 28A). Furthermore, mutant h-PDI also possessed chaperone-like activity (Fig. 28B). The mutant h-PDI prevented the aggregation of denatured polypeptides more efficiently than wild type h-PDI (Fig. 28B). This suggested that the

isomerase activity is responsible for promoting the aggregation. Coupled with my finding that the mutant h-PDI accelerates the folding of h-lysozyme *in vivo*, the chaperone-like activity of PDI could be another mode of function, other than its isomerase activity, involved in the step of protein folding in the cell.

DISCUSSION

Unexpectedly, mutant h-PDI without isomerase activity accelerated h-lysozyme folding to the same extent as wild type h-PDI. This suggested that a function of h-PDI besides oxidoreduction of proteins is responsible for the effect. PDI is a multi-functional protein that is identical to the β-subunit of prolyl 4-hydroxylase (137), glycosylation site binding protein (139), thyroid hormone binding protein (142, 143), and a component of the microsomal triglyceride transfer protein complex (138). Among them, the former three are involved in gene expression and the protein folding pathways. The former two play important roles in post-translational modification, however, their functions seem not to be responsible for the effect of PDI on h-lysozyme secretion in my system because h-lysozyme is modified with neither prolyl hydroxylation nor glycosylation in the cell. The participation of PDI in transcriptional activation, which is suggested in eukaryotic cells, was also excluded by Northern blotting as described in Fig. 27.

I then investigated the chaperone activity of recombinant PDI to explain the acceleration of h-lysozyme folding by PDI. PDI may be both an enzyme and a molecular chaperone (192). During the folding of nascent polypeptides, there is an aggregation pathway for folding intermediates, which competes with the correct folding and association pathways. A molecular chaperone temporarily binds to the intermediates possibly by hydrophobic interaction and prevents the intermediates from aggregation. Hydrolysis of ATP by chaperone leads to the release of the trapped intermediates and to their correct folding and association (3). The following findings suggest that PDI is a molecular chaperone. (i) PDI, as the b-subunit of prolyl 4-hydroxylase, prevents the misfolding and aggregation of the a-subunit in the *in vitro* cell-free synthesis system (197). (ii) PDI, as the subunit of the microsomal triglyceride-transfer protein complex, stabilizes the secondary structure of the 88-kDa subunit which tends to aggregate upon its dissociation from PDI (198). (iii) PDI nonspecifically binds to various peptides at the

carboxyl-terminal site, distinct from its catalytic sites (199). (iv) PDI associates with misfolded type I procollagen with a deletion (200) and misfolded mutant h-lysozyme (201) *in vivo*, which may lead to the retention of abnormal procollagen molecules within the ER and the ER-degradation of misfolded protein, respectively. (v) Seven ER-resident proteins have been detected, which bound selectively to denatured protein columns and which were specifically eluted by ATP, and one of these proteins was identified as PDI by immuno blotting analyses (193). Direct evidence that PDI has chaperone activity has been reported (194). These investigators have also found that PDI exhibits anti-chaperone activity in the oxidative refolding of hen egg lysozyme depending on the reaction conditions. My study showed that recombinant h-PDI has chaperone-like activity in the rhodanese, but not in the DHFR assay, suggesting that it has substrate-specificity in its chaperone activity. I suppose that proteins such as rhodanese, which are liable to aggregate and unable to refold spontaneously, are preferable substrates for PDI, whereas proteins such as DHFR or RNase A, which spontaneously refold, are not. From this perspective, h-lysozyme would not be a preferable substrate, which would explain why the effect of PDI described here was not so great as that described by Robinson *et al.* (202), in which the overexpression of PDI resulted in ten-fold higher levels of secretion of human platelet derived growth factor B and a four-fold increase in that of acid phosphatase. The alternative explanation is that the effect of PDI on foreign gene expression may depend on the cultivation conditions. In any case, the substrate specificity of PDI needs to be understood to apply PDI to an expression system for foreign genes. In addition, the factors that regulate the dissociation of the bound polypeptides from PDI remain to be elucidated. PDI has been found to have autophosphorylation activity (203). PPIase, another folding-assisting enzyme, has been also shown to possess chaperone activity in the folding of carbonic anhydrase (204). Whether or not such folding-assisting enzymes would actually function as molecular chaperones in the cell remains an issue to

be resolved. Ultimately, relationship between the effect of PDI and its chaperone-like activity described here remains obscure, however it could explain the effect.

The mutant h-PDI also had chaperone-like activity (Fig. 28B), indicating that protein disulfide isomerase activity is not responsible for chaperone-like activity. The suppression of aggregation of denatured rhodanese by the mutant was more efficient than that by the wild type h-PDI. This could be explained by its anti-chaperone activity (194). Wild type h-PDI partly prevented aggregation with its chaperone-like activity and partly promoted aggregation with its oxidation activity, of the intermolecular disulfide bonds of denatured and reduced rhodanese. However, the importance of its anti-chaperone activity during protein folding in the cell remains unclear.

Disruption of the PDI gene in yeast results in a recessive lethal mutation (205-208). However, the loss of PDI activity by site-specific disruption in both catalytic sites does not affect the viability of yeast cells (190). This indicates that the essential function of PDI does not reside in its isomerase activity but in its unidentified activity. On the other hand, the loss of PDI activity leads to a delay in the folding of carboxypeptidase Y (190). In this light, my study describes a new finding that the mutant h-PDI, lacking isomerase activity, accelerates h-lysozyme folding *in vivo*. Further genetic and biochemical analyses by means of the cell-free protein synthesis system coupled with *in vitro* translocation system (125) will identify the unknown function of PDI in the step of protein folding in the cell.

In my systems, the apparent effect of y-PDI on h-lysozyme folding was higher than that of h-PDI. Analyses of PDI activity showed that the increase of intracellular amount of y-PDI within the y-PDI integrated cells (HLP21) was 7 - 10 fold higher than that of h-PDI within the expressing cells (HLP11), although h-PDI and y-PDI were expressed under the same promoter in my system. PDI, which an ER-resident protein, has an ER-retention signal, consisting of -Lys-Asp-Glu-Leu (KDEL) sequence for mammal and -His-Asp-Glu-Leu (HDEL) sequence for yeast, at its carboxyl terminus. PDI resides

in the ER through binding to a receptor of the signal. The affinity of the HDEL sequence to the yeast receptor is higher than that of the KDEL sequence (136). The greater increase of intracellular PDI activity within HLP21 can be explained by the higher efficiency of y-PDI for retention within the ER.

The dose effect of h-PDI on h-lysozyme folding was studied as shown in Fig. 24. To maximize the expression level of h-lysozyme, an additional expression unit of y-PDI was tentatively integrated into the strain HLP21 and the higher expression level of y-PDI drastically inhibited cell growth (data not shown). I suppose that the capacity of the receptor for the ER-retention signal (HDEL receptor) would be saturated in the system because y-PDI itself was secreted to the medium possibly due to overflow. It was actually reported that overexpressed PDI is exported from the ER in rat exocrine pancreatic and Chinese hamster ovary cells (209, 210). Thus I suspect that competition of the receptor among certain ER-resident proteins, which are essential for the cell growth, would result from the overexpression of y-PDI. It can be explained that the overexpression of h-PDI with KDEL as the retention signal escaped the inhibition because of the relatively low affinity of the signal for the receptor (136). These observations indicated that more efficient retention of PDI within the ER by the additional integration of an expression unit of the ER-retention signal receptor would lead to further improvement of the expression systems for foreign genes. These system would mimic mammalian cells, which are secreting great amounts of disulfide-bonded proteins, such as activated lymphoid cells and fibroblasts (123).

Whereas mammalian PDI and y-PDI are thought to have diverged from a common ancestral gene during evolution (207), similarity between h-PDI and y-PDI in their primary structures is found only within their thioredoxin-like domains, which contain -Cys-Gly-His-Cys- sequences responsible for their oxidoreductase activities. The overall homology between h-PDI and y-PDI is no more than about 30 % (205, 206, 208), suggesting that h-PDI could have acquired another function, which y-PDI does not bear,

during evolution. It is noteworthy that mammalian PDI has another domain with internal homology, of which the function remains unknown. To determine whether or not the effect of h-PDI on h-lysozyme folding in both wild type and mutant coexpression systems resides in function specific to h-PDI, the effect of mutant y-PDI was also investigated. The expression unit of mutant y-PDI, in which both catalytic sites were replaced with -Ser-Gly-His-Cys-, was integrated into the yeast strain YPH499. The introduction of mutations into y-PDI resulted in the loss of the coexpression effect on the acceleration of h-lysozyme folding. However, there was a remaining increase in h-lysozyme secretion (approximately 16 %) (Fig. 29). This result indicated that the effect of y-PDI resided partly in an increase of intracellular PDI activity (Table 11) and that PDI actually accelerates disulfide bond formation by its intracellular thiol:protein oxidoreductase activity. It is also demonstrated that y-PDI, like h-PDI, assists protein folding in the cell through a function other than oxidoreduction. Whether this unidentified function of y-PDI is the same as that of h-PDI will be confirmed by further study.

Application of PDI to the expression of foreign genes was first challenged in the *E. coli* system, in which *E. coli* PDI, designated as DsbA (211, 212), was coexpressed with various antibody fragments including the single F_V fragment (213). Coexpression did not significantly change the folding limit of the functionally folded antibody fragments. DsbA protein is required for the *in vivo* folding of alkaline-phosphatase, β-lactamase, and OmpA protein (211, 212). However, its *in vivo* substrates might be limited to the resident proteins in the periplasmic space of *E. coli*, because DsbA protein is smaller than eukaryotic PDI, and lacks the domain in PDI that is responsible for the nonspecific peptide binding activity (199). Another explanation for the quite low effect of DsbA protein, is that aggregation steps in the periplasmic space, in which no molecular chaperone was found, would compete with protein folding and they would occur prior to disulfide bond formation (213). In this context, my application of PDI to the expression system of foreign genes suggests that the ER-resident chaperones and/or PDI itself would

promote the folding pathway, competing for the aggregation pathway of overexpressed proteins in yeast cells. My system will be helpful for studying the cooperativity among PDI, PPIase, and chaperones during protein folding in the cell and will be improved by the coexpression of some *sec* genes.

Table 10. Strains Used in this Study

Strain	Genotype
YPH499	*MATa, ura3, lys2, ade2, trp1, his3, leu2*
HL1	*MATa, ura3, lys2, ade2, trp1, his3::(HIS3, GAP-HLZM), leu2*
HL2	*MATa, ura3, lys2, ade2, trp1::(TRP1, GAP-HLZM), his3::(HIS3, GAP-HLZM), leu2*
HL10	*MATa, ura3, lys2, ade2, trp1::(TRP1, GAP-HLZM), his3::(HIS3, GAP-HLZM), leu2::(LEU2, GAP-Δ)*
HLP11	*MATa, ura3, lys2, ade2, trp1::(TRP1, GAP-HLZM), his3::(HIS3, GAP-HLZM), leu2::(LEU2, GAP-HPDI)*
HLP12	*MATa, ura3, lys2, ade2, trp1::(TRP1, GAP-HLZM), his3::(HIS3, GAP-HLZM), leu2::(LEU2, GAP-HPDIM)*
HLP21	*MATa, ura3, lys2, ade2, trp1::(TRP1, GAP-HLZM), his3::(HIS3, GAP-HLZM), leu2::(LEU2, GAP-YPDI)*
HLP22	*MATa, ura3, lys2, ade2, trp1::(TRP1, GAP-HLZM), his3::(HIS3, GAP-HLZM), leu2::(LEU2, GAP-YPDIM)*
HLP30	*MATa, ura3::(URA3, GAP-Δ), lys2, ade2, trp1::(TRP1, GAP-HLZM), his3::(HIS3, GAP-HLZM), leu2::(LEU2, GAP-HPDI)*
HLP31	*MATa, ura3::(URA3, GAP-HPDI), lys2, ade2, trp1::(TRP1, GAP-HLZM), his3::(HIS3, GAP-HLZM), leu2::(LEU2, GAP-HPDI)*
HP1	*MATa, ura3::(URA3, GAP-HPDI), lys2, ade2, trp1, his3, leu2::(LEU2, GAP-HPDI)*
HP2	*MATa, ura3::(URA3, GAP-HPDIM), lys2, ade2, trp1, his3, leu2::(LEU2, GAP-HPDIM)*

HPDIM and YPDIM represent h-PDI and y-PDI mutants, respectively.
Δ represents the integration of control plasmid pRSΔ305 or pRSΔ306.

Table 11. Correlation between the Coexpression of PDI and *in vivo* Folding of Lysozyme

Strain	Secreted h-lysozyme	Intracellular h-lysozyme	Intracellular PDI activity	
			RNase assay	TPOR assay
HL10	100	100	100	100
HLP11	128 (28)	250 (150)	121 (21)	112 (12)
HLP12	135 (35)	200 (100)	101 (1)	101 (1)
HLP21	149 (49)	550 (450)	243 (143)	248 (148)

Average values obtained from the experiments described in the legends to Figs. 23, 24, and 25 were used for the amount of secreted h-lysozyme. Values for intracellular h-lysozyme were estimated as the amount of h- lysozyme per total protein in the crude cell extracts. For the RNase and TPOR assays, crude cell extracts containing 760 μg of total proteins were used. Values for intracellular h-lysozyme, the RNase assay, and the TPOR assay represent the mean from two independent experiments. In all columns, values for HL10 are set to 100 %. Net increase of each value is represented in a parenthesis.

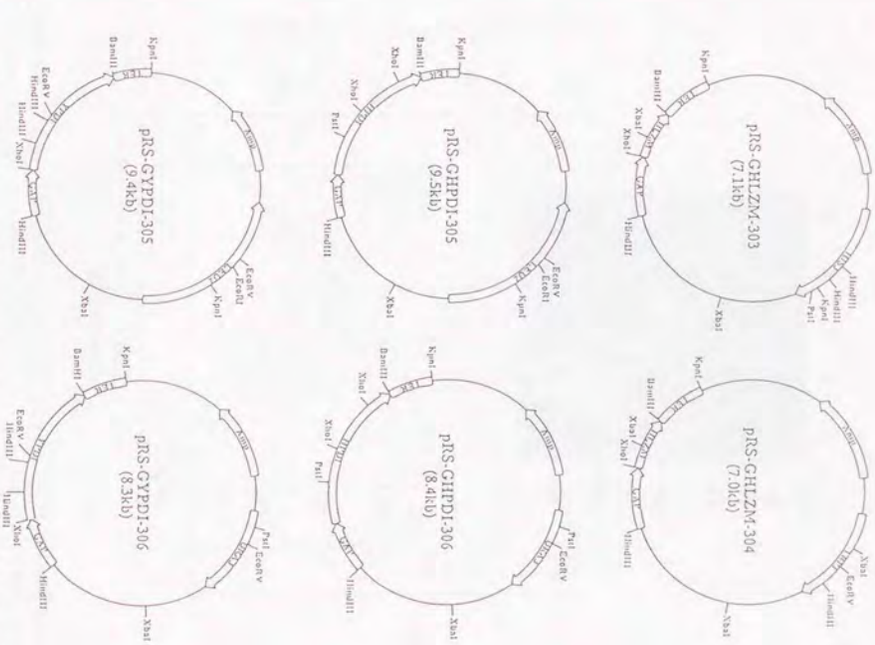
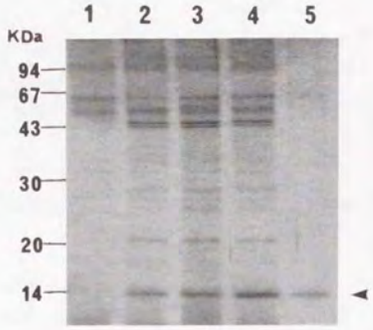


Fig. 22. Restriction maps of expression vectors of h-lysozyme, h-PDI, and y-PDI. HLZM, HPDI, and YPDI represent coding regions of h-lysozyme, h-PDI, and y-PDI, respectively. GAP and TER show the coding sequences of the yeast glyceraldehyde 3-phosphate dehydrogenase promoter and the yeast alcohol dehydrogenase terminator, respectively. Four selective marker genes (HIS3, TRP1, LEU2, and URA3) are shown in each vector. The vector sizes are represented in parentheses. Physical maps of the expression vectors of mutant h-PDI (pRS-GHPDIM-305) and y-PDI (pRS-GYPDIM-305) are the same as those of wild type h-PDI (pRS-GHPDI-305) and y-PDI (pRS-GYPDI-305) except that coding sequences of mutant h-PDI and y-PDI carry the mutations in their catalytic sites.

A



B

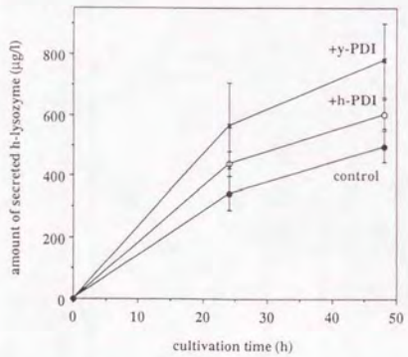


Fig. 23. The effect of h-PDI and y-PDI on h-lysozyme secretion. A. Yeast strains were grown in the YPD medium for 24 h at 30 °C and 750 ml of cultures were concentrated by ethanol precipitation after removing the cells by centrifugation. The resulting samples were resolved by 13.5 % SDS-polyacrylamide gel electrophoresis (SDS/PAGE) and the gel was stained with Coomassie Brilliant Blue R-250. The results of SDS/PAGE of typical clones are shown. Molecular weight standards are indicated on the left. The bands of h-lysozyme are denoted by arrow heads. Lane 1: yeast strain YPH499. Lane 2: HL10. Lane 3: HLP11. Lane 4: HLP21. Lane 5: 0.2 μg of purified h-lysozyme. B. Time course of the secretion of h-lysozyme from strains, HL10 (● : control), HLP11 (○ : +h-PDI), and HLP21 (× : +y-PDI). The amount of secreted h-lysozyme was estimated for cultures at 24 and 48 h by measuring the lytic activity of h-lysozyme. Ten independent clones were analyzed for each strain.

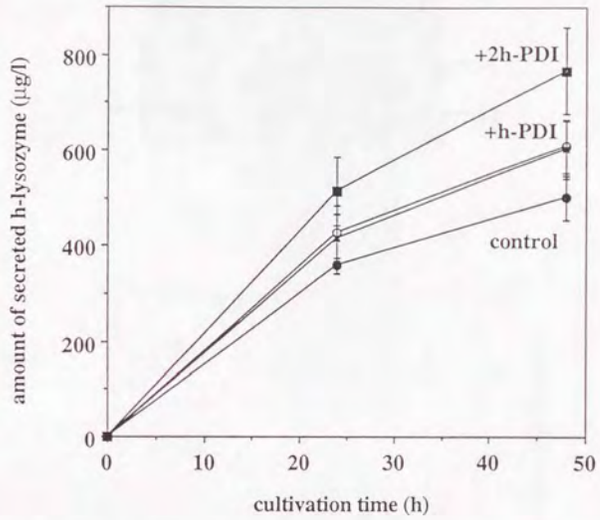
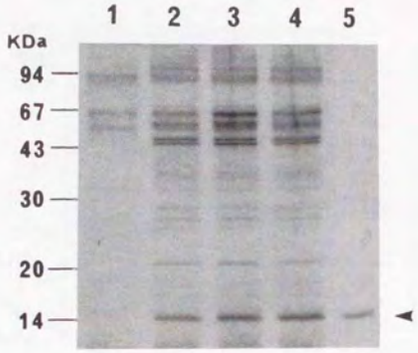


Fig. 24. The dose effect of h-PDI on h-lysozyme secretion. The dose effect of h-PDI on the secretion of h-lysozyme was investigated as described in the legend to Fig. 23. HL10 (● : control). HLP11 (○ : +h-PDI). HLP30 (×). HLP31 (■ : +2h-PDI).

A



B

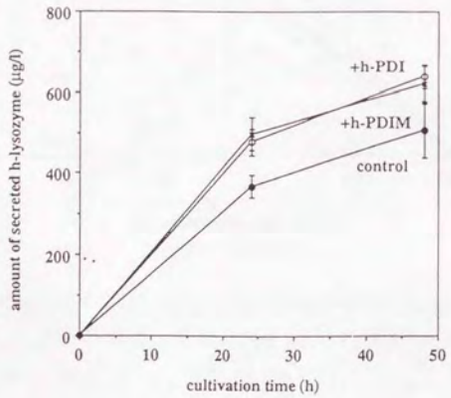


Fig. 25. The effect of mutant h-PDI on h-lysozyme secretion. The effect of mutant h-PDI, lacking isomerase activity, on the secretion of h-lysozyme was investigated as described in the legend to Fig. 23. A. Lane 1, YPH499. Lane 2: HL10. Lane 3: HLP11. Lane 4: HLP12. Lane 5: 0.2 μg of purified h-lysozyme. B. HL10 (● : control). HLP11 (○ : +h-PDI). HLP12 (× : +h-PDIM).

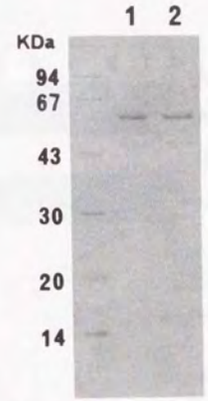


Fig. 26. SDS/PAGE of recombinant wild type and mutant h-PDIs. 3μg of purified recombinant wild type (lane 1) and mutant (lane 2) h-PDIs were resolved by 13.5 % SDS/PAGE, respectively, followed by staining with Coomassie Brilliant Blue. Molecular weight standards are shown on the left.

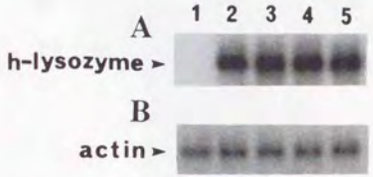


Fig. 27. The effect of h-PDI on the transcription of h-lysozyme gene. Total RNA (10μg) purified from yeast strains YPH499 (lane 1), HL10 (lane 2), HLP11 (lane 3), HLP12 (lane 4), and HLP21 (lane 5), was separated by 1 % agarose gel electrophoresis, transferred to a nylon membrane, and probed with a ^{32}P-labeled h-lysozyme coding DNA fragment (A). The membrane was reprobed with ^{32}P-labeled yeast actin gene (B).

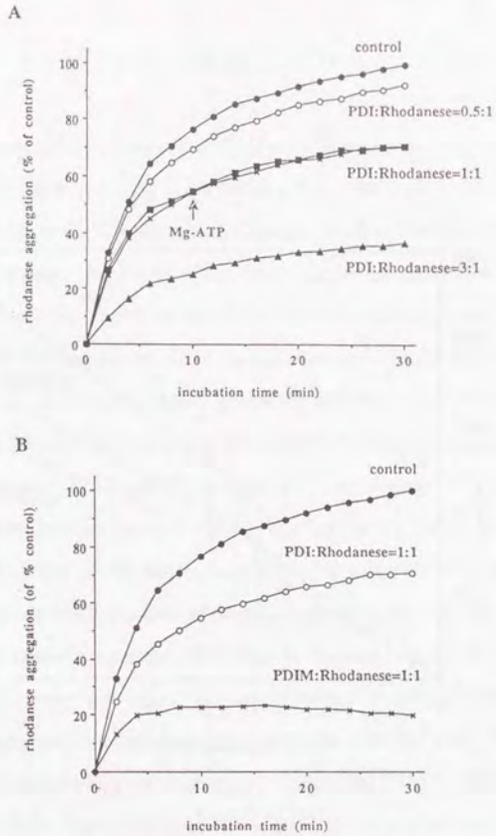


Fig. 28. Chaperone-like activity of h-PDI. A. The suppression of denatured rhodanese aggregation by h-PDI was investigated. Bovine rhodanese (800 μg) was denatured in 1 ml of buffer A at 25 °C for 1 h and diluted (0.46 μM final concentration) 100-fold into buffer C (30 mM Tris-HCl, pH 7.2, 50 mM KCl) containing 0 (●), 0.23 (○), 0.46 (×), and 1.38 μM (▲) recombinant h-PDI. The aggregation of denatured rhodanese was investigated by monitoring the increase in absorbance at 320 nm. The effect of Mg-ATP was also investigated. Ten min after the dilution of denatured rhodanese (0.46 μM) in buffer C containing 0.46 μM h-PDI, 1 mM ATP and 5 mM magnesium acetate at final concentration were added (■). Aggregation in the absence of h-PDI was set to 100 %. B. The effect of the mutant h-PDI on the suppression of denatured rhodanese aggregation was investigated. As described above, rhodanese was denatured and diluted in buffer C in the presence of 0.46 μM wild type h-PDI (○) and 0.46 μM mutant h-PDI (×). Aggregation in the absence of h-PDI (●) was set to 100 %.

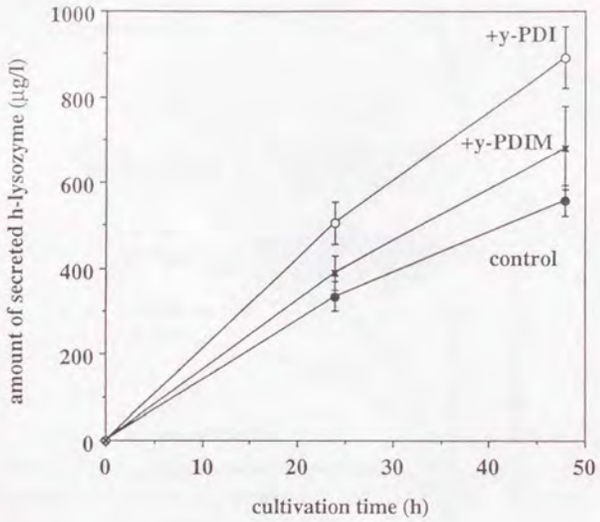


Fig. 29. The effect of mutant y-PDI on lysozyme secretion. The effect of mutant y-PDI, lacking isomerase activity, on the secretion of h-lysozyme was investigated as described in the legend to Fig. 2. HL10 (● : control). HLP21 (○ : +y-PDI). HLP22 (× : +y-PDIM).

3.3. PERSPECTIVE

PDI is now one of the best understood proteins which are believed to be involved in the pathway of protein folding in the cell and then is the most appropriate target to be studied for elucidation of the mechanism of protein folding. However, there are a lot of unanswered questions. Here I describe current problems about PDI in view of my findings described above and the recent studies reported by other groups.

Whereas PDI accelerates one of the rate-limiting steps during protein folding *in vitro*, its catalysis seems to be modest, comparing with that *in vivo*. Catalytic activity is 0.43 min^{-1} for the generation of native RNase A from reduced protein, 3 min^{-1} for the catalysis of folding of BPTI, and 12 min^{-1} for reduction of insulin (221). Discrepancy of the folding rates between *in vitro* and *in vivo* can be explained by the following possibilities. (i) There would exist a recycling system of PDI in the cell. When PDI catalyzes oxidative disulfide bond formation in proteins, its catalytic centers become reduced. The reoxidation system of PDI would be required for the turnover of PDI. It is proposed that the vitamin K cycle, which is involved in γ-carboxylation process in the ER, might act as a source of oxidizing equivalents for PDI (214). (ii) There would be a variety of PDI with distinct substrate specificities. ERp72 (215), ER60 (216) and P5 (218) are classified into a family of PDI-related proteins in terms of the sequence similarities around the catalytic sites (*a* and *a'* domains) to PDI. It is not known whether they have PDI activity, but overexpression of ERp72 in CHO cells does not lead to an increase in PDI activity (218). (iii) The catalytic action of PDI would be modulated by some components present within the ER, however, it is currently suggested that there are no synergistic effects due to the presence of other components in the cell from the observation that the specific activity of a total reticuloplasm extract in catalyzing refolding and reoxidation of BPTI was 11 % of that of homogeneous PDI and that PDI comprises 11 % of the extract (219).

On the other hand, there is a possibility that the actual activity of PDI in the cell is as modest as that *in vitro*. In this case, more efficient catalysis of PDI *in vivo* can be explained by a proposal that a number of combinations of disulfide bonds in polypeptides which are formed incorrectly *in vitro* is much greater than that *in vivo*. In the cell, nascent polypeptides are translocated into the luminal space of the ER through the membrane in linear forms and folding occurs simultaneously when the amino-termini of the nascent polypeptide chains emerge within the lumen of the ER, resulting in the restriction of the combinations of incorrect disulfide bonds formed in folding intermediates, while the combinations are free from the restriction during refolding of denatured proteins *in vitro*. Thus it may take shorter times for PDI to shuffle incorrect disulfide bonds *in vivo*.

In spite that a long time has passed since the method of purification of PDI was established and that it is easy to obtain a large amount of PDI due to its high content in the cell, analysis on its three-dimensional structure has not been successful. I have been also trying to determine its three-dimensional structure, collaborating with Dr. Inaka and Dr. Miki but have not succeeded in obtaining its crystal, possibly due to its high solubility in water. Now I am trying to crystallize it by changing crystallizing conditions and using PDIs of different origins. Based on the similarities of amino-acid sequences between PDI and thioredoxin, of which three-dimensional structure is known, the three-dimensional structures around the catalytic centers of PDI has been proposed. However, the determination of the whole structure of PDI is needed for understanding of its multiple roles, which thioredoxin does not have.

As described above, some of the multiple roles of PDI have been identified. Out of them, recent studies including my works described in the previous chapter have focused on the chaperone-like activity of PDI and have shown that PDI acts as a chaperone *in vitro*. However, there is a doubt about its *in vivo* function as a chaperone because a regulatory factor of the dissociation of PDI from bound folding intermediates remains obscure, while ATP is shown to modulate that of other well-established

chaperones such as HSP70 and HSP90. Further study will provide a picture of the chaperone-like function of PDI. Finally my studies on the multiple roles of PDI involved in the pathway of protein folding in the cell, i. e., catalysis of disulfide bond formation, induction of conformational change of folding intermediates, prevention of aggregation of folding intermediates, will lead to my advanced understanding of the mechanism of protein folding in the cell.

CHAPTER 4

Perspective of Research on Protein Folding in the Cell

Our understanding of various factors of direct relevance to protein folding *in vitro* has increased enormously during the past twenty or thirty years, however, important questions remain to be answered about the *in vivo* function of these factors. In this chapter, I describe some targets of research on protein folding in the cell that should be elucidated.

Studies on *in vitro* refolding of proteins has shown that some proteins are liable to aggregate and unable to refold spontaneously whereas others are able to spontaneously refold. However this classification of proteins cannot hold in the case of *in vivo* protein folding because there exists a quite difference between inside and outside of living organism. For example, there is no restriction in formation of incorrect disulfide bonds during *in vitro* refolding of a disulfide-bonded protein, while there exits restriction in that during *in vivo* folding because a nascent polypeptide is ready to fold just when a part of its amino-terminal translocates into the luminal space of the microsome. Here we can easily imagine that a number of possible dead-end intermediates *in vivo* resulting from the formation of incorrect disulfide bonds is quite less than that *in vitro*. Thus we should investigate systematically which protein can fold *in vivo* without the assistance of foldases and molecular chaperones and which protein cannot. We should also know why dead-end intermediates, which are destined to be degraded, cannot be recognized and refolded by foldases and molecular chaperones. These studies, coupled with elucidation of substrate specificities of PPIase, PDI, and chaperones, will lead to our advanced

understanding of the sequences or structural signals that dictate the *in vivo* folding pathway.

No information on the order of action of foldases and molecular chaperones in the pathway of *in vivo* protein folding is available. In the case of *E. coli* chaperones, such as GrpE, DnaK, and GroE, their order of action has been shown in *in vitro* reconstitution system. However, they could function in a different manner in the cell because the order might be controlled by their intracellular compartmentation. Genetical analysis will provide us much information on the hierarchy of their action. The coexpression system described in **CHAPTER 3.2** will be also helpful for studies on functional cooperativity of foldases and molecular chaperones.

Finally, I propose a new approach to analyze the pathway of *in vivo* protein folding and the mode of action of foldases and molecular chaperones. A cell-free translation system has been constructed using a continuous flow of the feeding buffer through the reaction mixture and a continuous removal of a polypeptide product (245). This system has an advantage in producing a target polypeptide in enough amount for the biochemical analysis under well-controlled condition. Simon *et al.* has constructed a system in which pancreatic microsome vesicles are fused to one side of a planar lipid bilayer separating two aqueous compartments. Although this system has been developed originally to investigate a protein-conducting channel of the ER membrane, I think that it can be applied as a model system for studies on translation-coupled translocation of a nascent polypeptide. And we know that hydrogen exchange analysis has given us much information on detailed structure of *in vitro* folding intermediates. Although all of these systems, of course, have been constructed and used for separate studies, I think that improvement and combination of them will bear a new approach to analyze directly the *in vivo* folding pathway of a nascent polypeptide and that to study the *in vivo* function of foldases and molecular chaperones.

Our further understanding of protein folding *in vivo* will enable us to predict whether a particular protein can be expressed in the desired manner in a variety of host-cell systems and to produce a great amount of a protein which is useful to biological research and medical application.

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