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Enzyme stabilization by domain insertion into a thermophilic protein

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Insufficient kinetic stability of exoinulinase (EI) restricts its application in many areas including enzymatic transformation of inulin for production of ultra-high fructose syrup and oligofructan, as well as fermentation of inulin into bioethanol. The conventional method for enzyme stabilization involves mutagenesis and therefore risks alteration of an enzyme's desired properties, such as activity. Here, we report a novel method for stabilization of EI without any modification of its primary sequence. Our method employs domain insertion of an entire EI domain into a thermophilic scaffold protein. Insertion of EI into a loop of a thermophilic maltodextrin-binding protein from *Pyrococcus furiosus* (PfMBP) resulted in improvement of kinetic stability (the duration over which an enzyme remains active) at 37°C without any compromise in EI activity. Our analysis suggests that the improved kinetic stability at 37°C might originate from a raised kinetic barrier for irreversible conversion of unfolded intermediates to completely inactivated species, rather than an increased energy difference between the folded and unfolded forms.

Keywords: exoinulinase/insertion/maltodextrin-binding protein/protein/stabilization

Introduction

Exoinulinase (EI) is a monomeric glycoside hydrolase catalyzing the release of the terminal fructose from the non-reducing end of the plant storage carbohydrate inulin (Pandey *et al*., 1999; Chi *et al*., 2009). Enzymatic hydrolysis of inulin by EI has a wide range of applications, including production of ultra-high fructose syrup and oligofructan, as well as fermentation into bioethanol (Pandey *et al*., 1999; Chi *et al*., 2009). The operating conditions for enzymatic EI processes are mild: aqueous media at ambient temperature and neutral pH (Pandey *et al*., 1999; Chi *et al*., 2009). The progress of enzymatic EI processes, where EI is secreted or supplied extracellularly, may be directly affected by the stability of the enzyme used (Rocha *et al*., 2006; Catana *et al*., 2007; Singh *et al*., 2008). Kinetic stability, the duration over which an enzyme remains active, is one of the critical attributes determining the economic feasibility of enzymatic processes (Eijsink *et al*., 2005; Polizzi *et al*., 2007). Enhanced kinetic stability can reduce operational and storage costs by extending the lifetime of an enzyme (Polizzi *et al*., 2007).

Successful attempts have been reported in the rational, combinatorial and data-driven design of kinetically stable enzymes (Declerck *et al*., 2003; Eijsink *et al*., 2004, 2005; Bommarius *et al*., 2006; Vázquez-Figueroa *et al*., 2007; Hernandez-Rocamora *et al*., 2008; Spadiut *et al*., 2009). These methods for enzyme stabilization require knowledge of 3D enzyme structures, understanding of forces and interactions affecting enzyme stability or construction and screening of diverse libraries. Enhanced stabilization achieved by these conventional methods involves changes in side chains of target protein residues, usually in the form of point mutations. These changes can often compromise an enzyme's intrinsic properties. Conventional approaches can sometimes result in identification of protein variants with improved stability without compromised activity (Eijsink *et al*., 2004, 2005; Bommarius *et al*., 2006), but these are in general quite rare and difficult to predict. Mutation of residues to those commonly found in naturally existing thermostable counterparts can improve stability of mesophilic proteins without activity loss (Lehmann and Wyss, 2001). Unfortunately, only a small fraction of thermophilic proteins in nature have been identified, and natural thermophilic proteins with desired activity and selectivity are not always available. Chemical modification and immobilization have been used for improving enzyme kinetic stability by reducing conformational flexibility (Abian *et al*., 2004; Srimathi *et al*., 2006; Villalonga *et al*., 2006). However, reduced conformational flexibility by modification and immobilization usually results in a significant loss of enzymatic activity (Fernández-Lafuente *et al*., 2001; Kranz *et al*., 2007). Molecular chaperones have also been used for improving kinetic stability of an enzyme *in vitro* (Millard *et al*., 2003; Miyawaki *et al*., 2008). For example, addition of GroES, GroEL and ATP *in vitro* increased kinetic stability of alcohol dehydrogenase at 50°C by 2-fold (Kohda *et al*., 1996). Similarly, the chaperone activity of αB-crystallin prevented unfolding and aggregation of citrate synthase at 45°C (Muchowski and Clark, 1998). However, addition of chaperones for stabilization is not practical due to the requirement of a relatively large dose. One may also stabilize an enzyme by domain fusion to chaperone. For example, the *in vitro* kinetic stabilization of an enzyme by end-to-end fusion to chaperonin along with supply of ATP has recently been reported (Bergeron *et al*., 2009).

Maltodextrin-binding proteins from various sources, such as the mesophile *Escherichia coli* and the hyperthermophile *Pyrococcus furiosus*, have been found to assist in the folding of end-to-end fusion partners which otherwise form inclusion bodies during intracellular production (Fox *et al*., 2003). A maltodextrin-binding protein from *E.coli* (EcMBP) also improved kinetic stability of end-to-end fused single-chain antibodies *in vitro* (Bach *et al*., 2001). EcMBP on its own without any fusion, when mixed in excess, increased the rate

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of functional refolding of denatured proteins *in vitro* (Richarme and Caldas, 1997). Based on these findings, we hypothesized that specific kinetic stabilization of EI might be achieved through domain fusion to a maltodextrin-binding protein.

Domain fusion requires no change of the primary sequence of target enzymes, so that their intrinsic properties might be largely maintained while enhancing stability, if fusion sites are adequately selected and relative domain orientation and connectivity are properly chosen. The easiest and most widely used fusion is an 'end-to-end' connection, where the N-terminus of one protein is connected to the C-terminus of the other. However, whether end-to-end fusion is optimal for stabilization of fusion partners has yet to be examined. Two protein domains can also be connected by 'insertional' fusion, in which one protein (e.g. a target enzyme) is inserted into the middle of the other (e.g. a scaffold protein). Since insertion involves more than one connection, the resulting fusion protein has the potential to form a more stable structure if an insertion site is properly selected (Doi and Yanagawa, 1999). Theoretical and experimental studies have demonstrated that the insertion of long sequences may destabilize a protein (Viguera and Serrano, 1997; Zhou, 2004), presumably by entropically disfavoring the occurrence of contact between residues leading to rapid folding (Zhou, 2004). Indeed, insertional fusion often results in formation of non-functional protein complexes such as inclusion bodies (Martineau *et al*., 1996; Betton *et al*., 1997). The success of domain insertion may depend on the stability of the scaffold protein. For this reason, we employed a thermostable maltodextrin-binding protein from the hyperthermophile *P.furiosus* (PfMBP) as a stabilizing scaffold into which the EI domain was fused. The 3D structure of EI used in the current study has yet to be determined. The protein termini of EI from *Aspergillus awamori* (Fig. 1A), which displays 37% sequence identity to EI used in our study, are in close proximity (Nagem *et al*., 2004), therefore it would be expected to produce functional fusions at a relatively high rate.

Here, we report kinetic, but not necessarily thermodynamic, stabilization of the EI domain by insertional fusion into PfMBP. An inserted EI domain displayed EI activity similar to the wild-type EI under various conditions. No kinetic stabilization of the EI domain was observed when co-incubated with equimolar PfMBP, nor upon end-to-end fusion to PfMBP, suggesting that stabilization was specific to domain insertion. We discuss the possible mechanism of kinetic stabilization by domain insertion into PfMBP and its potential applications.

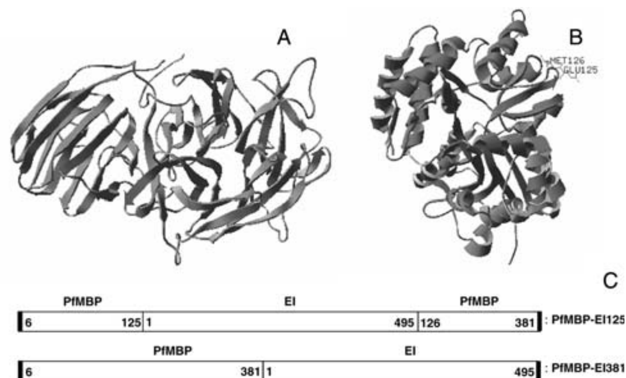

Fig. 1. Ribbon diagram of (**A**) EI from *A.awamori* (PDB code: 1Y9M) and (**B**) PfMBP (PDB code: 1ELJ). The residues 125 and 126 of PfMBP are shown. (**C**) Amino acid sequences of PfMBP-EI125 and PfMBP-EI381. The numbers indicate the amino acid numbers of given proteins. The first five residues of PfMBP numbered according to (Evdokimov *et al*., 2001) originate from the wild-type EcMBP, not the wild-type PfMBP (Evdokimov *et al*., 2001), and therefore were not included.

Materials and methods

Reagents

Oligonucleotides were purchased from Operon Biotechnologies Inc. (Huntsville, AL, USA). High fidelity Platinum Pfx DNA polymerase and Electromax DH5α-E cells were purchased from Invitrogen (Carlsbad, CA, USA). All DNA purification kits were purchased from Qiagen (Valencia, CA, USA). His-tag protein purification kits and columns were purchased from Novagen (Madison, WI, USA) and GE Healthcare (Buckinghamshire, England, UK), respectively. Restriction enzymes and T4 DNA ligase were purchased from New England Biolabs, Inc. (Ipswich, MA, USA). Inulin, antibiotics and biological reagents were purchased from Thermo Fisher Scientific (Waltham, MA, USA).

DNA construction

The plasmid, pREX12 (Kim *et al*., 2004), for expression of the wild-type EI was kindly provided by Dr S.I. Kim (Seoul National University, Seoul, Korea). PCR was used for replicating DNA sequences coding for the entire maltodextrin-binding protein from *P.furiosus* (PfMBP) from plasmid FLIPmal_Pf generously provided by Dr W. B. Frommer (Carnegie Institute of Plant Biology, Stanford, CA, USA). A six-histidine tag was genetically attached to the C-terminus of PfMBP for protein purification. The signal sequence of maltodextrin-binding protein from *E.coli* (EcMBP) (residues 1–30) (Puziss *et al*., 1992) was added to PfMBP for export of the protein to the periplasm of *E.coli*. Sequences of PfMBP and EcMBP were aligned beginning with the sixth residue of PfMBP (Evdokimov *et al*., 2001). The desired PCR products were purified by QIAquick PCR purification and QIAquick gel extraction kits.

For construction of PfMBP-EI125 and PfMBP-EI381, DNA sequences coding for the wild-type EI and parts of PfMBP were amplified by PCR from pREX12 and FLIPmal_pf, respectively. The purified DNA fragments were assembled into a full gene by overlap extension PCR. A six-histidine tag was genetically added to the C-terminus of each fusion complex. The signal sequence of EcMBP was included in PfMBP-EI125 and PfMBP-EI381. No additional linker was added between protein domains.

The DNA sequences coding for PfMBP, PfMBP-EI125 and PfMBP-EI381 were digested by *Bam*HI and *Spe*I restriction enzymes to create sticky ends needed for ligation. Plasmid pDIM-C8-MalE (Kim and Ostermeier, 2006) was digested with *Bam*HI and *Spe*I restriction enzymes, and purified by QIAquick gel extraction kit. The digested inserts and plasmids were then ligated using T4 ligase. Ligation products were then electroporated into 40 μl Electromax DH5α-E using a Bio-Rad Gene Pulser (Hercules, CA, USA). Electroporated cells were subsequently incubated for 1 h at

250 rpm and 37°C in a New Brunswick Scientific Innova TM4230 incubator (Edison, NJ, USA). Electroporated cells were then plated on LB agar plate supplemented with 50 μg/ml chloramphenicol and incubated for 16–24 h at 37°C. The colonies growing on LB agar plate supplemented with 50 μg/ml chloramphenicol were picked and re-cultured in test tubes containing 10 ml LB media and 50 μg/ml chloramphenicol. Plasmid DNA was extracted from re-cultured colonies using QIAprep spin mini-prep kit according to the manufacturer's protocol. The extracted DNA was then sequenced at Genewiz, Inc. (South Plainfield, NJ, USA).

Protein expression and purification

One liter of LB media containing 50 μg/ml chloramphenicol was inoculated with 2% overnight culture and shaken at 250 rpm at 37°C. Cells expressing the wild-type EI, PfMBP, PfMBP-EI125 and PfMBP-EI381 were grown at 37°C until the optical density at 600 nm was 0.6. Expression of the wild-type EI, PfMBP, PfMBP-EI125 and PfMBP-EI381 was then induced by adding 1 mM isopropyl-beta-D-1-galactopyranoside (IPTG). After induction, the cell culture was shaken at 250 rpm for another 16–24 h at 30°C for the expression of the wild-type EI. The other proteins were expressed in a same manner at 23°C. Induction of the wild-type EI at 23°C resulted in significant compromise in expression levels and therefore was not used. Cells were pelleted by centrifuging at 5000 rpm at 4°C for 20 min using a Beckman Coulter Avanti JE centrifuge (Fullerton, CA, USA). The pelleted cells were then stored at −75°C until ready for use. Expression levels of the wild-type EI, PfMBP-EI125 and PfMBP-EI381 were calculated to be 1000, 1200 and 20 U/g wet cell, respectively. The expression level of PfMBP-EI125 corresponded to ~7 mg/g wet cell, which was nearly same as that for PfMBP. Taken together, this indicates that domain insertion did not compromise the level of expression significantly. One unit of EI activity is defined as the amount of enzyme required to liberate 1 μmole of the reducing sugar per min at 37°C.

For protein purification, the pelleted cells were resuspended in 50 mM Tris–HCl buffer containing 0.5 M NaCl, pH 7.5 with a dilution ratio of ~10 ml per gram of cells. The cells were then lysed by French Press (Thermo-Fisher Scientific); the cell lysates were centrifuged at 20 000 rpm at 4°C. Supernatants containing the soluble proteins were then recovered and passed over a Ni^{2+} column. Bound proteins were eluted with imidazole solution and dialyzed at 4°C against at least 15 l of 50 mM Tris–HCl buffer, pH 7.5. Purified protein samples were stored at 4°C for less than 2 weeks prior to characterization of activity and kinetic stability as described below. No difference in terms of activity and kinetic stability of proteins was observed during storage at 4°C for less than 1 month (data not shown). The purities of the proteins were estimated by Coomassie Blue staining of sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) and were greater than 90%. Protein concentrations were determined using the Bradford assay (Bradford, 1976).

Enzyme assay

Hydrolysis of inulin by the wild-type EI, an equimolar mixture of PfMBP plus the wild-type EI, PfMBP-EI125 and PfMBP-EI381 was measured in 50 mM Tris–HCl buffer, pH 7.5 at 37°C, as described previously (Kim *et al*., 2004). The protein concentration of the wild-type EI, PfMBP, PfMBP-EI125 and PfMBP-EI381 in the assay buffer was 1 μM. Protein samples were incubated under designated conditions prior to addition of inulin. The final concentration of inulin was 500 μM in all assays, unless otherwise mentioned. For the measurement of inulin hydrolysis, a reaction mixture containing a protein and inulin was incubated at 37°C for 1 h followed by the addition of 3,5-dinitrosalicylic acid. The reaction mixture was then boiled for 10 min, and the amount of liberated reducing sugar was determined by absorbance at 550 nm (Melius, 1971). Michaelis–Menten kinetic parameters were determined from the initial rate of reaction at various substrate concentrations using Lineweaver–Burk plots.

In order to monitor irreversible inactivation of enzymes over time, samples were withdrawn at different time points of incubation and their enzymatic hydrolyses were measured at 37°C.

Circular dichroism spectroscopy

Secondary structures of proteins were determined using circular dichroism (CD), collected using a Jasco J-815 circular spectrometer (Easton, MD, USA) in the far-UV range with a 0.1 cm pathlength cuvette. The protein samples were withdrawn at several time points during incubation at 37°C. Ellipticity of samples containing 1 μM wild-type EI in the presence and absence of equimolar PfMBP at each wavelength was measured without dilution at 37°C. Ellipticity of samples containing 1 μM PfMBP-EI125 and PfMBP-EI381, respectively was measured in the same way. The spectrum of the background (buffer only) was measured and then subtracted from the sample spectrum.

Intrinsic tryptophan fluorescence

Intrinsic tryptophan fluorescence of protein samples was measured using a Photon Technology QuantaMaster QM-4 spectrofluorometer (Birmingham, NJ, USA). Excitation wavelength was 280 nm and emission was monitored at 337 nm. The protein samples were withdrawn at several time points during incubation at 37°C. Intrinsic tryptophan fluorescence of samples containing 1 μM of the wild-type EI with and without addition of equimolar PfMBP was measured without dilution. Intrinsic tryptophan fluorescence of samples containing 1 μM of PfMBP-EI125 and PfMBP-EI381, respectively was measured in the same way. The spectrum of the background (buffer only) was measured and then subtracted from the sample spectrum.

SDS–PAGE for determination of the amount of soluble proteins

Protein samples were incubated for the designated time period and then centrifuged. The supernatant of each sample was loaded in the SDS–PAGE gel. The bottom fraction remained after centrifugation was collected, resuspended in SDS and loaded in the SDS–PAGE gel. The gel was then stained with Coomassie Blue.

Results

Selection of scaffold protein and insertion site

Maltodextrin-binding protein from the hyperthermophile *P.furiosus* (PfMBP, Fig. 1B) is a 43 kDa periplasmic protein, highly stable against heat and chemical denaturation. PfMBP displays little loss in secondary structure at 85°C or in 6 M guanidine hydrochloride (Evdokimov *et al*., 2001), whereas the mesophilic maltodextrin-binding protein from *E.coli* (EcMBP) unfolds at 65°C or in 1 M guanidine hydrochloride (Ganesh *et al*., 1997). The thermophilic nature of PfMBP might permit insertion sites which would be unavailable in a mesophilic protein due to limited stability (Bloom *et al*., 2006). Structural information on PfMBP is available (Evdokimov *et al*., 2001). We selected the loop-forming residues 125 and 126 of PfMBP as our initial insertion site (Fig. 1B), as surface loops are in general more tolerant to mutations and insertions than other structural units (LaVallie *et al*., 1993; Feliu and Villaverde, 1998; Hiraga *et al*., 2004; Li *et al*., 2008). This loop region of PfMBP was chosen also because the corresponding location of EcMBP is known to be non-permissive (see the text below) so that we could test whether the high stability of PfMBP would allow exploration of larger sequence spaces for insertion.

Exoinulinase activity and structure of the EI domain inserted into PfMBP

We inserted the wild-type EI from *Bacillus sp. Snu-7* (Kim *et al*., 2004) between residues 125 and 126 of PfMBP to create a protein complex named PfMBP-EI125 (Fig. 1C-top) and measured its EI activity at 37°C. PfMBP-EI125 displayed nearly the same activity as the wild-type EI. The Michaelis–Menten kinetic parameters of PfMBP-EI125 for inulin hydrolysis at 37°C were almost identical to those of the wild-type EI (Table I), indicating no compromise in activity by domain insertion. To further assess the catalytic proficiency of the EI domain present in PfMBP-EI125, its EI activity was measured using 500 μM of inulin at different concentrations of urea. The ratio of EI activity of PfMBP-EI125 to wild-type EI was 0.96 ± 0.04 at 37°C with no denaturant. Both the wild-type EI and PfMBP-EI125 lost half of their activity at ~1.5 M urea (Fig. 2A), which is fairly low for an average globular protein (Chun *et al*., 1993; Viguera and Serrano, 1997; Bortoleto and Ward, 1999; Ibarra-Molero *et al*., 1999; Ramos *et al*., 1999; Chang and Li, 2002; Latypov *et al*., 2006). The wild-type EI and PfMBP-EI125 denatured at 4 M urea were rapidly 100-fold diluted into buffer and found to restore 30 ± 3 and 35 ± 4% of their respective original activity, suggesting the formation of a kinetically trapped intermediate or the existence of an off-pathway reaction during the refolding process. EI activities of the wild-type EI and PfMBP-EI125 during urea denaturation almost superimpose (Fig. 2A), suggesting that the insertion of the EI domain did not affect its relative thermodynamic stability. The wild-type EI and PfMBP-EI125 displayed similar heat denaturation trends as determined by EI activity (Fig. 2B), further supporting the lack of changes in unfolding energy of the EI domain following domain insertion. Heat denaturation was not reversible in either the wild-type EI or PfMBP-EI125 (Fig. 2C).

To examine the structural change of each domain upon insertional fusion, CD and tryptophan fluorescence of protein samples were measured. The EI protein from *A.awamori* is homologous to the EI domain we used in the current study, and is composed of a β-propeller fold and a β-sandwich-like structure in the N- and C-terminal domains, respectively (Fig. 1A; Nagem *et al*., 2004). PfMBP is composed of 19 α-helices and 17 β-strands. The signature of α-helical and β-strand structures was apparent in the shoulder at 208 nm and the minimum at 216 nm in CD spectra of PfMBP-EI125 and PfMBP-EI381 (Fig. 2D). Overall, the CD spectrum of PfMBP-EI125 was found to be similar to that of an equimolar mixture of PfMBP plus the wild-type EI (Fig. 2D), indicating no significant secondary structural changes of PfMBP and EI domains upon insertional fusion. Tryptophan fluorescence can be regarded as a probe to monitor the overall tertiary structure of proteins, in particular around tryptophan residues. The wild-type EI and PfMBP contain 15 and 10 tryptophan residues, respectively. Intrinsic tryptophan fluorescence spectra of PfMBP-EI125 and an equimolar mixture of PfMBP plus the wild-type EI were similar (Fig. 2E), further supporting the structural maintenance of PfMBP and EI domains upon insertional fusion. EI activity of the wild-type EI with and without equimolar PfMBP was statistically indistinguishable (the ratio of activity of an equimolar mixture to PfMBP-EI125 at 37°C with 500 μM of inulin was 0.97 ± 0.03).

Table I. Michaelis–Menten kinetic parameters of inulin hydrolysis of the wild-type EI, PfMBP-EI125 and PfMBP-EI381 at 37°C

	Protein		
	The wild-type EI	PfMBP-EI125	PfMBP-EI381
k_{cat} (min^{-1})	160 ± 7	180 ± 4	110 ± 3
K_m (mM)	3.4 ± 0.2	3.8 ± 0.3	7.8 ± 0.2
k_{cat}/K_m (min^{-1} mM^{-1})[a]	47 ± 3	47 ± 4	14 ± 1

[a]The errors were evaluated by the propagation of error method. The other errors represent uncertainty at 95% confidence levels.

Stabilization of the EI domain at 37°C by insertion into PfMBP

The wild-type EI was incubated at 37°C and its activity as a function of time was measured. All glassware, tubes and solutions were autoclaved and sterilized, to minimize the chance of contamination or proteolytic degradation during incubation. The wild-type EI lost activity irreversibly during incubation at 37°C (Fig. 3A). Decay in CD and intrinsic tryptophan fluorescence spectra of the wild-type EI mirrored the loss in activity (Fig. 3B and C), indicating that the structural loss is responsible for inactivation. Formation of precipitate was observed after 20-day incubation of the wild-type EI at 37°C (Fig. 4). The soluble and insoluble fractions of the wild-type EI solution after 20-day incubation at 37°C were run on SDS–PAGE for further characterization. An intense background streak was evident in the lane containing the insoluble fraction presumably due to the presence of precipitated insoluble material. The insoluble fraction of the wild-type EI solution after 20-day incubation at 37°C contained both monomeric EI and large SDS-resistant aggregates trapped in the loading well. The bands representing small fragments of proteins were observed in the lane containing the insoluble fraction of EI solution after 20-day

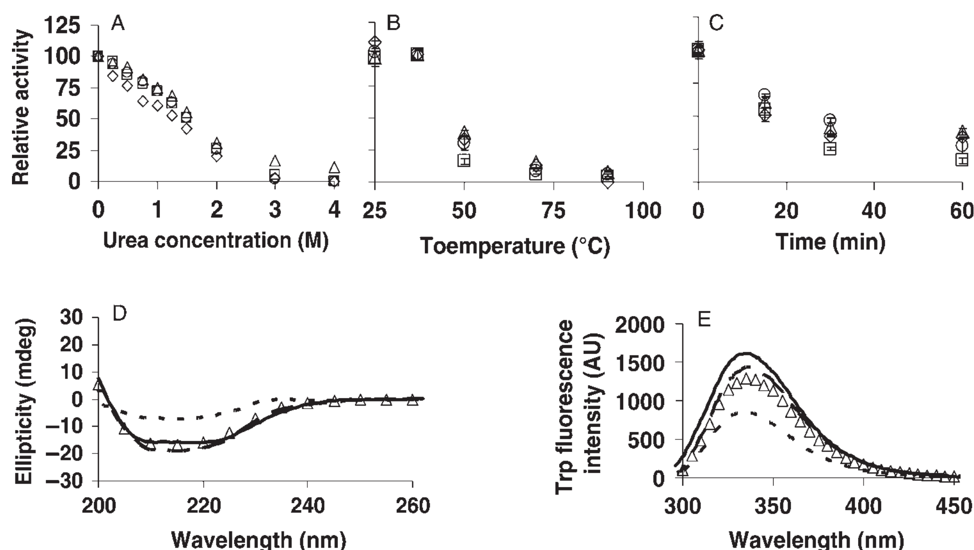

Fig. 2. EI activity of the wild-type EI (open square), PfMBP-EI125 (open circle), PfMBP-EI381 (open diamond) and an equimolar mixture of PfMBP plus the wild-type EI (open triangle) at various (**A**) urea concentrations, (**B**) temperatures and (**C**) time points of incubation at 50°C. Samples were incubated for 1 h at 37°C in (A) or designated temperatures in (B) prior to addition of inulin. EI activity was then measured using 500 μM of inulin at 37°C after 1 h incubation at the same temperature in both (A) and (B). EI activity of each protein sample was determined by the amount of liberated reducing sugar during inulin hydrolysis at 37°C for 1 h by measuring absorbance at 550 nm after reaction with 3,5-dinitrosalicylic acid (Melius, 1971). The relative activity (%) of each protein sample was defined as: (EI activity in a given urea concentration or at a designated temperature)/(EI activity in 0 M urea at 37°C) × 100. Note that specific EI activity of PfMBP-EI381 with 500 μM of inulin at 37°C was found to be ~35% of the wild-type EI at the same condition. (**C**) Samples were incubated at 50°C for designated time periods. Samples were then incubated for 1 h at 37°C followed by the addition of inulin for the determination of EI activity. The relative activity (%) of each protein sample was defined as: (EI activity after incubation at 50°C for designated time periods)/(EI activity at t = 0) × 100. CD (**D**) and tryptophan fluorescence (**E**) spectra of the wild-type EI (dotted line), PfMBP-EI125 (dashed line), PfMBP-EI381 (solid line) and an equimolar mixture of PfMBP plus the wild-type EI (open triangle) measured at 37°C. In (A)–(E), the concentrations of proteins were 1 μM.

incubation as well as fresh wild-type EI solution (Fig. 4). Note that these protein fragment bands seemed darker in the lane containing insoluble fraction because of the intense background streak. No additional small protein fragment bands were detected in both soluble and insoluble fractions of the wild-type EI after 20-day incubation at 37°C. No formation of precipitate or reduction of concentration in the soluble fraction was detected within ~8 days of incubation at 37°C (data not shown) during which the wild-type EI gradually lost half of its initial activity (Fig. 3A). No change in the mass of protein samples was detected during incubation at 37°C as analyzed by mass spectrometry (data not shown), indicating the lack of chemical modification under our experimental conditions. Taken together, the results suggest that the wild-type EI inactivated because of structural changes, which then induced the formation of large precipitates. Indeed, many proteins become irreversibly inactivated by partial or complete unfolding and aggregation (Eijsink *et al*., 2004, 2005).

The kinetic stability of PfMBP-EI125 was evaluated by measuring its activities as a function of time during incubation at 37°C. As desired, the kinetic stability of PfMBP-EI125 was much higher than that of the wild-type EI (Fig. 3A). No precipitation was observed in solutions of PfMBP-EI125 (Fig. 4) and its concentration in solution remained unchanged after 20-day incubation at 37°C (Fig. 4). No significant change in CD and intrinsic tryptophan fluorescence spectra of PfMBP-EI125 was observed (Fig. 3B and C), suggesting that the secondary and tertiary structures were largely maintained. At 50°C, irreversible inactivation of the wild-type EI and PfMBP-EI125 occurred at a similar rate (Fig. 2C). This suggests that kinetic stabilization by domain insertion into PfMBP might not be effective under the conditions causing denaturation of the inserted domain.

No stabilization of the EI domain by co-incubation with PfMBP or end-to-end fusion with PfMBP

To test whether the fusion of the protein domains was required for stabilization at 37°C, we evaluated the kinetic stability of an equimolar mixture of wild-type EI and PfMBP. Co-incubation with PfMBP at an equimolar ratio yielded no significant improvement in the kinetic stability of wild-type EI (Fig. 3) and did not prevent the precipitation observed after 20 days during incubation at 37°C (data not shown). This indicates that stabilization at 37°C was achieved by the linkage between EI and PfMBP, not by non-specific effects caused by the presence of PfMBP. We next examined whether the observed stabilization effect of domain insertion at 37°C could be achieved by end-to-end fusion. PfMBP-EI381, in which EI and PfMBP domains were end-to-end fused (Fig. 1-bottom), displayed CD and tryptophan fluorescence spectra similar to those of an equimolar mixture of PfMBP plus the wild-type EI (Fig. 2D and E), suggesting significant preservation of secondary and tertiary structures after fusion. However, the end-to-end fusion between PfMBP and EI domains within PfMBP-EI381 resulted in reduced activity (~30% of the wild-type EI, Table I) and no improvement of kinetic stability at 37°C (Fig. 3). The formation of precipitate was also observed after 20-day incubation of PfMBP-EI381 at 37°C (data not shown). Thus, simple end-to-end fusion was not sufficient for improving the kinetic stability of EI at 37°C. No stabilizing effect was observed upon either addition of equimolar

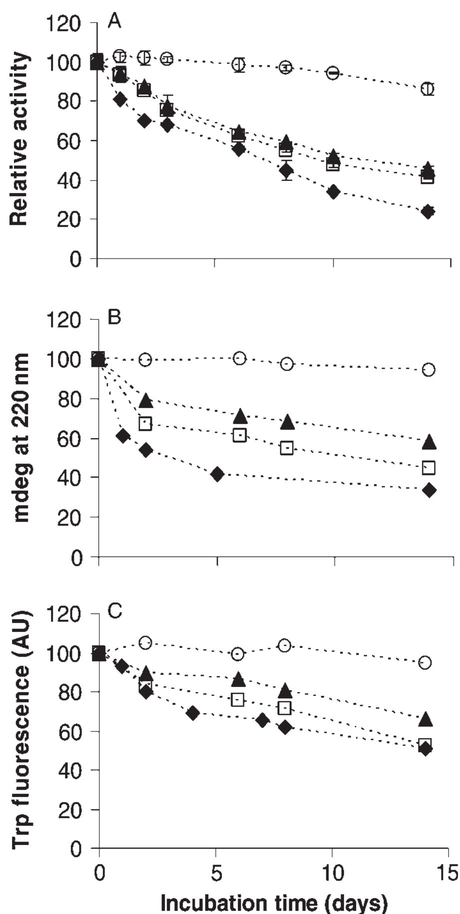

Fig. 3. Time course changes in (**A**) EI activity, (**B**) ellipticity at 220 nm and (**C**) tryptophan fluorescence of the wild-type EI (open square), PfMBP-EI125 (open circle), PfMBP-EI381 (closed diamond), and an equimolar mixture of PfMBP plus the wild-type EI (closed triangle) during incubation at 37°C. The protein concentration of the wild-type EI, PfMBP, PfMBP-EI125 and PfMBP-EI381 in the assay buffer was 1 μM. (A) Activity was measured using 500 μM of inulin at 37°C. Activity of each protein sample at time zero was set to 100%. Activities of the wild-type EI, PfMBP-EI125 and PfMBP plus the wild-type EI were all similar at the beginning of incubation. EI activity of PfMBP-EI381 under this condition was found to be ~35% of the wild-type. The errors represent one standard deviation ($n \geq 3$). The dotted lines are shown for easy reading of data. (B and C) Ellipticity and tryptophan fluorescence at time zero was set to 100% for each protein sample. Excitation wavelength was 280 nm and emission was monitored at 337 nm.

PfMBP or end-to-end fusion to PfMBP during urea and heat denaturation (Fig. 2A and B), as well as incubation at 50°C (Fig. 2C).

Discussion

Here, we report that the domain insertion into PfMBP can significantly improve kinetic stability of EI at 37°C. Unlike conventional stabilization methods of an enzyme that employ mutations and chemical modifications resulting in compromise in its intrinsic properties, the approach described here does not require any change on target protein residues. As a result, the activity of the EI domain was largely maintained. As stability is suggested to promote ‘evolvability’, the improved protein stability without any modification on a target protein might allow exploration of larger sequence spaces for evolution into variants with distinct properties (Bloom *et al*., 2006).

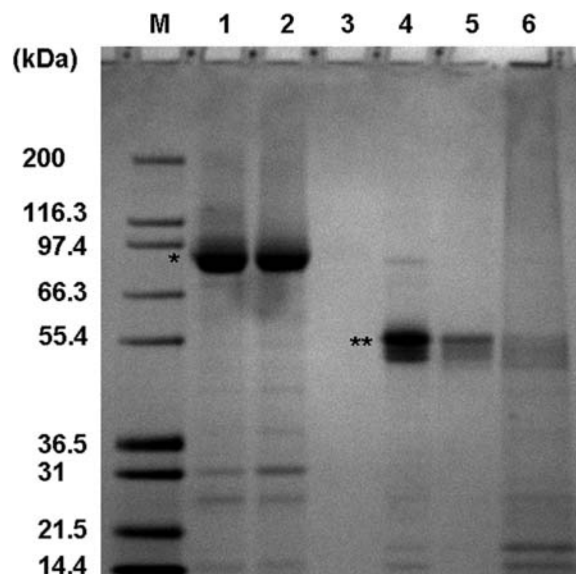

Fig. 4. The amount of soluble protein during incubation at 37°C. Protein samples were centrifuged after 0- and 20-day incubation at 37°C, and the supernatants and the bottom fractions were loaded in a SDS–PAGE gel. All samples contained 1 μM of proteins. M: molecular weight marker. 1: PfMBP-EI125 at the beginning of incubation. 2: PfMBP-EI125 in the supernatant after 20-day incubation. 3: PfMBP-EI125 in the bottom fraction after 20-day incubation. 4: the wild-type EI at the beginning of incubation. 5: the wild-type EI in the supernatant after 20-day incubation. 6: the wild-type EI in the bottom fraction after 20-day incubation. *PfMBP-EI125. **the wild-type EI.

Our results suggest that other intrinsic properties of the EI domain, such as EI activity, secondary structure, microenvironments around tryptophan residues and the relative thermodynamic stability, were largely maintained after insertion into PfMBP. Urea and heat denaturation as determined by EI activity was not reversible for the wild-type EI or PfMBP-EI125. This suggests that the unfolded states were kinetically trapped during the refolding process. Since both the wild-type EI and PfMBP-EI125 lost EI activity to a similar extent during renaturation, the formation of a kinetically trapped intermediate would be likely due to off-pathway folding of the EI domain.

Insertion of long sequences was found both theoretically and experimentally to destabilize proteins in a length-dependent manner (Viguera and Serrano, 1997; Zhou, 2004). A comparison of our results with that of previous insertion studies into EcMBP (Martineau *et al*., 1996; Betton *et al*., 1997) suggests that a stable scaffold domain is required for the inserted domain to acquire improved stability. The structures of PfMBP and EcMBP closely superimpose (Evdokimov *et al*., 2001) and residues 120 and 121 of EcMBP are structurally aligned with residues 125 and 126 of PfMBP (Evdokimov *et al*., 2001). The lengths of loops around these sites are same in both PfMBP and EcMBP (Sharff *et al*., 1992; Evdokimov *et al*., 2001). The location between residues 120 and 121 of EcMBP has previously been shown to be non-permissive (Betton and Hofnung, 1994). For example, insertion of a 263-residue β-lactamase domain into EcMBP between residues 120 and 121 with linkers resulted in the formation of inclusion bodies (Betton

et al., 1997). Induction of the same protein complex from the low copy plasmid pDIM-C8 under control of the *tac* promoter resulted in no detectable protein expression (data not shown), presumably due to proteolytic degradation as was the case with folding-defective EcMBP variants (Betton *et al.*, 1998). The formation of inclusion bodies was also observed in insertion of a short 13-aa peptide sequence into EcMBP at the same site (Martineau *et al.*, 1996). On the contrary, insertion of β-lactamase between residues 125 and 126 of PfMBP resulted in formation of a highly soluble and enzymatically active protein complex with no compromise in the expression level (data not shown). Together with our results on the EI domain inserted into PfMBP described above, this suggests that the inclusion body formation could primarily be due to incomplete folding of EcMBP, and the high stability displayed by PfMBP might allow for insertion into the sequence space, which is not available in the moderately stable EcMBP. Unfortunately, it remains unanswered whether the stability of the scaffold protein could differentiate the extent of *in vitro* kinetic stabilization of an inserted domain. Currently, insertion into previously known permissive sites of EcMBP (Betton *et al.*, 1993, 1997) and the corresponding location of PfMBP, as well as characterization of resultant protein complexes are underway to answer this question.

During early incubation (∼ up to 8 days) at 37°C, the observed activity loss of the wild-type EI was associated with its structural loss. There was no reduction of protein concentrations in the soluble fraction during this early incubation period. The formation of precipitate and reduction of concentration of soluble wild-type EI was only detectable after prolonged incubation (∼20 days at 37°C). Our results suggest that no chemical modification was involved in inactivation of the wild-type EI. To understand how domain insertion stabilizes the EI domain at 37°C, irreversible inactivation of the wild-type EI during incubation was analyzed in the context of the Lumry–Eyring model (Lumry and Eyring, 1954) or the equilibrium model (Peterson *et al.*, 2004; Eisenthal *et al.*, 2006): $N \overset{K_U}{\longleftrightarrow} U \overset{k}{\longrightarrow} I$ where N is the native state, U the unfolded state and I an inactivated state irreversibly formed from U. K_u is the unfolding constant ($=[U]/[N]$), and k is the forward rate constant of irreversible inactivation of the unfolded state(s). Unfolding-induced protein inactivation is quite common in many enzymes (Lumry and Eyring, 1954; Zale and Klibanov, 1983; Millard *et al.*, 2003; Lencki *et al.*, 2004; Meng *et al.*, 2004; Godoy-Ruiz *et al.*, 2006; Ladero *et al.*, 2006). Recent studies suggest that irreversible inactivation of most enzymes follow this model (Peterson *et al.*, 2004; Eisenthal *et al.*, 2006). Urea and heat denaturation of the EI domain was partially reversible, indicating the presence of an irreversible pathway for inactivation as well as a reversible route for restoration of activity. The time course loss of activity at 37°C can readily be explained by the occurrence of an irreversibly inactivated species I. According to this model, enhanced kinetic stability may be achieved through a decrease in K_u or k or both (Zale and Klibanov, 1983; Broering and Bommarius, 2005). In other words, both the unfolding transition between $N \leftrightarrow U$ and the kinetic barrier for $U \rightarrow I$ may determine the overall kinetic stability. Enzymes with a large energy difference between N and U would populate a very small amount of U and therefore barely irreversibly inactivate, unless the rate for $U \rightarrow I$ were high. On the contrary, EI activity of the wild-type EI was significantly reduced at relatively low concentrations of urea (Fig. 2A). This metastable characteristic of the EI domain readily permitted its alteration to the irreversibly inactivated species. Our results show that denaturation of the wild-type EI and PfMBP-EI125 followed almost identical trends as a function of urea concentrations or temperatures. In other words, the native state of the EI domain did not appear to be stabilized by insertion into PfMBP. Therefore, the observed kinetic stabilization at 37°C by domain insertion should mainly be attributed to enhancement of a kinetic barrier for the formation of I from U. This kinetic barrier would, however, be significantly reduced at 50°C regardless of domain insertion, consistent with the lack of a stabilizing effect of domain insertion under these more extreme conditions (Fig. 2C).

Our results indicate that the insertional fusion into PfMBP improved the kinetic stability of the EI domain at 37°C by preventing structural changes and formation of aggregate species. Maltodextrin-binding proteins are known to stabilize end-to-end fusion partners by enhancing solubility. Therefore, one may argue that the improved stabilization of EI by domain insertion into PfMBP could be due to simple enhancement of solubility. However, our results suggest that the improved stability of the EI domain might not merely be due to enhancement of solubility upon fusion to PfMBP for several reasons. The wild-type EI is highly soluble (no precipitation was observed from the wild-type EI solution during at least 8 days of incubation at 37°C), overall anionic (−25 net charge at pH 7.5) and expressed in a soluble form in *E.coli*. Therefore, the effect of solubility enhancement of the EI domain by fusion to PfMBP would be expected to be less than that for more hydrophobic proteins. In addition, end-to-end fusion to PfMBP, which has been known to increase the expression level of proteins by enhancing solubility (Fox *et al.*, 2003), did not improve the kinetic stability of the EI domain in our study.

Unique features associated with insertion compared to end-to-end fusion include the presence of two ‘tethers’ between the domains in the insertional fusion. Insertion of a protein domain reduces conformational flexibility of its N- and C- termini, as is the case with backbone cyclization. Indeed, backbone cyclization through N- and C-terminus connection has been used to improve stability of many proteins by reducing conformational entropy of unfolded states and/or bringing the residues that are distant in the primary sequence close to each other (Zhou, 2004). As a result, backbone cyclization has frequently resulted in changes in unfolding transition during chemical or heat denaturation (Scott *et al.*, 1999; Camarero *et al.*, 2001; Takahashi *et al.*, 2007), which was not observed in PfMBP-EI125 (Fig. 2A and B). However, it cannot be excluded that insertional fusion into other sites might affect thermodynamic stability. Unfolding leading to irreversible inactivation could occur locally (Eijsink *et al.*, 2005). In this context, mutations in protein regions whose unfolding initiates the irreversible inactivation process could improve kinetic stability (Eijsink *et al.*, 2004). Two tethers present in PfMBP-EI125 could prohibit the EI domain from forming irreversibly inactivated structures in a similar manner. An extensive study of backbone cyclization on kinetic stabilization would be required to test this.

Absence of stabilizing effects of PfMBP upon equimolar co-incubation with the wild-type EI suggests the importance of linkage between the two domains (Fig. 3). Both insertional and end-to-end fusions bring PfMBP and EI domains in close proximity; however, the significant difference between insertional and end-to-end fusions is a relative orientation of EI and PfMBP domains. Distinct structural arrangements between two domains could differentiate inter-domain interactions. The inter-domain interaction could be stabilizing the EI domain, as the structurally similar EcMBP can exert a non-specific chaperone activity *in vitro* when present in excess (Richarme and Caldas, 1997). Chaperone activity of PfMBP co-incubated in excess with EI under our experimental conditions was not tested, since high concentrations ($>$10–100 μM$\cong$0.4–4 mg/ml) of a protein could also stabilize other proteins in different ways (Mitsui and Mizuno, 1969), such as occupying surfaces of vessels to which unfolded proteins may be adsorbed (Lilie *et al.*, 1993). The inter-domain interaction would be understood better with structural information on PfMBP-EI125. Also, study of domain insertion into different sites of PfMBP would lead us to better understand the major determinants of stabilization of an inserted enzyme: reduced conformational entropy, inter-domain interactions or a combination of both.

The enzyme stabilization achieved by the described method can potentially be applied to other proteins. Neither of the proposed mechanisms of target enzyme stabilization (entropic effects mediated by multiple tethering of the insertional fusions; or inter-domain interactions with the 'chaperoning' PfMBP) depends precisely on properties of the target protein (although context-dependent effects are guaranteed). Insertional fusion can be readily achieved by site-specific and random methodologies (Ostermeier, 2005). The close proximity of the N- and C-termini of an inserted protein seems to increase the chance of successful insertion. Nearly 50% of single-domain proteins have their N- and C-termini proximal (Krishna and Englander, 2005), indicating the potential application of the described method to a wide range of proteins. The study of insertion of other proteins at various sites of PfMBP will be required to test the generality of this method.

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