Functional antibody production using cell-free translation: Effects of protein disulfide isomerase and chaperones

Lyubov A. Ryabova[1,2,3], Dominique Desplancq[1,4], Alexander S. Spirin[2], and Andreas Plückthun[1,*]

*[1]Biochemisches Institut, Universität Zürich, Winterthurerstr. 190, CH-8057 Zürich, Switzerland; [2]Institute of Protein Research, Russian Academy of Sciences, 142292 Pushchino, Moscow region, Russia. Present address: [3]Friedrich Miescher-Institut, PO Box 2543, CH-4002 Basel, Switzerland; [4]Département des Récepteurs et Protéines Membranaires, UPR 9050 du CNRS, ESBS, Pôle Universitaire d'Illkirch, Boulevard Sébastien Brandt, F-67400, Illkirch, France. *Corresponding author (e-mail: plueckthun@biocfebs.unizh.ch).*

Received 12 September 1996; accepted 26 November 1996

To create a rapid system to test the effect of sequence changes on recombinant antibody binding, we have developed a procedure for producing functional scFv fragments in an *Escherichia coli* cell-free translation system. Functional antibodies with antigen-binding activity are obtained only if disulfide formation and rearrangement is allowed to take place during the translation reaction. The inclusion of protein disulfide isomerase (PDI) leads to a threefold increase in yield over that obtained in the presence of glutathione redox systems. DsbA had no such effect, indicating that disulfide shuffling, and not net formation, is the crucial yield-limiting step. The addition of the molecular chaperones DnaK and DnaJ increased the amount of soluble protein but not the amount of functional scFv, which appears to be limited entirely by correct disulfide formation. None of these factors significantly influenced total protein synthesis. In the presence of PDI, chaperones, reduced glutathione and oxidized glutathione, 50% of the scFv produced (about 8 µg/ml in only 15 min) could be recovered from immobilized antigen.

Keywords: cell-free translation system, single-chain antibody, disulfide isomerase, molecular chaperones, protein folding

Antibody engineering depends on the availability of information regarding the effect of sequence changes on binding properties. Typical examples are the grafting of the antigen binding loops to human frameworks (humanization)[1], which frequently requires the back-mutation of individual residues to the murine type to restore full activity, changes in the antibody sequence to achieve an enhancement of binding affinity, or a loss of cross-reactivity or any more dramatic alterations engineered into in the antibody framework. Traditionally, such experiments have used site directed mutagenesis of antibody genes and their subsequent expression in *Escherichia coli*[2] or even transient expression in COS cells[3]. Whereas this methodology is simple and powerful, the time required for one complete cycle from designing a mutation to analyzing the effect on the protein is still significant. The production of antibodies by bacterial cells in large amounts is attractive, but not always necessary for rapidly testing functionality. The synthesis of antibodies in a cell-free system could thus provide a much faster access to binding data directly from the expression of the PCR-amplified gene and so accelerate the progress of antibody engineering. Furthermore, the bacterial construct may not always be the final format of the antibody, and bacterial expression may then be a detour.

Antibody domains are known to contain a conserved intramolecular disulfide bond that is critical for their stability and activity[4,5]. It was therefore imperative to find conditions under which the formation of these disulfide bonds takes place in good yields in a cell-free system. In the cytoplasm, where protein biosynthesis occurs in vivo, the redox potential is reducing[6].

In the endoplasmic reticulum, where the disulfide bonds of antibodies are formed, this reaction is catalyzed by protein disulfide isomerase (PDI) in the presence of glutathione[7,8] and disulfide rearrangement, not net formation[9–11], is known to be the crucial reaction in vivo. In the bacterial periplasm, these processes are catalyzed by DsbABCD[12–18].

Molecular chaperones are known to participate in protein folding in vivo[19], even though their precise mechanisms still need to be clarified. The chaperone proteins of the Hsp70 and Hsp60 classes bind to unfolded or partially folded polypeptides, thereby preventing aggregation, and may allow part of the folding to occur in the complex.

We used the scFv fragment[20] of the anti-hemagglutinin antibody 17/9 (ref. 21) to test the factors influencing the production of native antigen-binding antibody fragments. While some antigen-binding activity was reported for an in vitro translated immunotoxin after it has been dialyzed[22], no quantitative data have been given, and no attention was paid to control folding directly in the in vitro translation.

The bacterial cell-free translation system, in the presence of PDI, is capable of rapidly producing functionally active, correctly folded, disulfide-linked protein as a high proportion of the total polypeptide synthesized. The inclusion of molecular chaperones in the cell-free system has some effect on preventing the formation of aggregated material in the cell-free system, and increases the yield of functionally active molecules produced in the presence of PDI.

Results

Synthesis of an scFv fragment in an *E. coli* cell-free translation system. The mRNA, coding for an anti-hemagglutinin peptide (anti-hag) scFv, was obtained from the transcription of the corresponding plasmid and then translated in an *E. coli* S30 translation system without SH-containing compounds (see Experimental

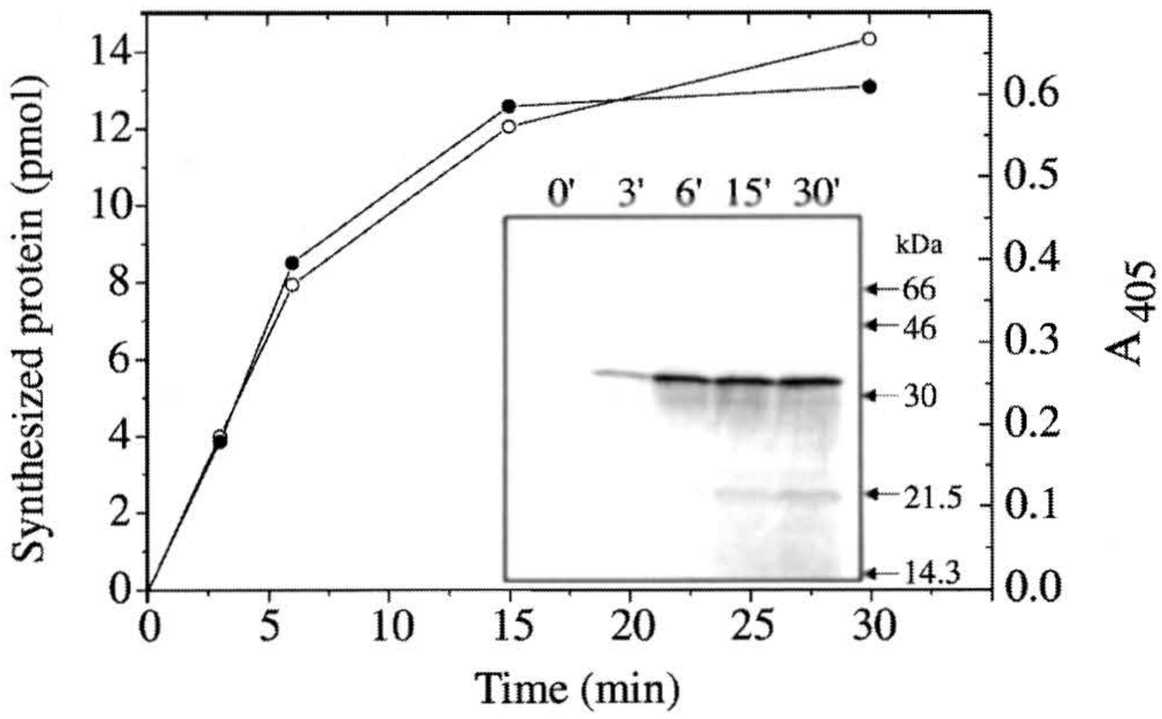

Figure 1. Production of scFv fragments in an *E. coli* cell-free translation system. [^{14}C]-labeled proteins were synthesized in vitro. Fifteen-microliter samples of the reaction mixture were analyzed by ELISA, 5-µl samples were analyzed by trichloroacetic acid precipitation assay and by electrophoresis (12% SDS-PAGE) with subsequent autoradiography. The amount of protein synthesized (-○-) and relative ELISA signals (-●-) are shown. Inset: PAGE of the [^{14}C]-labeled proteins synthesized at different incubation times.

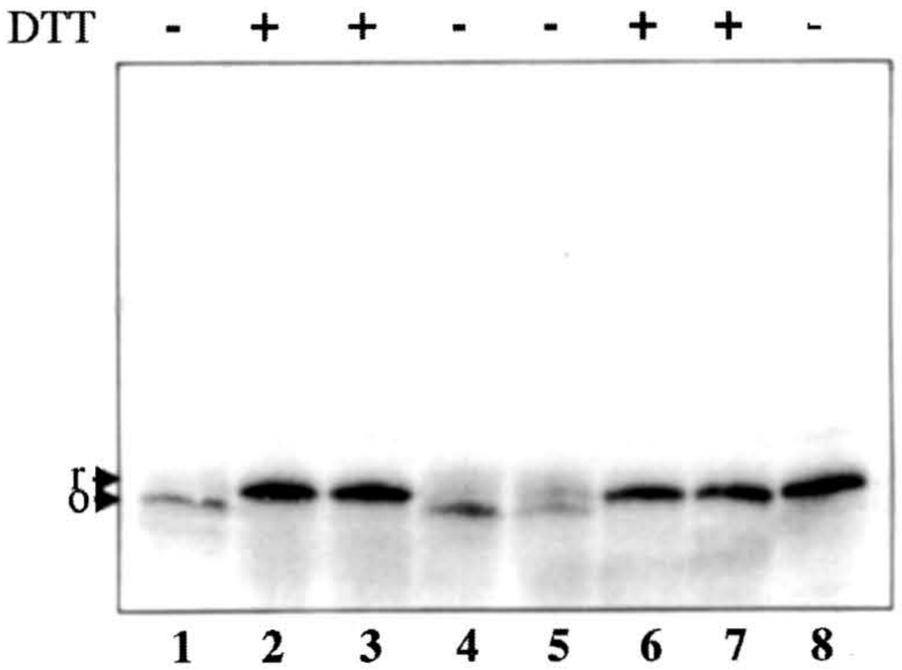

Figure 2. Influence of GSSG, GSH and DTT on disulfide bond formation during in vitro translation. Western blot analysis of the anti-hag scFv fragments. Without addition: lane 1, non-reducing PAGE; lane 2, reducing PAGE. With 0.1 mM GSH/ 1 mM GSSG: lane 3, reducing PAGE; lane 4: non-reducing PAGE. With 1 mM GSH/ 0.1 mM GSSG: lane 5, non-reducing PAGE; lane 6, reducing PAGE. With 1 mM DTT: lane 7, reducing PAGE; lane 8, non-reducing PAGE. DTT was added to reduced samples prior to electrophoresis where indicated. The oxidized protein (o) migrates slightly more rapidly than the reduced form (r).

protocol). The total amount of protein synthesized, as calculated from the specific radioactivity incorporated into the translation product, reaches a plateau of 14 pmoles of scFv per 50 µl of reaction mixture (8.3 µg/ml) in about 30 min (Fig. 1). That the radioactivity comes exclusively from the mRNA-directed synthesis is reflected in the appearance of the one major [^{14}C]leucine containing product with the molecular weight expected for the anti-hag scFv fragment (inset). The full-length product and the antigen-binding activity (measured by ELISA) both became detectable by the first time point, and the time courses of polypeptide synthesis and the functional activity are superimposable (Fig. 1), indicating that there is no measurable delay in the appearance of activity under these conditions.

At the same time, only 8% of the synthesized scFv molecules reached the active state (Table 1), as was estimated from a quantitative antigen-binding radioimmunoassay. An MS2 coat protein control mRNA showed a low background level of nonspecific adsorption of radioactive polypeptides (Table 1), and binding of scFv could be totally blocked with soluble antigen. We reasoned that the low antigen binding activity under these conditions must be due to protein aggregation and/or inefficient or erroneous disulfide formation.

Table 1. Yield of functional scFv fragments synthesized.

	mRNA	Additions	Hag-binding protein/ total protein synthesized (pmol/pmol)[a]	Yield of functional scFv (%)
(1)	MS2 RNA[b]	None	0.09/8.18	—
(2)	scFv RNA	None	0.76/9.02	8.4
(3)	scFv RNA	0.1 mM GSH/ 1.0 mM GSSG	0.80/8.70	9.2
(4)	scFv RNA	1.0 mM GSH/ 0.1 mM GSSG	0.62/8.85	7.0
(5)	scFv RNA	1 mM DTT	0.05/10.30	0.5
(6)	scFv RNA	DsbA (0.2 µM)	1.06/10.46	10.1
(7)	scFv RNA	PDI (0.9 µM)	2.40/7.80	30.7
(8)	scFv RNA	PDI added with 20 min delay	0.96/8.50	10.8
(9)	scFv RNA	chaperones[c]	0.86/8.62	9.9
(10)	scFv RNA	PDI, chaperones[c] and 1.0 mM GSH/ 0.1 mM GSSG	4.04/8.40	48.1

scFv fragments were synthesized in vitro. After 40 min of incubation at 37°C, the reaction mixture was centrifuged and the resulting supernatant was analyzed by RIA for the amount of [^{14}C]-labeled proteins that are capable of binding antigen. [a]The results are given for 50 µl of reaction mixture. [b]7 µg of MS2 RNA were used in a 50 µl of reaction to test for background binding. [c]DnaK (1 µM), DnaJ (0.4 µM), GrpE (0.4 µM), $GroEL_{14}$ (0.4 µM), and $GroES_7$ (0.4 µM).

In order to control the conditions for the formation of disulfide bonds more tightly, mixtures of reduced glutathione (GSH) and oxidized glutathione (GSSG) were introduced in the cell-free system. The antigen-binding activity of the scFv fragment is unaffected when the protein is synthesized in the presence of the "reducing" mixture of glutathione, while it is slightly increased when it is formed in the presence of the "oxidizing" mixture of glutathione (Table 1).

The importance of intrachain disulfide bonds for the activity of scFv fragments has been reported[5,23]. In accordance with these observations, the addition of 1 mM dithiothreitol (DTT) in the cell-free translation system resulted in a complete disappearance of antigen-binding activity of the polypeptides synthesized (Table 1), while the translation rate and yield were unaffected (data not shown).

The product synthesized in the cell-free system under four different redox conditions was analyzed by Western blot (Fig. 2). In SDS/PAGE under reducing conditions, or when the translation was performed in the presence of DTT, the product gave rise to one band of the size expected for the denatured full-length polypeptide, while the nonreduced polypeptides migrated somewhat faster. The antigen-binding activity of the product correlates with the presence of the oxidized (disulfide) form of the polypeptide (Table 1).

The samples from the translation mixture contain intermolecular cross-linked scFv aggregates that are too large to enter the gel, since the total intensities of the scFv bands in a nonreducing gel are significantly lower than in a reducing gel. These aggregates could be reduced by DTT so that the intensities of the bands in the reducing lanes represent the total amount of protein present in the sample (Fig. 2).

Effect of PDI on the yield of functional scFv fragments. When PDI is present in the translation reaction, the ELISA signal

Table 2. The effect of chaperones on the solubility of scFv fragments.

Additions[a]	[^{14}C]leucine incorporated[b] soluble protein/ total protein (%)	Phosphoimager analysis of electrophoretic scFv bands[c] total reaction mixture	supernatant	pellet
DTT (1 mM)	40	nd	nd	nd
no additions	75	1.00	0.74	0.24
DnaK	78	0.97	0.70	0.23
DnaJ	75	0.97	0.75	0.25
DnaK+DnaJ	92	0.95	0.92	0.10
DnaK+DnaJ+GrpE	95	0.95	0.95	0.07
GroEL	82	0.96	0.77	0.14
GroEL+GroES	83	0.98	0.79	0.14
Chaperones[a]	95	0.98	1.00	0.05
BSA (2 μM)	78	nd	nd	nd

scFv fragments were synthesized in vitro. After 40 min of incubation at 37°C, the 50 μl of reaction mixture was centrifuged at 14,000 rpm. In each case, 5 μl of total reaction mixture, supernatant and pellet dissoved in 12.5 μl of translation buffer were analyzed. [a]The concentrations are listed in Table 1. [b]The amount of the protein that is present in the total reaction mixture and in the supernatant was calculated directly from the amount of [^{14}C]leucine that is incorporated into the protein. Data from five experiments. [c]Quantification of [^{14}C]-labeled protein bands on SDS-PAGE using PhosphoimagerPC analysis. All intensities are normalized to the total protein in the assay with no additions. Data derived from six experiments. nd: not determined.

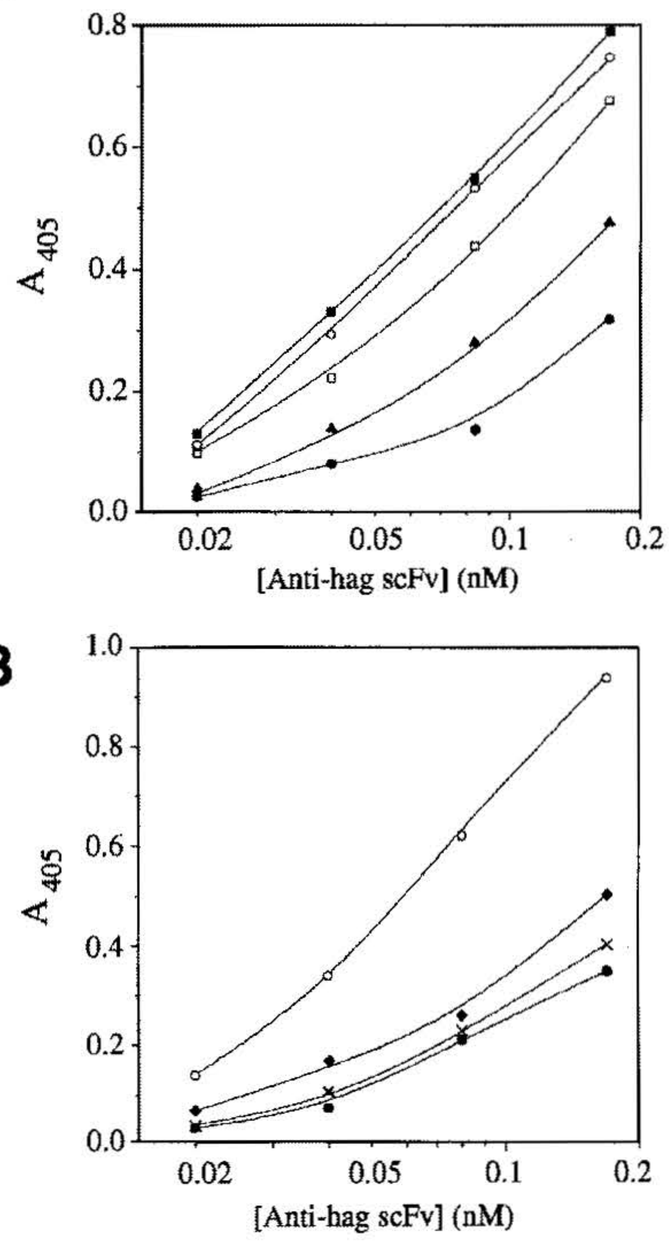

Figure 3. Effects of PDI DsbA and chaperones on the functional formation of scFv fragments. Relative antigen-binding ELISA signals of the in vitro synthesized anti-hag scFv antibodies. After 40 min of incubation at 37°C, the reaction mixtures were centrifuged and the resulting supernatant was analyzed for antigen-binding activity by serial dilution. (A) Translation in reaction mixture without additions (-●-), with PDI added at the beginning of translation (-○-), PDI added 20 min after the start of translation (-▲-), in the presence of PDI with 0.1 mM GSH/ 1 mM GSSG (-□-), and with 1 mM GSH/ 0.1 mM GSSG (-■-). (B) Translation without additions (-●-), in the presence of PDI (-○-), with a chaperone cocktail (DnaK, DnaJ, GrpE, GroEL, and GroES at concentrations given Table 1 (-×-) and, with DsbA (-◆-). The concentration of scFv fragment in the supernatant of the cell-free translation that was assayed (x-axis) was estimated from the material precipitated by trichloroacetic acid.

increased by up to threefold compared to a control translation lacking PDI (Fig. 3A). Similarly, a threefold higher production of functional scFv fragments was also found by the radioimmunoassay (Table 1). PDI exerts its effect only when present cotranslationally but not when added 20 min after the beginning of translation (Fig. 3A; Table 1).

The effect on the production of functional antibody fragments of adding a reducing glutathione mixture in the presence of PDI was marginal, whereas the addition of an oxidizing glutathione mixture was somewhat inhibitory (Fig. 3A). We suggest that the main role of PDI in this system is to catalyze disulfide rearrangements and not only net oxidation during protein synthesis[9–11] which appears to be necessary to allow scFv folding to proceed to the native state with the correct intradomain disulfide bonds formed.

The bacterial protein DsbA, which is not an efficient isomerization catalyst[17,24,25], has a comparatively weak effect on the yield of functionally active scFv fragments when included in the cell-free translation system (Fig. 3B; Table 1). The presence of reducing or oxidizing glutathione buffer together with DsbA does not change the amount of functional scFv fragments further (data not shown).

Aggregation of scFv fragments and the effects of chaperones. About 20–25% of the synthesized product was usually detected in the insoluble fraction under standard conditions, without DTT (Table 2). The presence of DTT during translation resulted in a much higher percentage of aggregated polypeptide, up to 60%, indicating that the reduced scFv is unfolded and aggregation-prone. Therefore, aggregation does not depend on the formation of intermolecular crosslinks. These large, sedimentable aggregates seem to contain various other molecules of the cell-free extract in addition to the scFv polypeptide and appear to be different from the interchain disulfide cross-linked aggregates discussed above.

The preparation of the crude *E. coli* extract may result in the depletion and/or inactivation of some cytoplasmic proteins, such as molecular chaperones. Therefore, the translation system was supplemented with purified DnaK, DnaJ, GrpE, GroEL and GroES, in various combination (Table 2). The presence of any or all of the molecular chaperones has no influence on the total protein synthesis. At the same time, the combinations of DnaK+DnaJ and DnaK+DnaJ+GrpE, and the complete chaperone cocktail significantly promote scFv solubility (Table 2). Comparable concentrations of BSA did not increase the amount of soluble protein, demonstrating that this increased solubility is not simply an effect of the protein concentration. The addition of GroEL, both in the absence and presence of its co-chaperone GroES, was largely unable to influence the aggregation process during protein synthesis, yielding only an additional 5% of soluble protein (Table 2).

The increased solubility of the synthesized scFv fragments is not accompanied by a significant increase in the proportion of correctly disulfide-bonded scFv fragments capable of binding antigen (Table 1; Fig. 3B). Thus, soluble but inactive scFv is produced in the presence of molecular chaperones by themselves, without taking measures to increase disulfide formation.

We then analyzed the oxidation state and distribution of the scFv into pellet and soluble fraction under several conditions. The addition of chaperones by themselves had a solubilizing effect on the scFv fragment (Fig. 4A) as seen by the decrease in the amount of scFv found in the large sedimental aggregates (Fig. 4B). The presence of PDI by itself, as well as in combination with the reduced form of glutathione, also led to much higher proportions of soluble scFv fragment (Fig. 4A), indicating that PDI significantly decreased the amount of intermolecular cross-linked aggregates incapable of

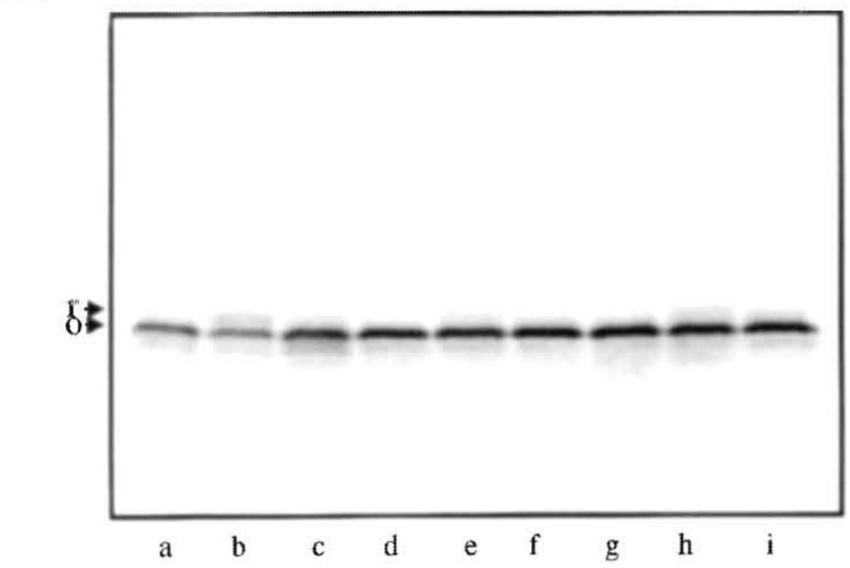

Figure 4. Western blot analysis, under non-reducing conditions, of supernatant and pellet fractions of the translation reaction. The scFv fragments were synthesized in a translation mixture without additions (a); in the presence of 1 mM GSH/ 0.1 mM GSSG (b); a chaperone cocktail containing DnaK, DnaJ, GrpE, GroEL, and GroES (c); PDI (d); PDI with 0.1 mM GSH/ 1 mM GSSG (e); PDI with 1 mM GSH/0.1 mM GSSG (f); PDI with a chaperone cocktail (g); PDI, a chaperone cocktail and 0.1 mM GSH/ 1 mM GSSG (h); and PDI, a chaperone cocktail and 1 mM GSH/ 0.1 mM GSSG (i); all present at the begining of translation. After 40 min of incubation at 37°C, the 50 µl reaction mixtures were centrifuged and 5 µl of the supernatant and 5 µl of the pellet (dissolved in 10 µl of translation buffer) were analyzed by SDS-PAGE. The samples were not reduced with DTT before electrophoresis. (A) Supernatant. (B) Pellet fraction. The oxidized protein (o) migrates slightly more quickly than the reduced form (r).

entering the gel. When both PDI and the oxidized form of glutathione were present in the translation mixture the amount of disulfide-containing scFv fragments in the pellet fraction increased (Fig. 4B), perhaps due to random intermolecular cross-linking of the scFv. Thus, mildly reducing conditions are required for formation of correct intrachain cross-linking, indicating the importance of disulfide rearrangement. In the soluble fraction only oxidized scFv fragment is visible, while in the insoluble fraction, scFv bands of both oxidized and reduced form are detected.

Joint effect of PDI and chaperones on the formation of functionally active scFv. In an attempt to maximize functional scFv production, we have tested the simultaneous inclusion of PDI and the chaperone mixture in the translation reaction. The presence of PDI, chaperones and the reduced mixture of glutathione in the translation reaction leads to the highest antigen-binding activity of the product in the supernatant (Table 3). Under these conditions, up to 48% of the total scFv synthesized during the *E. coli* cell-free translation could be recovered as active, antigen-binding material (Table 1).

Discussion

In order to maximize the amount of functional antibody synthesized in a cell-free translation system we identified factors that contribute to efficient folding and proper disulfide bond formation. The total yield of the protein synthesized was 10 to 14 pmol from a 50 µl incubation mixture (8 µg/ml) produced in 15 min at 37°C. However, only about 10% of the total amount of polypeptide synthesized was found to be functionally active when the strongly reducing compound DTT was omitted from the mixture, or when an oxidizing redox shuffle was introduced, or when the bacterial disulfide-forming catalyst DsbA was present. A massive intermolecular random cross-linking was observed in these cases. No functional protein at all was formed in the presence of reducing agents (1 mM DTT), and a large proportion of the scFv precipitated.

Table 3. The effect of PDI and chaperones on antigen binding activity.

Additions	Relative ELISA signal
PDI[b]	1.00[c]
PDI[b] + chaperones[a]	1.20
PDI[b] + chaperones[a], 0.1 mM GSH/ 1.0 mM GSSG	1.16
PDI[b] + chaperones[a], 1.0 mM GSH/ 0.1 mM GSSG	1.56

scFv fragments were synthesized in vitro. After 40 min of incubation at 37°C, the 50 µl of reaction mixture was centrifuged at 14,000 rpm and 15 µl of supernatant was analyzed by ELISA. Values at 1:40 dilutions are shown. [a]The concentrations are listed in Table 1. [b]0.9 µM. [c]Arbitrarily set to one.

A significant improvement was achieved when eukaryotic PDI was present during the cell-free translation. The proportion of functionally active antibody fragments increased by a factor of 3, reaching 30% of the total product. Eukaryotic PDI is known to be a much more efficient disulfide isomerization catalyst than DsbA[17,24,25], and its role is thought to be carried out by DsbC in bacteria[17]. The great importance of the disulfide isomerase activity, as opposed to the net oxidation activity, of PDI in vivo has been demonstrated[9–11].

If we define an equilibrium constant[27] between the reduced and oxidized form of PDI and glutathione, $K_{ox} = ([E\text{-}(SS)]\cdot[GSH]^2)/([E\text{-}(SH)_2]\cdot[GSSG])$, we can calculate the fraction of reduced enzyme as $[E\text{-}(SH)_2]/[E_{tot}] = g/(K_{ox} + g)$, where g is the ratio $[GSH]^2/[GSSG]$. Experiments were carried out at 1 mM GSH/ 0.1 mM GSSG ($g = 10^{-2}$ M) and 0.1 mM GSH/ 1 mM GSSG ($g = 10^{-5}$ M). Thus, in the "oxidizing" buffer, 1% of PDI was in the thiol form, whereas almost 90% of the PDI was in this form in the "reducing" buffer, assuming a K_{ox} of about 1 mM[26,27]. As in the case of oxidative folding of reduced ribonuclease in vitro[27], the successful net formation of correct disulfide bonds during in vitro translation proceeds only in the presence of significant amounts of reduced PDI. This indicates that the isomerization reaction, and not the net formation, is also crucial in the in vitro folding of scFv fragments. We cannot exclude any additional effect of direct binding of the scFv fragment to PDI, preventing it from premature aggregation. Such a chaperone-like activity has been proposed for PDI[28,29].

PDI was unable to affect the interchain multimers, and other incorrectly folded molecules, once they are formed in the system. It thus seems to act only on the nascent polypeptide chains, either during translation (cotranslationally), or immediately after release of the chain from the ribosome. Roth and Pierce[30] showed that PDI participates in the disulfide bond formation of immunoglobulins in vivo by interacting with nascent immunoglobulin light chains. However, in the B-cell, disulfide formation is separated from the ribosome by the endoplasmic reticulum membrane.

While the cocktail of molecular chaperones increased the amount of soluble scFv to almost the total amount of scFv synthesized, it had no influence on the amount of functional scFv fragment. The most significant influence on protein solubility (but not functionality) was observed from the Hsp70 system, DnaK and DnaJ. The addition of GrpE had no further influence. This suggests that no turnover of the chaperones, which would require the nucleotide exchange factor GrpE[31–33] takes place, but rather that DnaK and DnaJ may act as stoichiometric reagents. GroEL and

GroES by themselves had only a small effect on the amount of soluble protein. In a study of the folding of polypeptides emerging from ribosomes in a mammalian cell-free translation system, it was proposed[34] that Hsp70/Hsp40 interacted first with the elongating polypeptide. In the natural biosynthetic pathway of antibodies, however, they would be cotranslationally secreted. They may then interact with the antibody heavy chain binding protein (BiP), a Hsp70 homologue in the ER, and Hsp90[35,36].

At the same time, the complete chaperone cocktail did enhance the action of PDI somewhat, perhaps by extending the time during which PDI can act on the substrate. Specifically, the DnaK/DnaJ system may bind to either the nascent, ribosome-bound chains[37–39] or newly synthesized and released chains[32] and thus retard their precipitation to facilitate the action of PDI. Whether nascent chain binding occurs may depend on the particular protein translated[40].

It is therefore possible to synthesize active, disulfide-containing single-chain antibodies in the *E. coli* cell-free translation system. The formation of active molecules requires the catalysis and rearrangement of disulfide bond formation during the in vitro translation. When both PDI and chaperones are present in the system, almost 50% of the total amount of the synthesized polypeptide can be eluted from antigen-binding RIA, indicating that at least this amount of scFv synthesized is capable of specifically binding the antigen. Similar results have been obtained with several other unrelated antibodies (data not shown). The use of the cell-free system in the continuous-flow format[41] may be promising for the synthesis of functional antibodies on a larger scale, giving convenient access to directly labelled protein or even incorporating unnatural amino acids.

Experimental protocol

Construction of scFv fragment of hag binding antibody. The gene encoding the scFv fragment of the anti-hemagglutinin (hag) antibody 17/9 (ref. 21), in the orientation V_L-linker-V_H with a $(Gly_4Ser)_3$ linker, was obtained by gene synthesis[42] and cloned into pTFT74[43].

In vitro transcription. Plasmid DNA was isolated using a plasmid preparation kit from Qiagen (Chatsworth, CA) and transcribed[44] with T7 polymerase (Promega, Madison, WI). The mRNA was purified by phenol/chloroform (1:1) extraction, followed by precipitation with ethanol. Approximately 1.5 mg of mRNA was prepared from 20 μg of plasmid.

In vitro translation. An *E. coli* S30 extract (191 A_{260} units/ml) in acetate buffer (40 mM Tris-Ac, pH 8.2, containing 14 mM $MgAc_2$, 60 mM KAc, 100 μM EDTA, and 1 mM dithiothreitol) was prepared according to the procedure of Chen and Zubay[45]. The extract was then dialyzed overnight at 4°C against the same acetate buffer lacking dithiotreitol. The translation reaction was incubated at 37°C for 40 min in a 50 μl volume of 50 mM Tris-Ac, pH 8.2, containing 5 μl of *E. coli* S-30 crude extract, 7 μg scFv-mRNA, 8 μg of *E. coli* tRNA, 150 mM KAc, 15 mM $MgAc_2$, 100 μM EDTA, 30 mM acetyl phosphate[46], 500 μM ATP, 500 μM GTP, 10 μM [^{14}C]leucine (350.2 mCi/mmol (NEN, Boston, MA)) or 50 μM unlabeled leucine, and 100 μM each of the other 19 amino acids. Molecular chaperones (purified from *E. coli* overexpression plasmids, provided by S. Axmann and E. Pierpaoli), protein disulfide-isomerase (PDI) (from bovine liver, Pierce, Rockford, IL), and DsbA from *E. coli* (purified from an overexpression plasmid)[47] were used at concentrations indicated in Table 1. After translation and where indicated, samples were centrifuged in an Eppendorf Microfuge for 5 min at 14,000 rpm and 4°C to produce soluble (supernatant) and insoluble (pellet) fractions. Pellets from 50 μl reaction mixtures were resuspended in 10 μl of PAGE sample buffer[48], and 5 μl samples, as well as 5 μl samples of supernatant fractions and 5 μl aliquots from the total reaction mixture, were used for analysis by SDS-PAGE and for measuring the radioactivity precipitated by trichloroacetic acid. Fifteen microliters of supernatants were used for ELISA. The results were recalculated to pmol of protein synthesized in the original 50 μl reaction.

SDS-PAGE. Translation mixtures (5 μl) were applied to 0.1% SDS-12% PAGE[48] under reducing (SDS-PAGE sample buffer includes 100 mM DTT) and non-reducing conditions (sample buffer without DTT). The gels were stained, dried and exposed to X-ray film (Fuji) for 16–24 h. Gels were also analyzed using a PhosphorimagerPC (Molecular Dynamics, Sunnyvale, CA).

Western blot. Gels were blotted onto PVDF membrane using standard protocols, and the scFv fragments were detected using the anti-myctag Ab 9E10 (Dianova, Hamburg, Germany), followed by a goat anti-mouse IgG-peroxidase conjugated antibody (Sigma, St. Louis, MO).

ELISA. The hag binding activity of the protein products was determined by ELISA with the hag peptide KNSYPYDVPDYASLRS coupled to denatured and reduced human transferrin[49]. Bound scFv fragment was detected using the anti-myc Ab 9E10 followed by a goat anti-mouse IgG-peroxidase conjugated antibody. Specific binding to the antigen was confirmed by pre-incubation of the soluble scFv fraction dilutions with 100 μg antigen/ml, which almost completely abolished the ELISA signals. Samples from the translation mixtures without added mRNA were used to calculate the background.

Radioimmunoassay (RIA). The percentage of the synthesized protein which showed hag peptide binding activity was determined by RIA, similar to the ELISA described above. After blocking and incubation of the [^{14}C]-labeled proteins for 60 min the supernatant was removed from the well, and each well was washed three times with 100 μl of PBS containing 0.05% (v/v) Tween 20. The bound scFv fragments in each well were then eluted with 100 μl of 100 mM triethylamine (TEA) for 20 min, the wells washed three times more with TEA, all elution fractions were combined and TCA precipitable radioactivity was measured. The percentage of active antibody was calculated as the ratio between the amount of protein eluted from the antigen and total amount of protein synthesized. Under these conditions, essentially all the TCA-precipitable radioactivity is due to the antibody synthesized. All scFv proteins were shown to be eluted completely from the wells of the antigen-coated microtiter plate by the failure to detect any residual signal with the anti-myc Ab 9E10.

Acknowledgments

We are indebted to Esra V. Pierpaoli for providing DnaK, DnaJ, and GrpE, and to Sabine Axmann for GroEL and GroES. This work was supported by funds from Schweizerischer Nationalfonds 31-37720. 93, 31-37717. 93 and the Academy of Sciences of Russia. LR benefited from an EMBO Fellowship.

1. Hurle, M.R. and Gross, M. 1994. Protein engineering techniques for antibody humanization. *Curr. Opin. Biotechnol.* **5**:428–433.
2. Plückthun, A. 1992. Mono- and bivalent antibody fragments produced in *Escherichia coli*: engineering, folding and antigen binding. *Immunol. Rev.* **130**:151–188.
3. Trill, J.J., Shatzman, A.R., and Ganguly, S. 1995. Production of monoclonal antibodies in COS and CHO cells. *Curr. Opin. Biotechnol.* **6**:553–560.
4. Goto, Y. and Hamaguchi, K. 1979. The role of the intrachain disulfide bond in the conformation and stability of the constant fragment of the immunoglobulin light chain. *J. Biochem.* **86**:1433–1441.
5. Glockshuber, R., Schmidt, T., and Plückthun, A. 1992. The disulfide bonds in antibody variable domains: effects on stability, folding *in vitro*, and functional expression in *Escherichia coli*. *Biochemistry* **31**:1270–1279.
6. Holmgren, A. 1985. Thioredoxin. *Ann. Rev. Biochem.* **54**:237–271.
7. Freedman, R.B., Hirst, T.R., and Tuite, M.F. 1994. Protein disulfide isomerase: building bridges in protein folding. *Trends Biochem.* **19**:331–336.
8. Freedman, R.B. 1995. The formation of protein disulphide bonds. *Curr. Op. Struct. Biol.* **5**:85–91.
9. Laboissière, M.C.A., Sturley, S.L., and Raines, R.T. 1995. The essential function of protein-disulfide isomerase is to unscramble non-native disulfide bonds. *J. Biol. Chem.* **270**:28006–28009.
10. Tachibana, C. and Stevens, T.H. 1992. The yeast EUG1 gene encodes an endoplasmic reticulum protein that is functionally related to protein disulfide isomerase. *Mol. Cell. Biol.* **12**:4601–4611.
11. LaMantia, M.L. and Lennarz, W.J. 1993. The essential function of yeast protein disulfide isomerase does not reside in its isomerase activity. *Cell* **74**:899–908.
12. 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.
13. 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.
14. Guilhot, C., Jander, G., Martin, N.L., and Beckwith, J. 1995. Evidence that the pathway of disulfide bond formation in *Escherichia coli* involves interactions between the cysteines od DsbB and DsbA. *Proc. Natl. Acad. Sci. USA* **92**:9895–9899.
15. Kishigami, S., Kanaya, E., Kikuchi, M., and Ito, K. 1995. DsbA-DsbB interaction through their active site cysteines. Evidence from an odd cysteine mutant of DsbA. *J. Biol. Chem.* **270**:17072–17074.
16. Missiakas, D., Georgopoulos, C., and Raina, S. 1994. The *Escherichia coli* dsbC (xprA) gene encodes a periplasmic protein involved in disulfide bond formation. *EMBO J.* **13**:2013–2020.
17. Zapun, A., Missiakas, D., Raina, S., and Creighton, T.E. 1995. Structural and functional characterization of DsbC, a protein involved in disulfide bond formation in *Escherichia coli*. *Biochemistry* **34**:5075–5089.
18. Missiakas, D., Schwager, F., and Raina, S. 1995. Identification and characterization of a new disulfide isomerase-like protein (dsbD) in *Escherichia coli*. *EMBO J.*

14:3415–3424.
19. Hartl, F.U. 1996. Molecular chaperones in cellular protein folding. *Nature* **381:**571–579.
20. Huston, J.S., Levinson, D., Mudgett-Hunter, M., Tai, M.-S., Novotny, J., Margolies, M.N., et al. 1988. Protein engineering of antibody binding sites: recovery of specific activity in an anti-digoxin single-chain Fv analogue produced in *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* **85:**5879–5883.
21. Schulze-Gahmen, U., Rini, J.M., and Wilson, I.A. 1993. Detailed analysis of the free and bound conformations of an antibody. X-ray structures of Fab 17/9 and three different Fab-peptide complexes. *J. Mol. Biol.* **234:**1098–1118.
22. Nicholls, P.J., Johnson, V.G., Andrew, S.M., Hoogenboom, H.R., Raus, J.C.M. and Youle, R.J. 1993. Characterization of single-chain antibody (sFv)-toxin fusion proteins produced in vitro in rabbit reticulocyte lysate. *J. Biol. Chem.* **268:**5302–5308.
23. Proba, K., Ge, L., and Plückthun, A. 1995. Functional antibody single-chain fragments from the cytoplasm of *Escherichia coli*: influence of thioredoxin reductase (TrxB). *Gene* **159:**203–207.
24. Wunderlich, M., Otto, A., Seckler, R., and Glockshuber, R. 1993. Bacterial protein disulfide isomerase: Efficient catalysis of oxidative protein folding at acidic pH. *Biochemistry* **32:**12251–12256.
25. Darby, N.J. and Creighton, T.E. 1995. Catalytic mechanism of DsbA and its comparison with that of protein disulfide isomerase. *Biochemistry* **34:**3576–3587.
26. Darby, N.J. and Creighton, T.E. 1995. Functional properties of the individual thioredoxin-like domains of protein disulfide isomerase. *Biochemistry* **34:**11725–11735.
27. Lyles, M.M. and Gilbert, H.F. 1991. Catalysis of the oxidative folding of ribonuclease A by protein disulfide isomerase: dependence of the rate on the composition of the redox buffer. *Biochemistry* **30:**613–619.
28. Song, J.L. and Wang, C.C. 1995. Chaperone-like activity of protein disulfide isomerase in the refolding of rhodanese. *Eur. J. Biochem.* **231:**312–316.
29. Quan, H., Fan, G., and Wang, C.C. 1995. Independence of the chaperone activity of protein disulfide isomerase from its thioredoxin-like active site. *J. Biol. Chem.* **270:**17078–17080.
30. Roth, R.A. and Pierce, S.B. 1987. *In vivo* cross-linking of protein disulfide isomerase to immunoglobulins. *Biochemistry* **26:**4179–4182.
31. Liberek, K., Marszalek, J., Ang, D., Georgopoulos, C., and Zylicz, M. 1991. *Escherichia coli* DnaJ and GrpE heat shock proteins jointly stimulate ATPase activity of DnaK. *Proc. Natl. Acad. Sci. USA* **88:**2874–2878.
32. Langer, T., Lu, C., Echols, H., Flanagan, J., Hayer, M.K., and Hartl, F.U. 1992. Successive action of DnaK, DnaJ and GroEL along the pathway of chaperone-mediated protein folding. *Nature* **356:**683–689.
33. Buchberger, A., Schröder, H., Büttner, M., Valencia, A., and Bukau, B. 1994. A conserved loop in the ATPase domain of the DnaK chaperone is essential for stable binding of GrpE. *Nature Struct. Biol.* **1:**95–101.
34. Frydman, J., Nimmesgern, E., Ohtsuka, K., and Hartl, F.U. 1994. Folding of nascent polypeptide chains in a high molecular mass assembly with molecular chaperones. *Nature* **370:**111–117.
35. Munro, S. and Pelham, H.R.B. 1986. An Hsp70-like protein in the ER: identity with the 78 kd glucose-regulated protein and immunoglobulin heavy chain binding protein. *Cell* **46:**291–300.
36. Melnick, J., Dul, J.L., and Argon, Y. 1994. Sequential interaction of the chaperones BiP and GRP94 with immunoglobulin chains in the endoplasmic reticulum. *Nature* **370:**373–375.
37. Beckmann, R.P., Mizzen, L.E., and Welch, W.J. 1990. Interaction of Hsp 70 with newly synthesized proteins: implications for protein folding and assembly. *Science* **248:**850–854.
38. Nelson, R.J., Ziegelhoffer, T., Nicolet, C., Werner-Washburne, M., and Craig, E.A. 1992. The translation machinery and 70 kD heat shock protein cooperate in protein synthesis. *Cell* **71:**97–105.
39. Hendrick, J.P., Langer, T., Davis, T.A., Hartl, F.U., and Wiedmann, M. 1993. Control of folding and membrane translocation by binding of the chaperone DnaJ to nascent polypeptides. *Proc. Natl. Acad. Sci. USA* **90:**10216–10220.
40. Tokatlidis, K., Friguet, B., Deville-Bonne, D., Baleux, F., Fedorov, A.N., Navon, A., et al. 1995. Nascent chains: folding and chaperone interaction during elongation on ribosomes. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.* **348:**89–95.
41. Spirin, A.S., Baranov, V.I., Ryabova, L.A., Ovodov, S. Yu., and Alakhov, Yu. B. 1988. A continuous cell-free translation system capable of producing polypeptides in high yield. *Science* **242:**1162–1164.
42. Prodromou, C. and Pearl, L.H. 1992. Recursive PCR: a novel technique for total gene synthesis. *Prot. Eng.* **5:**827–829.
43. Freund, C., Ross, A., Guth, B., Plückthun, A., and Holak, T.A. 1993. Characterization of the linker peptide of the single-chain Fv fragment of an antibody by NMR spectroscopy. *FEBS Lett.* **320:**97–100.
44. Gurevich, V.V., Pokrovskaya, I.D., Obukhova, T.A., and Zozulya, S.A. 1991. Preparative *in vitro* mRNA synthesis using SP6 and T7 RNA polymerases. *Analyt. Biochem.* **195:**207–213.
45. Chen, H.-Z. and Zubay, G. 1983. Prokaryotic coupled transcription-translation. *Methods Enzymol.* **101:**674–690.
46. Ryabova, L.A., Vinokurov, L.M., Shekhovtsova, E.A., Alakhov, Yu, B., and Spirin, A.S. 1995. Acetyl phosphate as an energy source for bacterial cell-free systems. *Analyt. Biochemistry* **226:**184–186.
47. Lindner, P., Guth, B., Wülfing, C., Krebber, C., Steipe, B., Müller, F., and Plückthun, A. 1992. Purification of native proteins from the cytoplasm and periplasm of *Escherichia coli* using IMAC and histidine tails: a comparison of proteins and protocols. *Methods: A Companion to Meth. Enzymol.* **4:**41–56.
48. Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227:**680–685.
49. Kitagawa, T., Kanamaru, T., Wakamatsu, H., Kato, H., Yano, S., and Asanuma, Y. 1978. A new method for preparation of an antiserum to penicillin and its application for novel enzyme immunoassay of penicillin. *J. Biochem.* **84:**491–494.