The Effect of Folding Catalysts on the *In Vivo* Folding Process of Different Antibody Fragments Expressed in *Escherichia coli*

Achim Knappik, Claus Krebber and Andreas Plückthun*

Max-Planck-Institut für Biochemie, Protein Engineering Group, Am Klopferspitz, D-8033 Martinsried, Fed. Rep. Germany. *Corresponding author.

The F_v and F_{ab} fragment and both orientations of the single-chain F_v fragment (V_H-linker-V_L and V_L-linker-V_H) of an antibody can be expressed in functional form in the periplasm of *Escherichia coli*, but the yield of these correctly assembled proteins is limited by the periplasmic folding process. While the periplasmic *E. coli* disulfide isomerase DsbA is *required* for this assembly, its functional over-expression does not significantly change the folding limit. Similarly, the functionally over-expressed *E. coli* proline *cis-trans* isomerase does not change the amount of all but one of the antibody fragments, not even if DsbA is over-expressed as well. Therefore, aggregation steps in the periplasm appear to compete with periplasmic folding, and they may occur before disulfide formation and/or proline *cis-trans* isomerization takes place and be independent of their extent.

Received 9 September 1992; accepted 27 October 1992.

The expression process leading to native antibody fragments in *Escherichia coli* has been investigated, and it was shown that correctly folded and functional F_v and F_{ab} fragments can assemble if both chains are simultaneously secreted into the periplasmic space or linked to form a secreted single-chain F_v fragment[1-3], but that the yield of functional product appears to be limited by the folding process in the periplasm[4].

While protein folding is an exergonic process which occurs spontaneously *in vitro* (for reviews see refs. 5–7), and the final conformation is determined by the sequence, cellular factors seem to play a role in guiding this process (for reviews see refs. 8–10). Two structural elements have been discovered whose formation may be catalyzed by specific proteins: the formation and rearrangement of disulfide bonds and the *cis-trans* isomerization of prolyl peptide bonds. Additionally, cellular factors may be important in preventing side reactions such as aggregation[8-12].

Fischer[13] and co-workers discovered an enzymatic activity which is able to catalyze the *cis-trans* isomerization of proline imidic peptide bonds in oligopeptides. Because of the partial double bond character of the peptide bond, this process is slow[14] and can be a rate determining step in protein folding[14-16]. In oligopeptides, the equilibrium population of the *cis* form of Xaa-Pro bonds is about 10–30%[14,17,18]. In folded proteins, however, the conformation adopts to the requirements of the three-dimensional structure, and it is presumed that all members of a population of protein molecules have the same stereochemistry at every given bond. The frequency of *cis*-prolyl bonds in known structures is about 6.5%[19]. *In vitro* experiments have shown that the refolding of a number of proteins, but not all, containing *cis*-prolyl bonds can be somewhat accelerated by prolyl-*cis-trans* isomerase (PPIase) from various sources[20-24]. It was also shown previously[22] that the refolding of the light chain of the mouse monoclonal antibody Mab33, which shows slow refolding behavior due to *cis-trans* isomerization[25] can be catalyzed with PPIase *in vitro*. Furthermore, a fusion protein of a single-chain antibody with a toxin was obtained in slightly better yield, when *in vitro* refolding was carried out in the presence of PPIase[26]. Two *E. coli* proteins (PPIase A and B) have been discovered[27,28] which show sequence homologies to the cyclophilin group of eukaryotic PPIases[29], and they exhibit *cis-trans* isomerase activity[23,27]). PPIase B is a cytosolic enzyme, whereas PPIase A, the product of the *rot* gene[27], which has been demonstrated to act as a folding catalyst for RNAse T1 *in vitro*[23], is localized in the periplasm. However, no experiment has yet been reported to unequivocally demonstrate an *in vivo* role of this catalysis.

An enzymatic activity accelerating the isomerization of disulfide bonds was discovered in the microsomal fraction of various tissues[30,31]. It is now known as protein disulfide-isomerase (PDI) and has been characterized from various sources[32] and cloned and sequenced from several species[33]. In the eukaryotic cell, the disulfide bond isomerization is catalyzed by PDI in the endoplasmic reticulum (ER). In *E. coli*, the formation of disulfide bonds takes place in the periplasmic space[34]. Using genetic screens, an *E. coli* activity was recently discovered which facilitates the periplasmic formation of disulfide bonds of several proteins[35-37].

However, no *in vivo* experiments have been reported yet to demonstrate that these activities can overcome the limitation of the folding process of recombinant proteins, and the precise *in vivo* function of these putative folding catalysts remains unknown. As different recombinant proteins will have different rate-limiting steps in their folding, it is unlikely that a general effectiveness or ineffectiveness of these catalysts for all recombinant proteins will be found.

In this study we have used several fragments of the well characterized antibody McPC603. The 3D-structure[38] shows that it possesses 5 *cis*-proline and 5 disulfide bonds in the F_{ab} fragment. Its expression in *E. coli* was shown previously to be limited by the periplasmic folding process: most of the expressed antibody fragment is correctly transported and the signal peptide is cleaved off, but only a certain fraction of this material achieves the functional state. This fraction is dependent on the growth conditions of the cell, most notably the temperature[4]. We therefore wished to investigate at which steps the *in vivo* folding process diverts from the desired pathway and whether over-expressed folding catalysts may overcome such a block. We concentrate in this paper on the *E. coli* periplasmic proteins DsbA and PPIase A, as they have evolved to function in the periplasmic environment.

Results

Vector design for co-expression of PPIase. Plasmids were constructed to over-express the periplasmic *E. coli* PPIase together with various antibody fragments. We decided to introduce the *dsbA* and the *rot* genes into the antibody expression plasmid, as opposed to using a separate compatible plasmid, in order to minimize problems of quantitation due to loss or changes in copy number of one of the two plasmids. For this

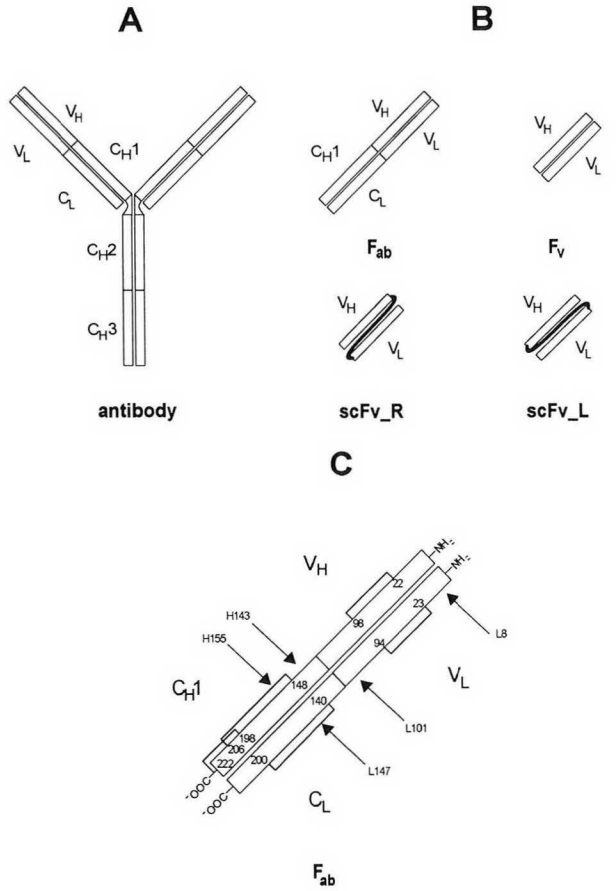
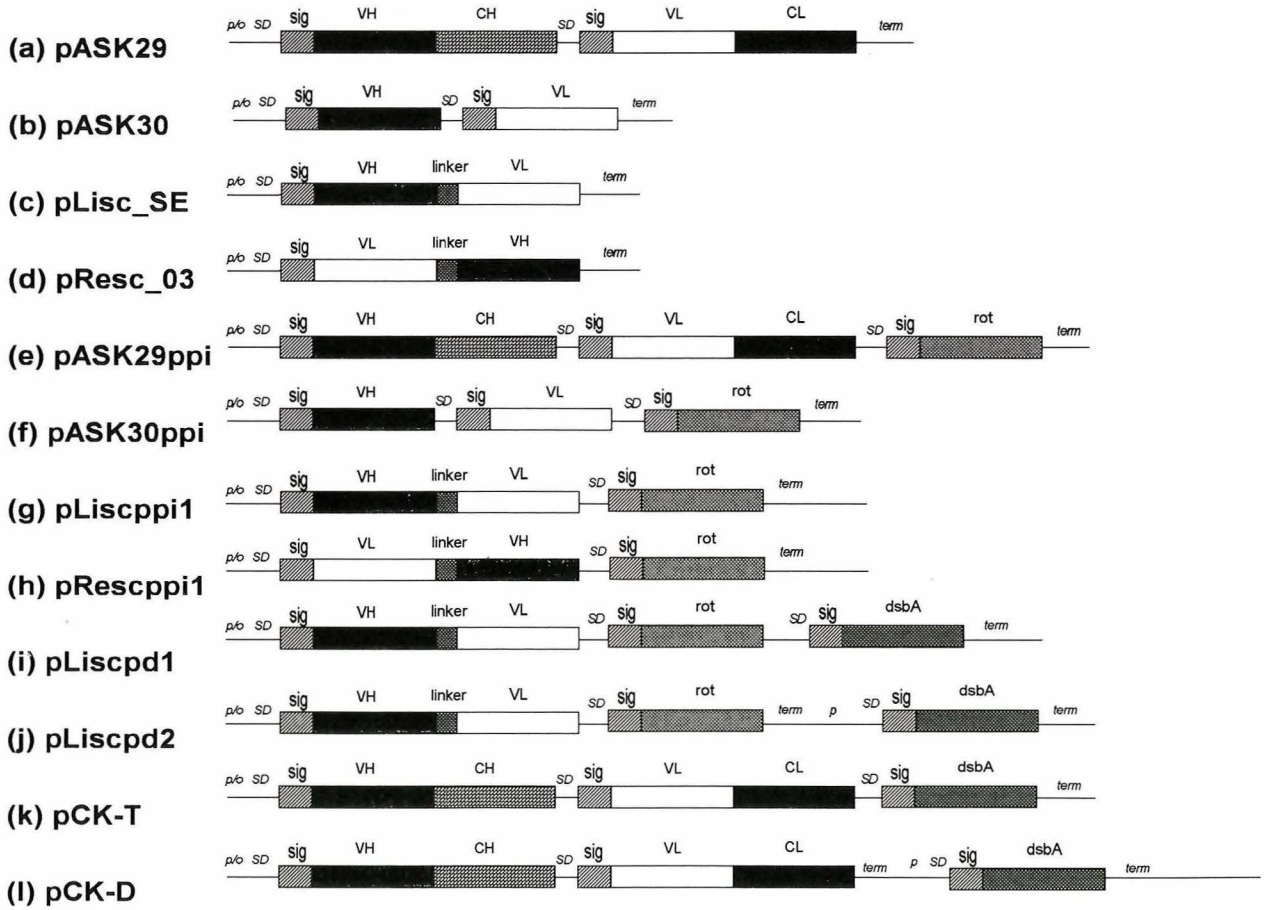


FIGURE 1. Antibody fragments used in this study. (A) A schematic drawing of a whole antibody. The different domains are indicated. (B) The various antibody fragments used in this study are shown in comparison with the whole antibody. (C) A more detailed view of the F_{ab} fragment showing the N- and the C-terminus, the position of the disulfide bonds and, indicated by arrows, the positions of the *cis* prolines.

purpose, the *rot* gene from plasmid pJLEC-2A (ref. 27) was introduced into plasmids encoding the F_{ab} fragment, the F_v fragment and two different single-chain fragments (scFV_L and scFv_R) of the antibody McPC603 (ref. 39) (Figs. 1, 2). The plasmid pResc_03 containing the single chain fragment in the arrangement V_L-linker-V_H was constructed in order to examine the effect of the *cis* prolyl residue L8 (Figs. 1, 3), which is preceded by the linker and the V_H domain in the scFv_L molecule, but lies close to the N-terminus of the scFv_R molecule and the unlinked F_v fragment or F_{ab} fragment. In all these constructs, the *rot* gene is part of the same operon as the antibody fragment and is thus under control of the inducible *lac* promoter.

Vector design for co-expression of DsbA. To design vectors for co-expression of DsbA together with antibody F_{ab} fragments, the *E. coli dsbA* gene was excised from the plasmid p12-7 (ref. 35) and introduced in two different ways into pASK29-L220 (ref. 4): DsbA was either expressed under the control of the *lac*-promoter in the same transcription unit with the antibody chains of the F_{ab}-fragment (pCK-T), or DsbA was expressed separately under control of its natural promoter (pCK-D, see Fig. 2) which leads to high constitutive expression. Thus, if a high concentration of DsbA was critical at the onset of induction, a difference

FIGURE 2. Plasmids used in this study. Abbreviations are: *p/o*: promoter/operator region, *p*: promoter, *SD*: Shine-Dalgarno sequence, *sig*: signal sequence, *term*: terminator. The genes encoding the various antibody domains are named according to Figure 1. The genes encoding the PPIase and DsbA are indicated as *rot* and *dsbA*, respectively.



between these expression strategies would be expected. To purify DsbA by immobilized metal ion affinity chromatography (IMAC), a fusion of six C-terminal histidine codons was constructed by site directed mutagenesis yielding the plasmid pCK-Th.

Vector design for co-expression of PPIase and DsbA. The *E. coli dsbA* gene was inserted into the plasmid pLiscppi1 resulting in the vectors pLiscpd1 and pLiscpd2, which contain the *dsbA* gene as a part of the artificial operon encoding the scFv gene and the *rot* gene or under its own promoter, respectively (Fig. 2), giving rise to constitutive expression.

Over-expression of putative folding catalysts. The over-expression of PPIase alone and together with DsbA was visualized by gel electrophoresis of the periplasmic proteins (Fig. 4A, B). The activity and the periplasmic localization of the enzymes was verified by measuring the activity in fractionated extracts (Fig. 5A). The level of PPIase over-expression was different in the different plasmid constructions, with the best expression obtained from the plasmid pLiscppi1, probably because of its very short intergenic region (Fig. 5B). However, in all cases a 10 to 60-fold over-expression of PPIase was seen compared with the level of genomically encoded PPIase in the periplasm of *E. coli*. If the level is compared to the molarity of the functional antibody fragment (Fig. 6), it is immediately apparent that an enormous molar excess of "catalyst" to "substrate" is present. Even if all antibody molecules were counted including the ones give rise to a precipitate[4], stoichiometric ratios of nascent antibody and PPIase should still be present at all times. Thus, while it is not possible to express PPIase at the exact same level in all constructs, a sufficient excess of PPIase relative to antibody should be present during expression in all experiments.

Additional over-expression of DsbA (Fig. 4B) did not diminish the amount of PPIase in the periplasm, indicating that simultaneous over-expression of four plasmid-encoded periplasmic proteins (PPIase, DsbA, β-lactamase and antibody fragment) does not exhaust the transport apparatus of *E. coli*. DsbA expressed as the last cistron of the operon yielded less enzyme than if the gene is expressed under its own promoter. The over-expression of folding catalysts did not change the growth behavior of induced cells. The possibility of differences in plasmid stability or general changes in cell physiology due to over-expression of these putative folding modulators could be ruled out by several methods, including the estimation of the amount of plasmid DNA in induced cells, containing the plasmid with or without the genes encoding the folding catalysts, and the estimation of the amount of plasmid-encoded periplasmic β-lactamase (see Experimental Protocol).

Yield of antibody fragments with co-expressed PPIase. The antibody F_{ab} fragment of McPC603, whose 3D-structure is known[38] contains 5 *cis*-proline residues (Figs. 1, 3). There is none in the V_H domain, two in V_L (and thus two in the F_v and in both scFv fragments), and two in C_H1 and one in C_L. For the F_{ab}, the F_v and the scFv_R antibody fragment, the effect of over-expressed PPIase on folding yields is marginal and lies within the range of experimental error. The folding yield of the scFv_L fragment is enhanced by the presence of more PPIase by a factor of about 1.8 (Fig. 7).

While the *total* expression level (including soluble and insoluble protein) of various antibody fragments, which are all under the control of the same promoter in the same vector is rather similar[4], the yield of folded material is much higher for the F_v and scFv fragments than for the F_{ab} fragment (details for the F_{ab} and the F_v fragments are given in ref. 4; the scFv fragments were found to behave similarly to the F_v fragments). Therefore, there seems to be a larger portion of F_{ab} fragment going to an unfolded and probably aggregated state in the periplasm after transport, which might conceivably be prevented from misfold-

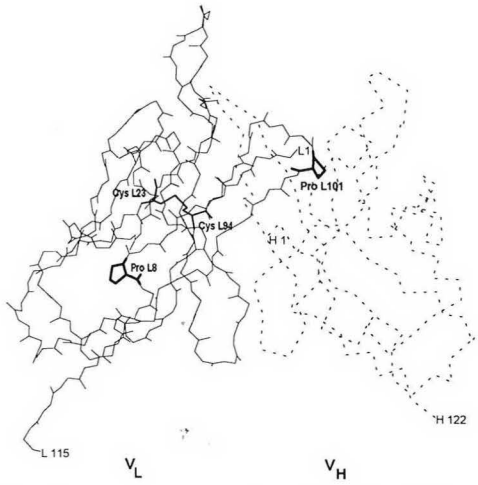


FIGURE 3. A three-dimensional model of the F_V fragment of McPC603 showing the backbone of the V_L domain (thin line), the α-carbon backbone of the V_H domain (broken lines) and the positions of the *cis* prolines and the disulfide bond in the V_L domain (thick lines).

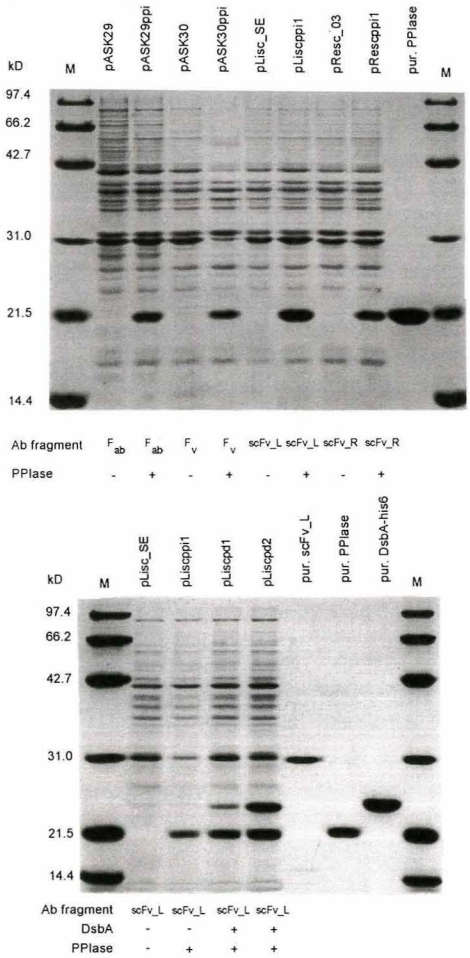


FIGURE 4. (A) Coomassie stained 15% SDS gel of periplasmic extracts, prepared as described in the Experimental Protocol, showing the PPIase over-expression of the various constructs (see Fig. 2 for plasmid names). Purified PPIase is also shown for comparison. M: Molecular weight standard. (B) Coomassie stained 15% SDS gel showing the over-expression of three different plasmid encoded proteins in periplasmic extracts prepared as described in the Experimental Protocol. For comparison, purified single-chain antibody, PPIase and DsbA-his$_6$ fusion protein are also shown. The band at 31 kD in the periplasmic extracts is plasmid encoded β-lactamase. See text for further details.

ing by more efficient catalysis of folding. Yet, there is no significant effect of PPIase observed on the yield of the F_{ab} fragment, although more than 40 active PPIase molecules are present per folded F_{ab} fragment in the periplasm. Therefore, the insufficient catalysis of proline-*cis-trans* isomerization does not appear to limit the folding yield of the F_{ab} fragment in the periplasm.

In the F_v fragment and the scFv fragments, the possible gain in yield is likely to be smaller, since a larger percentage of the protein is already achieving the native state without over-expressed PPIase[4]. Yet, the effect on the F_v fragment, and the scFv_R fragment are negligible. This suggests that insufficient catalysis of proline *cis-trans* isomerization does not limit the folding for these molecules, either. The yield of scFv_L was found to be higher by about a factor 1.8 in duplicate experiments. Yet, there is still precipitated material found in the periplasm, and the effect of catalysis is still undramatic for this protein.

Comparison of the scFvs. Interestingly, the amount of functionally expressed scFv_R was rather similar to the yield of scFv_L (about 75%). This is different from the observation of Anand et al.[40], who reported a dramatic increase in yield when the single-chain F_v fragment is expressed in the order V_L-linker-V_H. To investigate the two types of F_v fragments in more detail, scFv_R and scFv_L were purified from the periplasm of *E. coli*. The equilibrium denaturation of the two fragments was determined by fluorescence. Very similar denaturation curves were obtained for both proteins independently of whether fluorescence excitation was carried out at 280 nm (Fig. 8) or 290 nm (not shown). This is consistent with the scFv fragments behaving as a single co-operative unit in equilibrium denaturation experiments, similar to observations on another scFv fragment using different linkers[41]. The free energy of folding extracted from these data using a two-state model[42] are 4.5 kcal/mol for scFv_L and 4.7 kcal/mol for scFv_R, respectively; the midpoint of denaturation is at about 3 M urea.

Yield of F_{ab} fragment with co-expressed DsbA. The L220 mutant of the F_{ab} fragment of McPC603 contains 5 intramolecular disulfide bridges[4]. In every domain there is the disulfide bond typical for the immunoglobulin fold and there is an additional one in the C_H1 domain (see Fig. 1). The co-expression of DsbA, either polycistronically or under control of its own promoter, did not lead to an increase in yield of functional F_{ab} fragment (Fig. 7), as estimated from the *in vivo* folding assay (see Experimental Protocol).

Yield of F_v fragment expressed in a *dsb*⁻ strain. If the F_v fragment encoded by the plasmid pASK30 (see Fig. 2) was expressed in the *E. coli dsb*⁻ strain JCB472, the amount of soluble antibody protein in periplasmic extracts was less then 5% compared with the expression yield obtained with the isogenic *dsb*⁺ strain JCB473. If the *dsb*-gene was introduced in the plasmid pASK30, however, the amount of soluble antibody fragment expressed in JCB472 was fully restored to wild-type levels, as could be quantitated by densitometric monitoring of immunoblots (data not shown). This demonstrates that DsbA is needed for the folding process in the periplasm and that it is active *in vivo*, even when it is encoded on the plasmid. However, a higher amount of DsbA does not diminish aggregation of the antibody.

Yield of scFv_L with co-expressed PPIase and DsbA. The amount of soluble scFv_L in the periplasm, expressed with the plasmids pLiscpd1 and pLiscpd2, containing both the *rot*- and the *dsbA* gene, was estimated by Western blots and ELISA. To supply a reducing agent in the periplasm for facilitating disulfide reshuffling, 5 mM reduced glutathione was added to the culture prior to induction in an additional experiment. Under no conditions could we observe an effect on the yield of scFv fragment when co-expressing both folding catalysts together (data not shown). It also makes no difference in yield if reduced glutathione was added to the culture and if the *dsbA* gene was part of the inducible operon or if it was expressed under its own promoter. Hence it must be concluded that neither the *cis-trans* isomerization of prolyl-peptide bonds nor the formation and

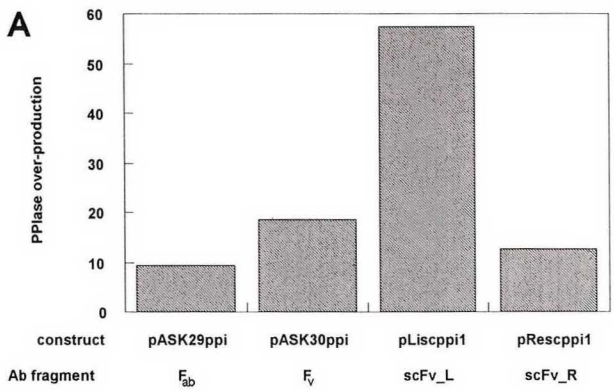


FIGURE 5. (A) Over-expression of PPIase. PPIase activity was measured in the periplasmic extract of induced cells harboring the various constructs (see Fig. 2 for nomenclature) according to Liu and Walsh[27]. The calculated PPIase amount was normalized to periplasmic protein content, corrected for genomically expressed PPIase and compared with the amount of PPIase expressed in cells without plasmid. (B) Intergenic regions of the different constructs encoding the antibody fragments and PPIase in the same operon. The name of the construct (see Fig. 2) is given together with the nucleotide sequence of the intergenic part of the artificial operon. The stop codon of the antibody genes and the start codon of the *rot* gene are boxed, the Shine-Dalgarno sequence of the rot gene is represented by the filled box.

rearrangement of disulfide bonds play the yield-limiting role in the folding process of over-expressed antibody fragments in the periplasm of *E. coli*.

Discussion

Previous experiments showed that the folding and/or assembly process in the periplasm might limit the yield of functional antibody fragments in *E. coli*. This hypothesis was derived from the observation that an increase in promoter strength does not increase the amount of functional material significantly, but does increase the amount of processed, but insoluble protein[4]. In all these cases, the amount of precursor protein is small, indicating that the transport process of the antibody protein is not limiting, but folding of the antibody protein in the periplasm becomes saturated only at a much higher flux rate through the membrane than can be folded. We cannot rigorously exclude that proteolysis also acts on these proteins along their path from the ribosome, although the lack of influence of promoter strength on the amount of functional material argues against cytoplasmic proteolysis limiting the amount of correctly assembled antibody fragment.

The linking of the two chains of the F_v fragment to give a scFv fragment (in the orientation V_L-linker-V_H or V_H-linker-V_L) does not increase the amount of active fragment compared to the unlinked F_v fragment[3]. Thus, at least for this antibody, the association of the heterodimeric F_v fragment does not seem to constitute a kinetic problem in *E. coli*. However, since different F_v fragments will have rather different V_L-V_H equilibrium association constants[3], some F_v fragments may not associate well for thermodynamic reasons.

Hence, it is reasonable to suppose that the *in vivo* folding bottlenecks occur during the folding of individual domains, albeit to different extents in the F_{ab} fragment and the various versions of the F_v fragment. Since the F_{ab} fragment is diverted from the desired path to a greater extent, and has a larger number of disulfide bonds and *cis*-prolines, we investigated the effect of catalyzing these processes *in vivo*. Both the activity of over-expressed proline *cis-trans* isomerase and of over-expressed DsbA could be measured in, or after purification from, periplasmic extracts. The *in vivo* activity of DsbA encoded on a plasmid could be directly demonstrated since it can fully substitute the chromosomal copy of *dsbA*: in a *dsbA*$^-$ strain, antibody production was found to be almost completely abolished. Thus, these proteins are made as functional enzymes from these over-expression vectors. Furthermore during *in vitro* folding experiments, effects of human PDI and of proline *cis-trans* isomerase were seen on an scFv-toxin fusion protein, suggesting that these processes may at least play some role in limiting the yield of the folding process under certain conditions[26]. However, only 5–10% of the material could be refolded *in vitro*, indicating that other processes such as aggregation may compete with the folding process *in vitro* as well.

Using single co-expression vectors, we could not detect significant influences of over-expressed DsbA or PPIase. The same result was obtained when human PDI was over-expressed together with the F_{ab} fragment of McPC603 (data not shown) or when PPIase was over-expressed together with the single-chain variable regions of a T-cell receptor (C. Wülfing and A. Plückthun, unpublished results). This suggests that other processes limit the folding process in the periplasm. Since the amount of soluble F_v fragment in the periplasm of *E. coli* was small if the expression was carried out in the *dsb*$^-$ strain JCB472, but was within the normal range when the isogenic *dsb*$^+$ strain JCB473 was used, DsbA activity *is* directly or indirectly involved in the folding process which leads to functional antibody fragments in *E. coli*. A similar phenomenon was previously described by Yu et al.[37] for enterotoxin formation in *E. coli*.

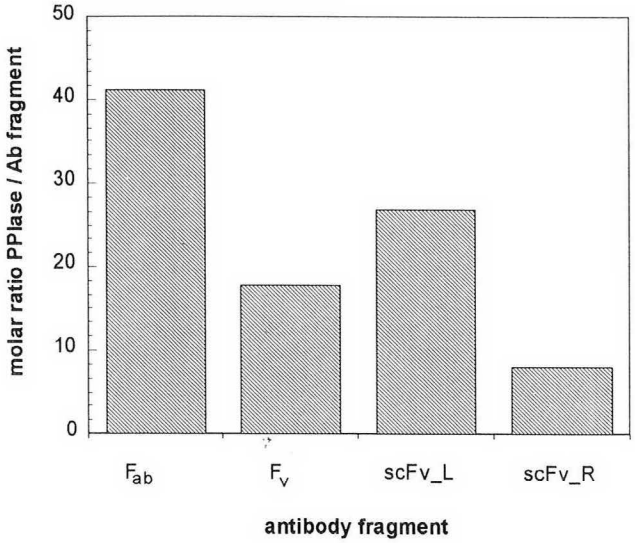


FIGURE 6. Molar ratio of PPIase per antibody fragment. The molar amount of PPIase over-expressed in the different constructs was estimated by measuring the activity in periplasmic extracts according to Liu and Walsh[27] and correcting the values for wild-type PPIase. This value is compared with the molar amount of functional antibody fragment to show the molar excess of "enzyme" over "substrate". The values are normalized to the amount of protein per liter of culture and OD_{550} of 1.

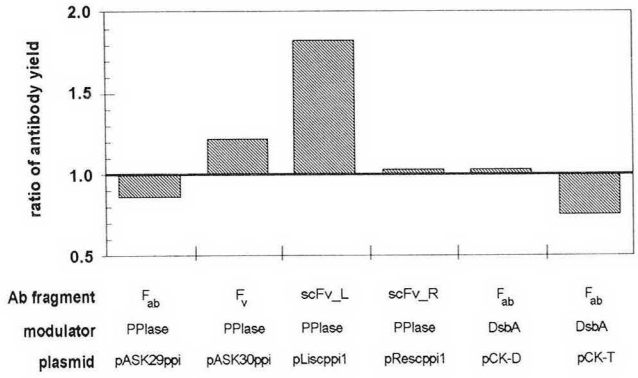


FIGURE 7. Effect of over-expression of the folding modulator on the yield of the different antibody fragments. The yield without folding modulator over-expression is taken as one and compared with the yield of antibody fragment when folding modulators are co-expressed. For plasmid names, see Figure 2, for further details, see text and Experimental Protocol.

Yet DsbA over-expression is not able, even when over-expressed, to overcome the folding block which leads to insoluble antibody protein. This is most clearly seen for the F_{ab} fragment, for which there is a large fraction still ending up as insoluble periplasmic protein. Most likely, aggregation processes of folding intermediates compete with the completion of folding of the domains. These over-expressed folding modulators do not seem to work on aggregated proteins.

We cannot exclude rigorously, however, that the thermodynamic solubility limit has been reached for any antibody fragment in the periplasm, because there is uncertainty about the volume of the periplasm. This thermodynamic model would also be consistent with a requirement of DsbA for the folding but lack of influence on the yield when folding catalysts are over-expressed. However, the insoluble protein can only be rescued by using denaturants and therefore it is not likely that the native antibody is aggregated. Furthermore, we cannot rigorously

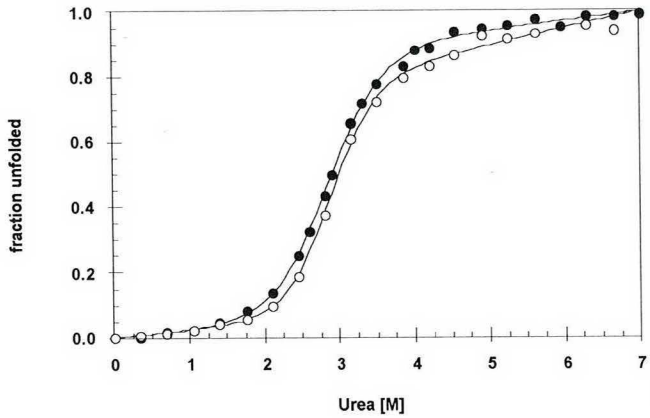


FIGURE 8. Equilibrium denaturation experiment of scFv_L (●) and scFv_R (○). The fraction of unfolded protein is plotted against the concentration of denaturant, as determined by fluorescence measurements (excitation at 280 nm, emission at 370 nm). The data were corrected for aggregation, which occurred in intermediate urea concentrations, and fitted to the two-state equation of Pace[42].

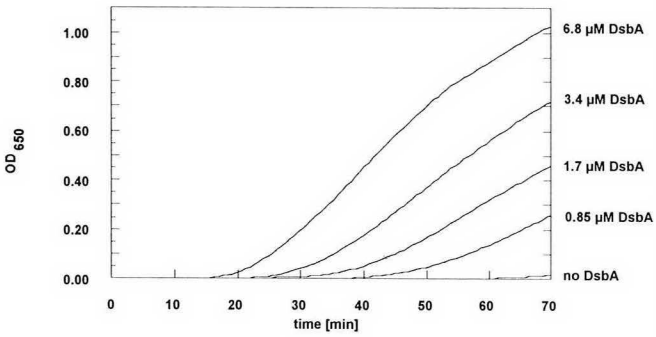


FIGURE 9. Influence of different concentrations of *Dsb*A-his$_6$ fusion protein on the rate of reduction of insulin. The assay mix only differed in the amount of DsbA, purified from antibody producing *E. coli*. The optical density, indicating aggregation of reduced insulin, is plotted as a function of time. See text and Experimental Protocol for further details.

exclude some denaturation after the native state has been reached. Our observations suggest that it is possible to vary the amount of insoluble protein by reducing the promoter strength or the amount of the inducer IPTG and therefore increase the *relative* amount of functional antibody fragment, but the *absolute* amount of native antibody protein seems to be fairly constant for a given type of antibody fragment. Only the reduction of temperature increases the absolute amount of all antibody fragments.

The only significant effect on folding was seen with PPIase on the scFv_L construct. While its equilibrium folding behavior is not significantly different from scFv_R (Fig. 8), this construct gives rise to a hindered isomerization of the L8 peptide bond, since the N-terminus is no longer free, but preceded by V_H and the linker. Nevertheless, the overall yield of folding is still quite similar between scFv_L and scFv_R. However, the level of functionally expressed antibody fragment scFv_R does not reach the level of functionally expressed scFv_L with or without co-expressed PPIase, and thus the folding of the chains in this particular fragment (V_L-linker-V_H) may involve other rate determining steps which are still not understood.

In conclusion, it appears that pathways leading to aggregation limit the folding of antibody molecules in *E. coli* by diverting the native folding pathway before disulfide isomerization and proline *cis-trans* isomerization occurs, or which are at least independent of the extent of disulfide isomerization and proline *cis-trans* isomerization. The further understanding and manipulation of these other processes is expected to be of general importance for the production and the engineering of complex proteins in bacterial hosts.

Experimental Protocol

***E. coli* strains.** The *E. coli* K12 strain JM83 (*ara*Δ(*lac*-proAB) *rps*L φ80 *lac*ZΔM15) was used for all antibody expression experiments if not indicated otherwise. *E. coli* RB791 (W3110 *lac*I^{q}L$_8$) was used for overexpressing PPIase A intracellulary for purification. *E. coli* JCB472 (F′(*tra*D30 *pro*AB *lac*I^q *lac*ZΔM15) Δ(*lac pro*) *thi str*A *sbc*B *hsd*R$^-$ *dsb*A1 zih-12::*Tn*10 (ref. 35)) and the isogenic *dsb*$^+$ variant JCB473 were used for investigation of a DsbA requirement of antibody formation.

Vector construction. Vectors were constructed using standard protocols for cloning and site-directed mutagenesis. Plasmids pASK29ppi and pASK30ppi were constructed by subcloning the *rot* gene (a kind gift from J. Liu and C. T. Walsh, Harvard Medical School) into pASK29 and pASK30, respectively[4,39]. Plasmid pLisc_SE was constructed by introducing an *EcoRI* site at the 3′-end of the V_L gene of pLisc[39] using site-directed mutagenesis[43]. Plasmid pResc_03 was constructed by inserting a 98-mer oligonucleotide encoding the linker region into the 3′-end of the pLisc-V_L-his gene[39] using site-directed mutagenesis, and subsequently cloning the V_H-gene into this vector. Plasmids pLiscppi1 and pRescppi1 were constructed by subcloning the *rot* gene into pLisc_SE and pResc_03. In all constructs, the *rot* gene was part of an artificial operon under control of the *lac* promoter. Vectors for co-expression of F_{ab} fragment with DsbA were constructed by subcloning the *dsb*A gene (a kind gift from J. C. A. Bardwell and J. Beckwith, Harvard Medical School) in two different ways into pASK29-L220[4] resulting in the plasmid pCK-T, where the *dsb*A gene is part of the inducible F_{ab} operon, and in the plasmid pCK-D, which contains the whole *dsb*A transcription unit behind the F_{ab} operon. Plasmids pLiscpd2 and pLiscpd1, which contain the gene for the scFv_L fragment together with the genes for PPIase and DsbA were constructed by inserting the *dsbA* gene with and without its own promoter, respectively, into pLiscppi1 (see Fig. 2). All constructs were verified by dideoxynucleotide sequencing. The protein sequence of DsbA reported by Bardwell et al.[35] and Kamitani et al.[36] differs by a frameshift at the C-terminus (nt 564 is missing in the sequence of Kamitani et al., using the numbering of Bardwell et al.). We have evidence that the sequence of Bardwell et al. is correct, since the fusion of a C-terminal histidine tail to this sequence allowed the purification of the protein by IMAC and is thus expressed in the correct frame.

PPIase assay. Over-expression of PPIase was confirmed by performing SDS-PAGE on the periplasmic fraction of induced cells containing the appropriate plasmid (Fig. 4). Furthermore, PPIase activity was measured in fractionated cell extracts according to Liu and Walsh[27] to confirm that the over-expressed enzyme was active and most if not all of the enzyme was localized in the periplasm.

β-Lactamase assay. The activity of the plasmid encoded β-lactamase was measured in fractionated cell extracts by the method of O'Callaghan et al.[44]

DsbA assay. The activity of DsbA was measured according to Holmgren[45] except that the reaction was carried out in a volume of 700 µl. The DTT concentration used in this assay was varied between 0.14 mM and 1.4 mM DTT. Samples were assayed in a low salt buffer, since otherwise no or only minor precipitation of the reduced insulin B-chain could be observed. Therefore, DsbA purified by IMAC was dialyzed against a buffer containing 0.1 M potassium phosphate (pH 6.5) and 2 mM EDTA. Periplasmic extracts were desalted and concentrated in a Centricon 10 (Amicon) using the same buffer.

Purification of PPIase. PPIase was purified from crude extracts of *E. coli* RB791 harboring the plasmid pJLEC-2B (ref. 27) using a procedure similar to Liu and Walsh[27].

Purification of DsbA-his$_6$. *E. coli* JM83 harboring the plasmid pCK-Th was grown for 24 hours at 21 °C in LB medium containing 100 µg/ml ampicillin. The cells were harvested by centrifugation, resuspended in 0.8 mM imidazole, 1 M NaCl, 50 mM Tris-HCl, pH 7, and passed twice through a French Press. The lysate was centrifuged at 31000xg (SS34, 30 minutes at 4 °C) and the supernatant was passed through a 0.45 µm filter (Millex). The cleared solution was directly applied to a Ni^{2+}-NTA-column (Diagen), washed with the same buffer, pre-eluted with 8 mM imidazole, 1 M NaCl, Tris-HC1, pH 7, and eluted with a gradient of 8 to 70 mM imidazole. The DsbA containing fractions were pooled, dialyzed against 10 mM MOPS, pH 7 and concentrated. The concentrate was then applied to a DEAE-CL6B column (Pharmacia) and eluted with a gradient of 0 to 200 mM NaCl in 10 mM MOPS, pH 7.

Equilibrium denaturation of scFv fragments. The equilibrium stability of the two different types of scFv fragment was calculated from equilibrium denaturation experiments in urea. (0.4 µM) was

incubated for 5 hours at 25°C in various concentrations of urea (0–7 M) without reducing agent, and the fluorescence emission at 370 nm was measured. The wavelength of excitation was 290 nm. The data were corrected for aggregation, which occurred in intermediate urea concentrations, and fitted to the two-state equation of Pace[42].

Preparation of periplasmic fractions. *E. coli* JM83 cells containing the appropriate plasmid were grown in 30 ml LB medium containing 100 μg/ml ampicillin at 37°C or at room temperature. After induction with 1 mM IPTG and a further incubation for 1 hour or 3 hours, 10 ml of the culture was harvested and resuspended in BBS containing 1 mM EDTA giving a final OD_{550} of 10. The cells were then shaken at 4°C overnight and the periplasmic fraction was separated from the spheroplasts by centrifugation. The activity of β-lactamase was measured in the medium after removing the cells by centrifugation and in the periplasmic fraction in order to determine the distribution of periplasmic proteins.

***In vivo* folding assay:** A 2 l culture of *E. coli* JM83 harboring the plasmid encoding the respective antibody fragment and the putative folding catalyst and, as a control, cells with the same plasmid but without the *rot* or the *dsbA* gene, were incubated in LB medium containing 100 μg/ml ampicillin at room temperature until an OD_{550} of 0.5 was reached. IPTG was added to a final concentration of 1 mM, and the incubation was continued for an additional 3 hours. The cells were harvested and resuspended in 10 ml BBS (pH 8.0). The cells were then lysed by two passages through a French Press at 1.38×10^5 kPa. After separation of the cell debris by centrifugation (31000xg, 25 minutes) and sterilization of the crude extract by filtration through a sterile filter, the functionally produced antibody fragments were prepared using a one-step purification procedure: the crude extract was applied to a phosphorylcholine-sepharose affinity column[1,3] equilibrated with 200 ml BBS, and non-binding material was eluted with the same buffer. Antibody fragment binding to the column was eluted with 5 mM phosphorylcholine in BBS. The concentration of the antibody fragments was estimated spectroscopically and compared with the fragment concentration from the control experiment. All experiments were carried out at least in duplicate.

Control experiments. To estimate the accuracy of the purification procedure as a quantitation of active antibody fragments, experiments with two cultures containing the same plasmid were performed. The yield of purified antibody fragments lay in the same range with a maximum deviation of ±20%. The possibility of different plasmid copy numbers due to reduced plasmid stability was excluded by comparing the amount of plasmid DNA purified from an aliquot of the culture after harvesting the cells. Additionally, the activity of the plasmid encoded β-lactamase was measured.

ELISA. As an alternative for measuring the amount of soluble antibody fragments in the periplasm, an immunoassay was performed. The periplasmic fraction was serially diluted into microtiter plates and incubated overnight at 4°C. After removal of non-bound material by washing the wells with PBS/Tween and blocking the remaining binding sites with 5% skim milk in PBS, the bound antibody fragments were either detected with an antiserum raised against the antibody McPC603 or, for the F_{ab} fragment, directly with a ProteinA-peroxidase conjugate.

Acknowledgments

We would like to thank J. Bardwell and J. Beckwith (Harvard Medical School) for *dsb*⁻ strains and the *dsb* gene and helpful discussions and J. Liu and C. Walsh (Harvard Medical School) for the *rot* gene. We would also like to thank Barbara Munz for help in some experiments.

References

1. Skerra, A. and Plückthun, A. 1988. Assembly of a functional immunoglobulin F_v fragment in *Escherichia coli*. Science **240:**1038–1041.
2. Better, M., Chang, C. P., Robinson, R. R. and Horwitz, A. H. 1988. *Escherichia coli* secretion of an active chimeric antibody fragment. Science **240:**1041–1043.
3. Glockshuber, R., Malia, M., Pfitzinger, I. and Plückthun, A. 1990. A comparison to stabilize immunoglobulin F_v-fragments. Biochemistry **29:**1362–1367.
4. Skerra, A. and Plückthun, A. 1991. Secretion and *in vivo* folding of the F_{ab} fragment of the antibody McPC603 in *Escherichia coli*—influence of disulfides and *cis*-prolines. Protein Eng. **4:**971–979.
5. Anfinsen, C. B. 1973. Principles that govern the folding of protein chains. Science **181:**223–230.
6. Creighton, T. E. 1978. Experimental studies of protein folding and unfolding. Prog. Biophys. Mol. Biol. **33:**231–297.
7. Jaenicke, R. 1987. Folding and association of proteins. Prog. Biophys. Mol. Biol. **49:**117–237.
8. Fischer, G. and Schmid, F. X. 1990. The mechanism of protein folding. Implications of *in vitro* refolding models for de novo protein folding and translocation in the cell. Biochemistry **29:**2205–2212.
9. Lorimer, G. H. 1992. Role of accessory proteins in protein folding. Curr. Opin. Struct. Biol. **2:**26–34.
10. Jaenicke, R. 1991. Protein folding: local structures, domains, subunits, and assemblies. Biochemistry **30:**3147–3161.
11. Gething, M. J. and Sambrook, J. 1992. Protein folding in the cell. Nature **355:**33–45.
12. Kiefhaber, T., Rudolph, R., Kohler, H. H. and Buchner, J. 1991. Protein aggregation *in vitro* and *in vivo*: a quantitative model of the kinetic competition between folding and aggregation. Bio/Technology **9:**825–829.
13. Fischer, G., Bang, H. and Mech C. 1984. Nachweis einer Enzymkatalyse für die *cis-trans*-Isomerisierung der Peptidbindung in prolinhaltigen Peptiden. Biomed. Biochem. Acta **43:**1101–1111.
14. Brandts, J. F, Halvorson, H. R. and Brennan, M. 1975. Consideration of the possibility that the slow step in protein denaturation reactions is due to *cis-trans* isomerism of proline residues. Biochemistry **14:**4953–4963.
15. Schmid, F. X. and Baldwin, R. L. 1978. Acid catalysis of the formation of the slow-folding species of RNase A: evidence that the reaction is proline isomerization. Proc. Natl. Acad. Sci. USA **75:**4764–4768.
16. Schmid, F. X., Grafl, R., Wrba, A. and Beintema, J. J. 1986. Role of proline peptide bond isomerization in unfolding and refolding of ribonuclease. Proc. Natl. Acad. Sci. USA **83:**872–876.
17. Grathwohl, C. and Wüthrich, K. 1976. NMR studies of the molecular conformations in the linear oligopeptides H-(*l*-Ala)$_n$-*l*-Pro-OH. Biopolymers **15:**2043–2057.
18. Juy, M., Lam-Thanh, H., Lintner, K. and Fermandjian, S. 1983. Conformation and mobility of tyrosine side chain in tetrapeptides. Int. J. Pept. Prot. Res. **22:**437–449.
19. Stewart, D. E., Sarkar, A. and Wampler, J. E. 1990. Occurence and role of *cis* peptide bonds in protein structures. J. Mol. Biol. **214:**253–260.
20. Kiefhaber, T., Quaas, R., Hahn, U. and Schmid, F. X. 1990. Folding of ribonuclease T1. 1. Existence of multiple unfolded states created by proline isomerization. Biochemistry **29:**3053–3061.
21. Fransson, C., Freskgard, P. O., Herbertsson, H., Johansson, A., Jonasson, P., Martensson, L. G., Svensson, M., Jonsson, B. H. and Carlsson, U. 1992. *Cis-trans* isomerization is rate-determining in the reactivation of denatured human carbonic anhydrase-II as evidenced by proline isomerase. FEBS Letters **296:**90–94.
22. Lang, K., Schmid, F. X. and Fischer, G. 1987. Catalysis of protein folding by prolyl isomerase. Nature **329:**268–270.
23. Schönbrunner, E. R., Mayer, S., Tropschug, M., Fischer, G., Takahashi, N. and Schmid, F. X. 1991. Catalysis of protein folding by cyclophilins from different species. J. Biol. Chem. **266:**3630–3635.
24. Lin, L.-N., Hasumi, H. and Brandts, J. F. 1988. Catalysis of proline isomerization during protein-folding reactions. Biochim. Biophys. Acta **956:**256–266.
25. Goto, Y. and Hamaguchi, K. 1982. Unfolding and refolding of the constant fragment of the immunoglobulin light chain. J. Mol. Biol. **156:**891–910.
26. Buchner, J., Brinkmann, U. and Pastan, I. 1992. Renaturation of a single-chain immunotoxin facilitated by chaperones and protein disulfide isomerase. Bio/Technology **10:**682–685.
27. Liu, J. and Walsh, C. T. 1990. Peptidyl-prolyl *cis-trans*-isomerase from *Escherichia coli*: a periplasmatic homolog of cyclophilin that is not inhibited by cyclosporin A. Proc. Natl. Acad. Sci. USA **87:**4028–4032.
28. Hayano, T., Takahashi, N., Kato, S., Maki, N. and Suzuki, M. 1991. Two distinct forms of peptidylprolyl-*cis-trans*-isomerase are expressed separately in periplasmic and cytoplasmic compartments of *Escherichia coli* cells. Biochemistry **30:**3041–3048.
29. Harrison, R. K. and Stein, R. L. 1990. Substrate specificities of the peptidyl prolyl *cis-trans* isomerase activities of cyclophilin and FK-506 binding protein: evidence for the existence of a family of distinct enzymes. Biochemistry **29:**3813–3816.
30. Venetianer, P. and Straub, F. B. 1963. The enzymic reactivation of reduced ribonuclease. Biochim. Biophys. Acta **67:**166–168.
31. Goldberger, R. F., Epstein, C. J. and Anfinsen, C. B. 1963. Acceleration of reactivation of reduced bovine pancreatic ribonuclease by a microsomal system from rat liver. J. Biol. Chem. **238:**628–635.
32. Freedman, R. B., Bulleid, N. J., Hawkins, H. C. and Paver, J. L. 1989. Role of protein disulphide-isomerase in the expression of native proteins. Biochem. Soc. Symp. **55:**167–192.
33. Parkkonen, T., Kivirikko, K. I. and Pihlajaniemi, T. 1988. Molecular cloning of a multifunctional chicken protein acting as the prolyl 4-hydroxylase β-subunit, protein disulphide-isomerase and a cellular thyroid-hormone binding protein. Biochem. J. **256:**1005–1011.
34. Gilbert, H. F. 1990. Molecular and cellular aspects of thiol-disulfide exchange. Adv. Enzymol. **63:**69–172.
35. Bardwell, J. C. A., McGovern, K. and Beckwith, J. 1991. Identification of a protein required for disulfide bond formation *in vivo*. Cell **67:**581–589.
36. Kamitani, S., Akiyama, Y. and Ito, K. 1992. Identification and characterization of an *Escherichia coli* gene required for the formation of correctly folded alkaline-phosphatase, a periplasmic enzyme. EMBO J. **11:**57–62.
37. Yu, J., Webb, H. and Hirst, T. R. 1992. A homologue of the *Escherichia coli* DsbA protein involved in disulphide bond formation is required for enterotoxin biogenesis in *Vibrio cholerae*. Mol. Microbiol. **6:**1949–1958.
38. Satow, Y., Cohen, G. H., Padlan, E. A. and Davies, D. R. 1986. Phosphocholine binding immunoglobulin F_{ab} McPC603. J. Mol. Biol. **190:**593–604.
39. Skerra, A., Pfitzinger, I. and Plückthun, A. 1991. The functional expression of antibody F_v fragments in *Escherichia coli*: improved vectors and a generally applicable purification technique. Bio/Technology **9:**273–278.
40. Anand, N. N., Mandal, S., MacKenzie, C. R., Sadowska, J., Sigurskjold, B., Young, N. M., Bundle, D. R. and Narang, S. A. 1991. Bacterial expression and secretion of various single-chain F_v genes encoding proteins specific for a *Salmonella* serotype B O-antigen. J. Biol. Chem. **266:**21874–21879.
41. Pantoliano, M. W., Bird, R. E., Johnson, S., Asel, E. D., Dodd, S. W., Wood, J. F. and Hardman, K. D. 1991. Conformational stability, folding, and ligand-binding affinity of single-chain-F_v immunoglobulin fragments expressed in *Escherichia coli*. Biochemistry **30:**10117–10125.
42. Pace, C. N. 1990. Measuring and increasing protein stability. Trends Biotechnol. **8:**93–98.
43. Kunkel, T. A., Roberts, J. D. and Zakour, R. A. 1987. Rapid and efficient site specific mutagenesis without phenotypic selection. Methods Enzymol. **154:**367–382.
44. O'Callaghan, C. H., Morris, A., Kirby, S. M. and Shingler, A. H. 1972. Novel method for detection of β-lactamases by using chromogenic cephalosporin substrate. Antimicrob. Agents Chemother. **1:**283–288.
45. Holmgren, A. 1979. Thioredoxin catalyzes the reduction of insulin disulfides by dithiothreitol and dihydrolipoamide. J. Biol. Chem. **254:**9627–9632.