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Functional antibody single-chain fragments from the cytoplasm of *Escherichia coli*: influence of thioredoxin reductase (TrxB)

(Cytoplasmic expression; disulfide bond formation; redox potential; screening system)

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Functional antibody single-chain fragments from the cytoplasm of *Escherichia coli*: influence of thioredoxin reductase (TrxB)

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SUMMARY

The cytoplasmic expression of a functional antibody (Ab) fragment, containing the correct intradomain disulfide bonds, was investigated in *E. coli*. We used a single-chain Fv (scFv) fragment of the levan-binding Ab ABPC48, which was shown to be functional only in the presence of the disulfide bonds. Significant amounts of functional, disulfide-containing scFv could be produced in the cytoplasm of *E. coli* in the absence of thioredoxin reductase (TrxB) activity. The amount of soluble protein remained largely unchanged by this null mutation. A stronger promoter did not result in further improved yields of functional Ab fragment, despite much higher protein production, suggesting that inefficient disulfide formation was still limiting the yield of active scFv. This method of expressing functional Ab fragments in the cytoplasm of *E. coli* may be important for screening and selection systems.

INTRODUCTION

Disulfide bonds are one of the hallmarks of the immunoglobulin superfamily (Hunkapiller and Hood, 1989). They are conserved within all domains of the family, such as constant and variable Ab domains, T-cell receptors, and other related molecules, like many of the surface proteins of lymphoid cells. Apparently, disulfide bonds serve an important function in stabilizing the β-barrel that makes up the immunoglobulin fold.

The stabilizing effect of the disulfide bonds in Ab domains has been tested experimentally (Goto and Hamaguchi, 1979). Mutagenesis experiments with variable Ab domains have shown that in general it may not be possible to obtain the folded structure in the absence of the disulfide bonds (Glockshuber et al., 1992), because the total free energy is then insufficient.

For efficient functional expression of Ab fragments in bacteria, the disulfide forming machinery of the periplasm (Bardwell et al., 1991; Kamitani et al., 1992) has to be used, and indeed such an assembly is possible with a wide variety of Ab fragments (Plückthun, 1992). However, the functional Ab expression in the cytoplasm may potentially be of great interest. First, since the in vivo folding of the Ab seems to limit the overall production (Plückthun, 1992), and since there is probably no ATP-dependent molecular chaperone in the periplasm (Wülfing and Plückthun, 1994), recruitment of the cytoplasmic folding machinery is tempting. Second, using Ab

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Abbreviations: *A*, absorbance (1 cm); aa, amino acid(s); Ab, antibody (ies); ABSE, acetate-buffered saline EDTA (50 mM Na-acetate buffer pH 5.2/150 mM NaCl/10 mM EDTA); CDR, complementarity determining regions; DTT, dithiothreitol; ELISA, enzyme linked immunosorbent assay; Fab, Ab-binding fragment; Fv, variable fragment or Ab; H, heavy chain; IPTG, isopropyl-β-D-thiogalactoside; nt, nucleotide; PAGE, polyacrylamide-gel electrophoresis; PVDF, polyvinyldifluoride; PVP, polyvinyl-pyrrolidone; scFv, single-chain Fv fragment; SB, super broth; SDS, sodium dodecyl sulfate; TBS, Tris-buffered saline (25 mM Tris·HCl pH 7.5/150 mM NaCl/3 mM KCl); TrxB, thioredoxin reductase; V_H, variable heavy chain; wt, wild type;

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in catalysis (Lerner et al., 1991), metabolic selection schemes would be an attractive means to enhance their activities, and more possibilities exist for cytoplasmic than periplasmic reactions. Ultimately, such Ab might be used in the engineering of metabolic pathways with new activities. Third, direct coupling of Ab-antigen interaction to transcription may become possible, thereby linking affinity to bacterial growth (via an antibiotic-resistance-encoding gene) or detection (via a colorigenic enzyme reaction).

Functional Ab molecules from the cytoplasm (however, without investigating the disulfide state) have been reported for yeast (Carlson, 1988) and higher eukaryotes (Biocca et al., 1990). Previous experiments with *E. coli* (Cabilly, 1989) have shown that at low temperature a certain fraction of an Fab fragment, which was cytoplasmically expressed, gave rise to positive ELISA signals. Unfortunately, the disulfide-bond formation in these molecules was also not investigated. We have now revisited this question with recombinant scFv fragments, which we knew only reacted with the antigen when the disulfide bonds were formed, and we have characterized the influence of deleting the *trxB* gene, a mutation which has recently been found to enhance the formation of disulfide bonds in the cytoplasm (Derman et al., 1993).

EXPERIMENTAL AND DISCUSSION

(a) Experimental system

As a model system for investigating the limits of cytoplasmic expression, we used the levan-binding Ab ABPC48 (Lieberman et al., 1975). This Ab contains a Cys to Tyr mutation in position 98 of the heavy (H) chain (CysH98Tyr), shown both by nt (Auffray et al., 1981) and aa sequencing (Rudikoff and Pumphrey, 1986). Periplasmic expression of a scFv fragment of this Ab neither yields any soluble protein nor any positive antigen-ELISA signal, but both are restored as soon as CysH98 is introduced (K.P. and A.P., unpublished).

The scFv fragment in the orientation V_L-linker-V_H of ABPC48 was obtained by total gene synthesis, using a method based on that of Kolbinger et al. (1993). The antigen of ABPC48 is bacterial and plant levan (poly-β-2,6-fructose) (Feingold and Gehatia, 1957). The protein carries the sequence EQKLISEEDLNHHHHH at the C terminus for purification by IMAC (Lindner et al., 1992) and detection by the anti-myc Ab 9E10 (Munro and Pelham, 1986). The experiments described here were all carried out with a mutant of ABPC48 in which the V_H disulfide bond is restored, which was missing in the wt. The mutant, thus, is a 'normal' scFv fragment.

For the expression experiments, two different promoter systems were used. The *lac*-based system is based on our secretory plasmids (Skerra et al., 1991; Ge et al., 1994) from which the signal sequence has been deleted. The T7-based system (Studier and Moffatt, 1986; Freund et al., 1993) flanks the Ab-encoding gene with the T7 promoter and T7 transcription terminator. The secretion plasmids with the tag sequences have been described (Ge et al., 1994).

To investigate the effect of the *trxB* mutation with the *lac* system, both *E. coli* A304 (*fhuA*22, *garB*10, *trxB*15::*kan*, *ompF*627, *supD*32, *fadL*701, *relA*1, *pit*-10, *spoT*1, *mcrB*1, *phoM*510) (Russel and Model, 1986) and *E. coli* BL21DE3 *trxB*$^-$ (see section **b** below) were used. These strains are deficient in TrxB, due to a Tn*kan*^{R} insertion within *trxB*. As *trxB*$^+$ strains, *E. coli* JM83 (λ$^-$, *ara*, Δ(*lac-proAB*), *rpsL*, *thi*, ϕ80d*lacZ*ΔM15) (Yanisch-Perron et al., 1985) and *E. coli* BL21DE3 {F$^-$, *ompT*$^-$, $r_B^- m_B^-$, (λimm21, *lacI*, *lac*UV5, T7 pol, *int*$^-$)} (Studier and Moffatt, 1986) were used. Since the two BL21 strains are isogenic and only differ in the *trxB* gene, they serve as control for any potential differences other than the *trxB* gene between JM83 and A304, which might contribute to the observed effects.

To investigate the effect with the T7 system, the *trxB* mutation was moved from *E. coli* A304 to *E. coli* BL21DE3, to generate *E. coli* BL21DE3 *trxB*$^-$. This was achieved by P1-phage-mediated transduction following a standard protocol (Miller, 1972), selection for *kan*^{R}, and verifying the destruction of *trxB* in the transductants by measuring the TrxB activity (Holmgren, 1984).

The expression experiments were carried out at 37 °C and 24 °C. At 37 °C the total amount of cytoplasmically expressed protein was significantly higher, but no or only very small amounts of functional protein could be detected (data not shown). The duration of induction had no dramatic effect, although the amount of functional protein reached the maximum after overnight induction (data not shown). For sample preparation we took care to avoid formation of disulfide bonds in vitro after cell disruption or any consecutive steps, by using a degassed low-pH buffer (ABSE, see legend to Fig. 1).

(b) Expression with the *lac*-based system

When the scFv fragment was expressed cytoplasmically under the control of the *lac* promoter at 24°C, in both *trxB*$^+$ (JM83 and BL21DE3) and *trxB*$^-$ (A304 and BL21DE3*trxB*$^-$) strains, about 50–75% of the scFv protein was soluble (Fig. 1A). The total amount of expressed protein was similar between the strains A304 and both versions of BL21DE3, but significantly lower for JM83. However, in both *trxB*$^-$ strains we found a significant amount of disulfide-containing scFv fragment (as evidenced by SDS-PAGE under non-reducing conditions,

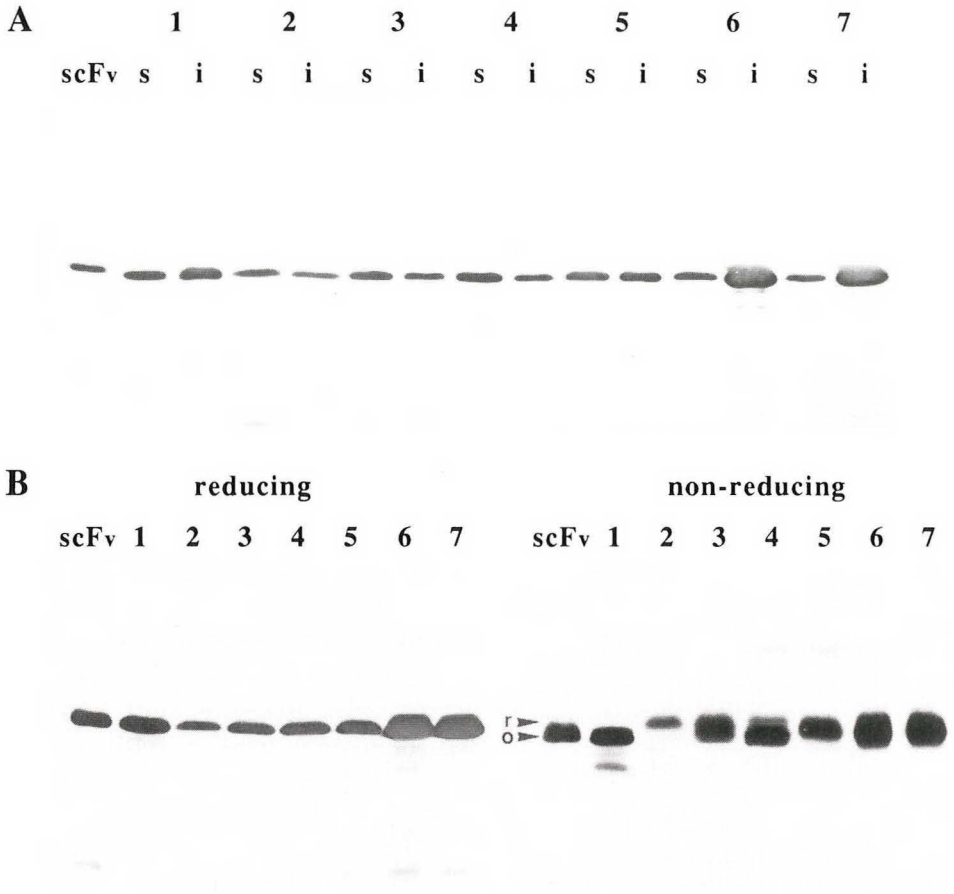

Fig. 1. Western blot analysis of crude extracts of different cultures expressing the same anti-levan scFv fragment. For numbering of cultures, see Table I. (**A**) Soluble and insoluble fractions of crude extracts under reducing conditions. **s**, soluble fraction, **i**, insoluble fraction. **scFv**, purified periplasmically expressed scFv fragment of ABPC48. (**B**) Total extracts under reducing and nonreducing conditions. The oxidized protein (**o**) migrates slightly faster than the reduced form (**r**), (**arrow**). The non-reducing lanes **2** and **5** (*trxB*$^+$ strains/*lac* system) show only reduced scFv fragment. In the non-reducing lanes **3** and **4** (*trx*B$^-$ strains/*lac* system), scFv bands of both oxidized and reduced form are visible. Because of the bulk of insoluble protein, splitting of the bands is not visible with the T7 system (non-reducing lanes **6** and **7**). **Methods:** Expression experiments were carried out as follows: 10 ml of SB media (20 g tryptone/10 g yeast extract/5 g NaCl/2.5 g K_2HPO_4/1 g $MgSO_4 \cdot 7H_2O$/0.1 mg biotin/1 mg thiamine/100 mg ampicillin; all per litre) were inoculated with an overnight culture to give an initial A_{600} of 0.05. Expression was induced at $A_{600} = 0.5$ with IPTG (1 mM final concentration) and continued for 5 h or overnight. The experiments were carried out at 24 and 37°C. After centrifugation, cell pellets were resuspended in varying volumes of ABSE (about 1/5 of the original volume ABSE (degassed)) in order to normalize the cell densities of the resulting suspensions. Cell disruption was achieved by French Press lysis. After centrifugation of the crude extracts, supernatants were removed (soluble fraction) and the resulting pellets were resuspended again in ABSE (insoluble fraction). Cytoplasmic and periplasmic expression experiments were carried out under identical conditions. Samples of the crude extracts and whole cells were applied to 0.1% SDS-12% PAGE under reducing (5 × SDS-PAGE sample buffer includes 500 mM DTT) and nonreducing conditions (SDS-PAGE sample buffer without DTT). Gels were blotted on PVDF membrane using standard protocols, and the scFv fragments were detected using the anti-myc Ab 9E10 followed by an anti-mouse IgG-peroxidase conjugate.

Fig. 1B), whereas in the *trxB*$^+$ strains no oxidized protein was detectable. The signal in functional ELISA increases in parallel with the disulfide containing species (Table I).

Using a different scFv fragment, which recognizes tryptophan synthase (L.G., A. Lupas, S. Peraldi-Roux, S. Spada and A.P., data not shown), again much higher antigen-binding ELISA values in cytoplasmic expression were found in the *trxB*$^-$ strain A304 than in the *trxB*$^+$ strain JM83 (L.G. and A.P., unpublished).

The data in Fig. 1 and Table I show that the *trxB* null mutation has no or only very little influence on the amount of soluble protein, but does dramatically increase the amount of disulfide-containing (and thus functional) antibody in the cytoplasm. Thus, the disulfide-containing, functional scFv molecules are only a variable fraction of a more or less constant amount of soluble cytoplasmic scFv.

(c) Expression with the T7-based system

In the *trxB*$^-$ strains, 50% and more of the available antibody protein is oxidized, but the amounts remain relatively small (approx. 2 μg/l in a standard shake flask culture). In order to examine the effect of an increased antibody expression, we used the T7-promoter in an attempt to maximize total protein production. These experiments were carried out with strains BL21DE3 and

TABLE I

Relative ELISA signals produced by different cultures expressing the same anti-levan scFv fragment

No.[a]	Location[a]	Promoter	Strain[b]	TrxB[c]	Relative ELISA signal[d]
1	peri	*lac*	JM83	+	1.00[e]
2	cyto	*lac*	JM83	+	0.05
3	cyto	*lac*	A304	−	0.42
4	cyto	*lac*	BL21DE3*trxB*$^-$	−	0.82
5	cyto	*lac*	BL21DE3	+	0.10
6	cyto	T7	BL21DE3*trxB*$^-$	−	0.34
7	cyto	T7	BL21DE3	+	0.05

[a]The numbers are referenced in Fig. 1. peri denotes periplasmic protein (expressed with signal sequence), cyto denotes cytoplasmic protein (expressed without signal sequence).

[b] For strain description see section **a**.

[c] Symbol + denotes presence of TrxB, − denotes TrxB deficiency.

[d] For ELISA experiments, bacterial levan from *Erwinia herbicola* (Sigma) was used. Polystyrene plates were coated overnight at 4°C with a solution of 10 μg/ml levan in H_2O. The following steps were carried out at room temperature and incubation times were 60 min. Blocking was achieved with 1% (w/v) polyvinyl-pyrrolidone (PVP-40) in TBS (25 mM Tris·HCl pH 7.5/150 mM NaCl/3 mM KCl) 0.05% (v/v) Tween-20, and plates were then incubated with a 1 : 50 dilution in ABSE (see legend to Fig. 1) of the soluble fractions (see legend to Fig. 1). Bound scFv fragment was detected with the anti-myc Ab 9E10 followed by an anti-mouse IgG-peroxidase conjugate. Specific binding to the antigen was confirmed by adding 100 μg antigen/ml to the dilutions of the soluble fractions, which almost completely inhibited the ELISA signals.

[e]The periplasmic system (corresponding to about 2.5 mg purified scFv protein per litre culture) was arbitrarily set to 1.0.

BL21DE3 *trxB*$^-$ (see section **a**), which encode the T7 polymerase in the chromosome under control of the *lac* promoter (Studier and Moffat, 1986). With both strains, the total amount of Ab protein was about an order of magnitude higher, with an enormous increase of insoluble material. The amount of soluble protein even slightly decreased compared to strain A304 (Fig. 1A). Similar to the *lac*-based system, the yield of functional material was significantly increased by the presence of the *trxB* mutation (as evidenced by antigen-ELISA, Table I), but the amount of ELISA-reactive material was lower in BL21DE3 *trxB*$^-$ than in A304, in agreement with the Western blot results (Fig. 1A).

(d) Conclusions

(*1*) We conclude that in these functional cytoplasmic expression experiments, neither transcription nor translation are limiting processes. Rather, folding coupled with disulfide formation is the limiting step. It seems that in the case of the T7-based system, these slow steps have to compete with fast and facile concentration-dependent aggregation and precipitation steps, favored by the initial high concentration of reduced and incorrectly folded scFv fragment.

(*2*) The *trxB* mutation is tolerated by *E. coli*, suggesting that it only has a moderate effect on the overall redox state in the cytoplasm. A truly oxidizing environment in the cytoplasm may be toxic, since too many cytoplasmic proteins require free sulfhydryls for their function. It is unclear by which mechanism the observed cytoplasmic disulfides are formed, since there is no disulfide forming machinery in the *E. coli* cytoplasm (to the best of our knowledge, there are no permanent disulfides in natural cytoplasmic *E. coli* proteins); the 'illegitimate' use of naturally occurring oxidized glutathione or thioredoxin and/or its homologs is likely. A direct involvement of molecular oxygen remains a possibility as well.

(*3*) It is possible to obtain significant amounts of disulfide-containing, functional Ab fragments in the *E. coli* cytoplasm, in the presence of a *trxB* null mutation. However, the inefficient oxidation process, despite being significantly improved by the *trxB* mutation, did not lead to a higher expression level of functional scFv fragment than in the periplasmic expression system.

(*4*) In periplasmic expression, yields are frequently limited by aggregation processes during periplasmic folding (Plückthun, 1992), but recently mutations have been identified which lead to extremely well expressing antibodies in the periplasm (Knappik and Plückthun, 1995). It remains to be investigated whether these beneficial folding mutations also affect cytoplasmic folding similarly, or whether the inefficient cytoplasmic disulfide formation is solely limiting the yield.

In summary, our results clearly indicate the applicability of *trxB*$^-$ strains for the expression of significant amounts of functional and disulfide containing scFv fragments. Therefore, such systems might become especially attractive for screening and selection systems for Ab and similar molecules.

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EXPERIMENTAL AND DISCUSSION, or RESULTS AND DISCUSSION should point to the data shown in the figures and tables, and briefly discuss their significance. Divide this section into subsections marked, (a), (b), (c), etc., with short subtitles (lower-case, Roman, not underlined). Do not prepare a separate DISCUSSION section; instead, the last subsection subtitled, (x) Conclusions, should be short and divided into points, (*1*), (*2*), (*3*)... [see, e.g., *Gene* 100 (1991) 48–49, or 110 (1992) 5–6].

REFERENCES should be assembled alphabetically and typed double-spaced. They should be referred to by name and year (Harvard System). More than one citation from the same author(s) and year must be identified by the letters, a, b, c, etc., placed after the year of publication. In the text, when referring to a paper by more than two authors, only the name of the first author should be given, followed by a non-italicized 'et al.'. When referring to a personal communication or unpublished paper, all initials and name(s) of all author(s) should be cited, and written permission from those authors should be submitted to your Editor (with a copy to the Editor-in-Chief). Literature references must be in accord with the following examples (also consult recent *Gene* issues):

Westmoreland, B.C., Szybalski, W. and Ris, H.: Mapping of deletions and substitutions in heteroduplex DNA molecules of bacteriophage lambda by electron microscopy. Science 163 (1969) 1343–1348.

Rodriguez, R.L. and Tait, R.C.: Recombinant DNA Techniques: An Introduction, Addison-Wesley, Reading, MA, 1983.

Daniels, D.I., Schroeder, J.L., Szybalski, W., Sanger, F. and Blattner, F.R.: A molecular map of coliphage lambda. In: Hendrix, R.W., Roberts, J.W., Stahl, F.W. and Weisberg, R.A. (Eds.), Lambda II. Cold Spring Harbor Laboratory, Cold Spring Habor, NY, 1983, pp. 469–676.

References must be accurate (rechecked in the library) and follow *Gene*'s style. Journal titles should be abbreviated according to the 'List of Serial Title Word Abbreviations' [1985; ISBN 2-904939-02-8; available from your library or International Serials Data System (ISDS), CIEPS, ISDS International Centre, 20, rue Bachaumont, 75002 Paris (France)]. For reviews with a great number of references, employ the system used in *Gene* Vol. 100 (1991) 13–26.

TABLES should be numbered, I, II, III, etc., and, at the top, bear a short descriptive title. Detailed footnote(s) identified by superscript, $^{a,b,c,\ldots}$, usually one for each major column, should be typed double-spaced under the table (for Methods, see FIGURES).

FIGURES (line drawings, including graphs) should be prepared as laser-quality computer printouts. Send *original* printouts, not photos. Only if a laser-quality printer is not available (dot matrix printers are unsatisfactory), should you prepare professionally drawn figures in black ink on white paper. Always use large and bold lettering, and heavy smooth lines, as to permit photographic reduction. Be sure that all symbols in the figure are large and match the explanations in the legend. Sequence figures should be either 60 nucleotides (or amino acids) in width (to fit into a single printed column), or 120–150 nt (or aa) wide (to fit across the entire printed page). Please make figures compact, but still readable. Half-tone illustrations (e.g., gel photos) should be submitted as black-and-white glossy prints and have as much contrast as possible. Add bp, nt, kb or kDa symbols above the numerals in marker lanes. The minimum size of figures is 10 × 12 cm and the maximum is 20 × 25 cm. Legends should be typed double-spaced, on separate pages. All pertinent technical details should only be specified in the figure legends and/or table footnotes, and be **detailed** enough **to permit repetition** of the experiments; when appropriate, add a bold subtitle, **Methods.**, to such a description of the experimental details. Please add to legends (for figures) or to footnotes (for tables) all details [temp., time(s), voltage, concentrations, type and % of gel, markers, sizes (bp, kb, kDa), etc.]. **Proofreading** should be done carefully. Please note that it is the Publisher's policy that only printer's errors may be corrected: no changes in, or additions to, the edited manuscript will be accepted.

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