A single-chain antibody fragment is functionally expressed in the cytoplasm of both *Escherichia coli* and transgenic plants

Paraskevi Tavladoraki[1,*], Alessandra Girotti[1], Marcello Donini[1], Francisco Javier Arias[1], Camillo Mancini[1,2], Veronica Morea[3], Roberta Chiaraluce[4], Valerio Consalvi[4] and Eugenio Benvenuto[1]

[1]*ENEA, Dipartimento Innovazione, Divisione Biotecnologie e Agricoltura and* [2]*Dipartimento Ambiente, Divisione Protezione dell'Uomo e degli Ecosistemi. C. R. Casaccia, Roma, Italy;* [3]*IRBM, 'P. Angeletti', Pomezia, Roma, Italy;* [4]*Università di Roma 'La Sapienza', Dipartimento di Scienze Biochimiche 'A. Rossi-Fanelli', Piazzale A. Moro 5, Roma, Italy*

Despite the well-known crucial role of intradomain disulfide bridges for immunoglobulin folding and stability, the single-chain variable fragment of the anti-viral antibody F8 is functionally expressed when targeted to the reducing environment of the plant cytoplasm. We show here that this antibody fragment is also functionally expressed in the cytoplasm of *Escherichia coli*. A gel shift assay revealed that the single-chain variable fragment (scFv) accumulating in the plant and bacterial cytoplasm bears free sulfhydryl groups. Guanidinium chloride denaturation/renaturation studies indicated that refolding occurs even in a reducing environment, producing a functional molecule with the same spectral properties of the native scFv(F8). Taken together, these results suggest that folding and functionality of this antibody fragment are not prevented in a reducing environment. This antibody fragment could therefore represent a suitable framework for engineering recombinant antibodies to be targeted to the cytoplasm.

Keywords: scFv fragment; intrabody; disulfide bond; cytosol.

Antibodies can be ectopically expressed in a wide variety of cells and recent reports have shown the feasibility of the procedure referred to as 'intracellular immunization' to interfere with the activity of a target molecule inside the various intracellular compartments [1]. Intracellular antibodies (intrabodies) are extremely interesting for a wide variety of applications, in that they have the potential to modify diverse biological processes, for example by neutralizing intracellular antigens, stabilizing protein–protein interactions or performing catalytic function themselves.

A fundamental issue for many such applications is antibody targeting to the cytoplasm, an environment in which full-length antibodies and Fab fragments are generally not functional, despite some successful studies [2–7]. Antibody engineering and in particular the design of single-chain variable fragments (scFv) has extended the array of antibody molecules that can be functionally expressed in the cytoplasm modulating intracellular functions [8–12]. However, not all scFv antibodies can be efficiently expressed in the cytoplasm [13–15]. The difficulties associated with obtaining functional antibodies in the cytoplasm have often been ascribed to the reducing properties of this environment, which does not allow the formation of disulfide bonds. The contribution of the disulfide bonds to antibody folding and stability was demonstrated by mutagenesis experiments [16] and is validated by the almost total conservation of disulfide bonds within the whole immunoglobulin superfamily (Kabat database).

Antibody stability varies as a consequence of sequence variations within both frameworks and complementarity determining regions, and the primary sequence determines the distribution of folding intermediates between the folding and aggregation pathways [17,18]. This evidence may explain why some antibodies can be functionally expressed under reducing conditions and why some others can even tolerate the lack of a disulfide bond. One of the few antibodies naturally lacking a disulfide bond is the levan-binding antibody ABPC48, in which a tyrosine residue is present rather than cysteine in position 92 (Kabat numbering) of the heavy-chain variable domain [19]. This molecule was analyzed as a model system to dissect immunoglobulin folding and stability and is considered a potential starting material for engineering of recombinant antibody with improved frameworks for cytoplasmic expression [19,20].

Ideally, antibodies targeted to the cytoplasm should be intrinsically capable of efficient and functional folding in this environment. A molecule with such characteristics is the single chain variable fragment scFv(F8) deriving from a monoclonal antibody raised against the coat protein of the plant artichoke mottle crinkle virus. This is one of the few antibodies which has been functionally expressed in the plant cytoplasm through a construct lacking any secretory signal-sequence and it has been shown to interfere with virus replication *in vivo* [9].

We studied the redox state of the signal-sequenceless scFv(F8) expressed in transgenic plants and demonstrated that this antibody, accumulating in the plant cytoplasm in a soluble and functional form, bears free cysteine residues. Most importantly, the same results were obtained expressing the

Correspondence to E. Benvenuto ENEA, Dipartimento Innovazione, Divisione Biotecnologie e Agricoltura, C. R. Casaccia, PO Box 2400, 00100 Roma, Italy. Fax: +39 06 30484741, Tel.: +39 06 30486347, E-mail: benvenutoe@casaccia.enea.it

Abbreviations: Fab, 50-kDa antigen-binding fragment derived from papain digestion of immunoglobulin molecules; scFv, single-chain variable fragment; IDA, iodoacetamide; DTT, dithio-DL-threitol; GdmCl, guanidinium chloride.

**Present address*: Università Roma Tre, Dipartimento di Biologia, Viale Marconi 446, 00146 Roma, Italy.

signal-sequenceless scFv(F8) in *E. coli*. This antibody fragment shows a remarkable half-life *in vivo* and can be successfully refolded *in vitro* in a reducing environment. The refolded product shows spectral and binding properties identical to the native scFv(F8). These observations indicate that this molecule may represent a breakthrough in the search of a stable framework for recombinant antibody technology, and in particular for antibody targeting to the cytoplasm. The study of this molecule might very well provide new insights into the factors governing antibody stability and folding.

EXPERIMENTAL PROCEDURES

Analysis of disulfide bond formation in scFv(F8) expressed in transgenic plants

Fresh leaves from transgenic plants (line 67i) were vacuum-infiltrated with degassed infiltration solution (100 mM Tris/HCl pH 8.0, 10 mM EDTA) containing 100 mM iodoacetamide (IDA) and homogenized in the presence of liquid N_2. Degassed extraction buffer (100 mM Tris/HCl pH 8.0, 0.5 mM EDTA, 0.05% Tween-20) containing 100 mM IDA and protease inhibitors (1 mM phenylmethanesulfonyl fluoride, 0.1 $mg \cdot mL^{-1}$ pepstatin A, 1 $\mu g \cdot mL^{-1}$ leupeptin, 2 $\mu g \cdot mL^{-1}$ aprotinin) was added to the homogenate inside an anaerobic hood under N_2 flux. The extract was centrifuged and the clear supernatant was tested for the presence of functional scFv(F8) by ELISA performed according to Tavladoraki *et al*. [21]. The redox state of the cysteines in scFv(F8) was evaluated after immunoprecipitation and Western blot analysis on a nonreducing SDS/PAGE. As an oxidized control, scFv(F8) was extracted from transgenic plants as described above, omitting IDA. ScFv(F8) was also extracted from transgenic plants in the presence of 100 mM 2-mercaptoethanol in the extraction buffer. In this case, the infiltration step was omitted and anaerobic conditions were not applied. The extract was tested for the presence of functional scFv(F8) by ELISA and for the presence of free sulfhydryl groups by Western blot analysis of the crude extract. In this case the boiling step before loading was omitted.

Expression of scFv fragments in bacteria and analysis of disulfide bond formation

Expression of the anti-viral scFv(F8) and the anti-lysozyme scFv(D1.3) [22] (kind gift of G. Winter, MRC, Cambridge, UK) in *E. coli* was induced according to Tavladoraki *et al*. [21]. When preservation of the redox state of the sulfhydryl groups was necessary, bacteria were subsequently transferred to prechilled Luria–Bertani medium containing 200 mM IDA. This step allows penetration of IDA into the bacterial cell and trapping of free sulfhydryl groups before cell lysis [23]. After 20 min on ice, bacteria were pelleted and subcellular fractionation was carried out. The periplasmic fraction (fraction 1) was obtained by a mild osmotic shock [21]. A second osmotic shock preparation (fraction 2) was subsequently obtained by resuspending the pellet in 16 mL of ice-cold 5 mM $MgSO_4$ per litre of initial culture. After 30 min on ice, the extract was centrifuged at 3000 ***g*** for 15 min and the supernatant was stored at 4 °C. The pellet was resuspended in 16 mL of 50 mM Tris/HCl pH 7.8 per litre of initial culture. Cell lysis was initiated by three freezing/thawing cycles and completed by hen-egg white lysozyme (1 $mg \cdot mL^{-1}$). After 30 min on ice, the viscous extract was treated with 20 $\mu g \cdot mL^{-1}$ DNase I and 10 $\mu g \cdot mL^{-1}$ RNase A for 15 min at 4 °C and centrifuged at 20 000 ***g*** for 20 min. The clear supernatant corresponding to the cytoplasmic fraction (fraction 3) was stored. The pellet was resuspended in 16 mL of 50 mM Tris/HCl pH 7.8, 300 mM NaCl per litre of initial culture. After sonication, the homogenate was cleared by centrifugation (fraction 4). In the case of bacteria harboring the scFv(D1.3) constructs, lysis by lysozyme was omitted to avoid any interference in ELISA and the two cytoplasmic fractions were obtained by sonication. Protease inhibitors (see above) were present throughout the whole fractionation procedure. Where preservation of the redox state of the sulfhydryl groups was necessary, 50 mM IDA was added to all buffers during cell fractionation. The presence of functional scFv in the various bacterial fractions was detected by ELISA and the redox state of the cysteines in the scFv(F8) was evaluated after immunoprecipitation and Western blot analysis on nonreducing SDS/PAGE. Periplasmic extract (fraction 1) from bacteria harboring the secretory scFv(F8) construct was boiled for 5 min in the presence of 36 mM dithio-DL-threitol (DTT), immediately treated with 100 mM IDA and immunoprecipitated to be used as carboxamidomethylated marker (B_1 IDA/+DTT). In parallel, an oxidized (noncarboxamidomethylated) control was prepared, in which DTT was omitted during boiling (B_1 IDA/−DTT). ScFv(F8) was purified in large scale from the periplasm (fractions 1 and 2) of bacteria expressing the secretory scFv(F8), according to Tavladoraki *et al*. [21].

Immunoprecipitation and Western blot analysis

Immunoprecipitation was performed with the anti-(c-*myc* tag) monoclonal antibody 9E10 [24] linked to Sepharose. Bacterial or plant extracts were incubated with 9E10–Sepharose overnight at 4 °C in batch. Sepharose was collected by centrifugation and washed four times with 10 mM phosphate pH 7.2, 0.65 M NaCl, 0.05% Tween-20 and twice with 5 mM Tris/HCl pH 7.8. Bound scFv was eluted by the addition of sample preparation buffer (30 mM Tris/HCl, pH 6.8, 2% SDS, 15% glycerol, 0.025% bromophenol blue), either in the presence or in the absence of 2-mercaptoethanol, and boiling for 5 min. Samples were centrifuged and an aliquot of the supernatant was analyzed by 12% SDS/PAGE. Western blot analysis was performed with the monoclonal antibody 9E10 according to Tavladoraki *et al*. [21].

Pulse-labeling of protoplasts and cell suspensions

Protoplasts were isolated from control and transgenic plants (line 67i) according to Zhu and Negrutiu [25] and resuspended in 0.5 M mannitol, 0.1% Mes. Subsequently, a mixture of [^{35}S]L-methionine and [^{35}S]L-cysteine (70% and 30%, respectively) was added to a final concentration of 200 $\mu Ci \cdot mL^{-1}$ (200 nM). After 2 h, unlabeled methionine (1.4 mM) and cysteine (0.6 mM) were added. The protoplasts were centrifuged and resuspended in 0.5 M mannitol, 0.1% Mes containing unlabeled methionine and cysteine as above. Aliquots of protoplasts containing the same amount of living cells (9×10^5) were withdrawn at various time intervals. Protoplast pellets were frozen in liquid N_2 and kept at −80 °C.

Liquid suspension cell lines were initiated from callus cells in Murashige and Skoog medium containing 3% sucrose, 1 $mg \cdot L^{-1}$ 2,4-dichlorophenoxyacetic acid and 0.1 $mg \cdot L^{-1}$ 6-benzylaminopurine. Incorporation of [^{35}S]L-methionine and [^{35}S]L-cysteine into cell suspensions was performed as described above. To detect labeled scFv(F8) molecules, protoplast and cell suspension samples were subjected to two freezing/thawing cycles, resuspended in NaCl/P_i

14321033, 1999, 2, Downloaded from https://febs.onlinelibrary.wiley.com/doi/10.1046/j.1432-1327.1999.00443.x by Universidad De Valladolid, Wiley Online Library on [21/01/2025]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

containing 300 mM NaCl, 2% polyvinylpolypyrrolidone, 0.05% Tween-20, protease inhibitors and homogenized. After centrifugation at 20 000 ***g*** for 20 min, supernatants were analyzed for the presence of labeled scFv(F8) by immunoprecipitation, followed by 12% SDS/PAGE and fluorography.

Spectroscopy

Fluorescence and light-scattering measurements were performed on a LS50B Perkin-Elmer spectrofluorimeter using 1-cm quartz cuvettes. Intrinsic fluorescence emission spectra were recorded at 300–400 nm at 20 °C, with the excitation wavelength set at 295 nm. Right-angle light scattering was measured at 20 °C with both excitation and emission wavelength set at 480 nm and using the same spectral bandwidth of 4 nm. Fluorescence quenching by acrylamide [26] was performed at 10 $\mu g \cdot mL^{-1}$ protein concentration in NaCl/P_i at 20 °C and acrylamide concentration was varied over 0–100 mM. The excitation wavelength was 278 nm and emission spectra were recorded at 300–400 nm 10 min after the addition of the quencher. The values of effective quenching constants were obtained from modified Stern–Vollmer plots analyzing $F_0/\Delta F$ vs. 1/[acrylamide] (15 data pairs) [27]. CD spectra were recorded at 20 °C on a Jasco J-720 spectropolarimeter: far-UV CD spectra (200–250 nm) were recorded using a 0.5-cm pathlength quartz cuvette at 0.15 $mg \cdot mL^{-1}$ protein concentration, near-ultraviolet CD measurements (250–320 nm) were performed using a 1.0-cm pathlength quartz cuvette at a protein concentration of 1.2 $mg \cdot mL^{-1}$. The results were expressed as the mean residue ellipticity ([Θ]) assuming a mean residue weight of 110 per amino-acid residue.

Unfolding and refolding studies

Equilibrium unfolding experiments were performed at 20 °C by incubating scFv(F8) (32 $\mu g \cdot mL^{-1}$, 1.2 μM) at increasing guanidinium chloride (GdmCl) concentrations (0–4 M) either in NaCl/P_i or in 20 mM Tris/HCl, pH 9.0, 0.15 M NaCl, in the absence or in the presence of 2.0 mM DTT and 0.1 mM EDTA. After 3 h, intrinsic fluorescence emission spectra were recorded at 20 °C. To test the reversibility of the unfolding, scFv(F8) (0.65 $mg \cdot mL^{-1}$, 24.1 μM) was unfolded at 20 °C in 4 M GdmCl, either in NaCl/P_i or in 20 mM Tris/HCl, pH 9.0, 0.15 M NaCl in the absence or in the presence of 18 mM DTT and 1 mM EDTA. After 3 h, refolding was started by 24-fold dilution at 20 °C into solutions of the same buffer used for unfolding containing decreasing GdmCl concentrations (± 2 mM DTT and 0.1 mM EDTA final concentration). After 3 h, intrinsic fluorescence emission spectra were recorded at 20 °C and samples refolded in the presence of 0.23 M GdmCl, 20 mM Tris/HCl, pH 9.0, 0.15 M NaCl (± DTT) were tested for functionality by ELISA.

Characterization of the scFv(F8) refolded under reducing conditions was performed after a 20-fold dilution at 20 °C in 20 mM Tris/HCl pH 9.0, 0.15 M NaCl of unfolded and reduced scFv(F8) (0.2 $mg \cdot mL^{-1}$, 3 h in 4.0 M GdmCl in 20 mM Tris/HCl, pH 9.0, 20 mM DTT, 1 mM EDTA). After 3 h, the refolding mixture was first concentrated and dialyzed against NaCl/P_i under N_2 on an Amicon ultrafiltration cell using a 43-mm YM 10 membrane and then analyzed by acrylamide fluorescence quenching, CD spectra and ELISA.

Data analysis

The changes in intrinsic fluorescence emission spectra at increasing GdmCl concentrations were quantified as the intensity-averaged emission wavelength, $\bar{\lambda}$ [28], calculated according to the following equation:

$$\bar{\lambda} = \Sigma(I_i \lambda_i)/\Sigma(I_i) \quad (1)$$

where λ_i and I_i are the emission wavelength and its corresponding fluorescence intensity at that wavelength. Baseline and transition region data for scFv(F8) GdmCl denaturation were fit to a two-state [29] linear extrapolation model according to the following equation:

$$\Delta G_{unfolding} = \Delta G^{H_2O} + m_g[\text{GdmCl}] = -RT\ln K_{unfolding} \quad (2)$$

where $\Delta G_{unfolding}$ is the free energy change for unfolding for a given denaturant concentration, ΔG^{H_2O} is the free energy change for unfolding in the absence of denaturant and m is a slope term which quantitates the change in $\Delta G_{unfolding}$ per unit concentration of denaturant, R is the gas constant, T is the temperature and $K_{unfolding}$ is the equilibrium constant for unfolding. The model expresses the signal as a function of denaturant concentration:

$$y_i = \frac{y_N + m_N[X]_i + (y_D + m_D[X]_i)\cdot e^{[(-\Delta G^{H_2O} - m_g[X]_i)/RT]}}{1 + e^{[(-\Delta G^{H_2O} - m_g[X]_i)/RT]}} \quad (3)$$

where y_i is the observed signal, y_N and y_D are the native and denatured baseline intercepts, m_N and m_D are the native and denatured baseline slopes, $[X]_i$ is the denaturant concentration after the ith addition, ΔG^{H_2O} is the extrapolated free energy of unfolding in the absence of denaturant, m_g is the slope of a ΔG unfolding vs. [X] plot, R is the gas constant and T is the temperature. The $[\text{GdmCl}]_{0.5}$ is the denaturant concentration at the midpoint of the transition and, according to Eqn (2), is calculated as:

$$[\text{GdmCl}]_{0.5} = \Delta G^{H_2O}/m_g \quad (4)$$

RESULTS

Redox state of cysteines in the signal-sequenceless scFv(F8) expressed in plants

The anti-viral scFv(F8) can be functionally expressed in transgenic *Nicotina benthamiana* plants through a signal-sequenceless construct [9]. To analyze disulfide-bond formation in the scFv(F8) accumulating inside the plant cytoplasm, a gel-shift assay was performed. This is based on the observation that disulfide-containing proteins migrate faster in nonreducing SDS/PAGE than their reduced counterpart [23]. The scFv(F8)

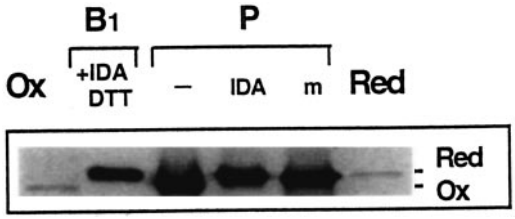

Fig. 1. Redox state of cysteines in scFv(F8) expressed in transgenic plants. ScFv(F8) molecules were extracted from transgenic plants both in the presence (P IDA) and in the absence (P^-) of IDA. After immunoprecipitation, samples were subjected to nonreducing SDS/PAGE and detected by immunoblotting. P m, P^- extract analyzed in the presence of 2-mercaptoethanol in the sample preparation buffer (reducing conditions); Red, purified scFv(F8) subjected to SDS/PAGE under reducing conditions (reduced control); Ox, purified scFv(F8), analyzed under nonreducing conditions. B_1 IDA/+DTT: carboxamidomethylated marker (see Experimental procedures). Bars indicate the gel shift.

14321033, 1999, 2, Downloaded from https://febs.onlinelibrary.wiley.com/doi/10.1046/j.1432-1327.1999.00443.x by Universidad De Valladolid, Wiley Online Library on [21/01/2025]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

Table 1. Relative ELISA signals produced by extracts from transgenic plants expressing the signal-sequenceless scFv(F8) construct. ScFv(F8) was extracted from transgenic plants: in the presence of 100 mM IDA (P IDA); in the presence of 100 mM 2-mercaptoethanol (I m); in the absence of IDA and 2-mercaptoethanol (P^-). $P^{\bullet}$ IDA represents P^- extract in which 100 mM IDA was added after air oxidation of cysteines. Crude extracts were tested on ELISA after normalization to the same amount of total soluble protein. P^- extract was arbitrarily set to 1.0. Values represent the mean of triplicate trials with standard deviations.

Experimental conditions	Relative ELISA signal
P^-	1.00
P IDA	0.53 (± 0.13)
I m	1.00 (± 0.10)
$P^{\bullet}$ IDA	0.64 (± 0.06)

was extracted from plant tissues both in the presence and in the absence of IDA, which alkylates free cysteines and irreversibly blocks disulfide formation, thus preventing spontaneous air oxidation during extraction. In nonreducing SDS/PAGE the scFv(F8) molecules extracted from plants in the presence of IDA and under anaerobic conditions (P IDA) migrated more slowly than those extracted in the absence of IDA (P^-) forming a single retarded band (Fig. 1). Moreover, the mobility of IDA-extracted scFv(F8) molecules corresponded to that of the carboxamidomethylated marker (B_1 IDA/+DTT) and the reduced controls (P m and Red). Conversely, the scFv(F8) molecules extracted from plants in the absence of IDA (P^-) migrated similarly to the oxidized control (Ox). These data demonstrate that the signal-sequenceless scFv(F8) molecules present in the transgenic plants possess free sulfhydryl groups. These groups are spontaneously converted to their oxidized form during extraction, unless IDA is included in the extraction buffer.

P^- and P IDA extracts were also tested for binding in ELISA (Table 1). P IDA extract showed binding activity, indicating that the carboxamidomethylated scFv(F8) is active. However, the ELISA value of this extract was lower than that of the P^- extract. A similar decrease was also observed if IDA was added to an air-oxidized plant extract ($P^{\bullet}$ IDA) before the ELISA, thus ruling out the involvement of cysteine alkylation in the diminished binding activity. The same results were obtained when IDA was added to purified scFv(F8) (data not shown). Furthermore, scFv(F8) was extracted from the transgenic plants with 2-mercaptoethanol, analysed for the presence of free sulfhydryl groups by SDS/PAGE (data not shown) and tested for binding in ELISA. Table 1 shows that the presence of 2-mercaptoethanol in this plant extract (I m) does not influence binding activity, showing scFv(F8) functionality under reducing conditions.

ScFv(F8) expression in the cytoplasm of *E. coli* and analysis of disulfide bond formation

To further assess scFv(F8) functionality under reducing conditions, we also tested scFv(F8) solubility and activity in the cytoplasm of a prokaryotic cell. Two constructs for scFv(F8) expression in *E. coli* through the heat-inducible λ PL promoter were prepared, one bearing the bacterial pelB signal sequence for secretion and the other lacking any signal-sequence [9,21]. Secretory and cytoplasmic constructs for bacterial expression of the anti-lysozyme scFv(D1.3) antibody under the same promoter were also used as a control. After heat induction, the bacterial cells were fractionated in two periplasmic fractions (1 and 2) and two cytoplasmic fractions (3 and 4), as indicated in Fig. 2. ELISA revealed a substantial amount (≈120 $\mu g \cdot L^{-1}$ of culture) of soluble and functional scFv(F8) in the cytoplasm of bacteria expressing the intracellular construct (I-bacteria) (Fig. 2a). However, this amount is much lower (≈100-fold) than that accumulated in the bacteria bearing the secretory construct (B-bacteria). Western blot analysis also showed that the total amount of soluble scFv(F8) protein accumulating in the cytoplasm of I-bacteria was much lower than that accumulated in B-bacteria, according to the ELISA values (data not shown). These results indicate low accumulation levels rather than low activity of the cytoplasmic antibody. Furthermore, no scFv(F8) was detected in the insoluble fraction of I-bacteria (data not shown) providing evidence that the low level of soluble scFv(F8) found in I-bacteria is probably due to an increased proteolytic degradation in the cytoplasm rather than to aggregation processes. In the same experimental approach, no functional scFv(D1.3) antibody was detected in bacteria expressing the corresponding signal-sequenceless construct (Fig. 2b), while Western blot analysis detected a small amount of soluble protein (data not shown). These results indicate that intracellular expression of functional scFv(D1.3) is not feasible.

The redox state of the scFv(F8) targeted to the cytoplasm of bacteria was also tested. ScFv(F8) extracted from the cytoplasm

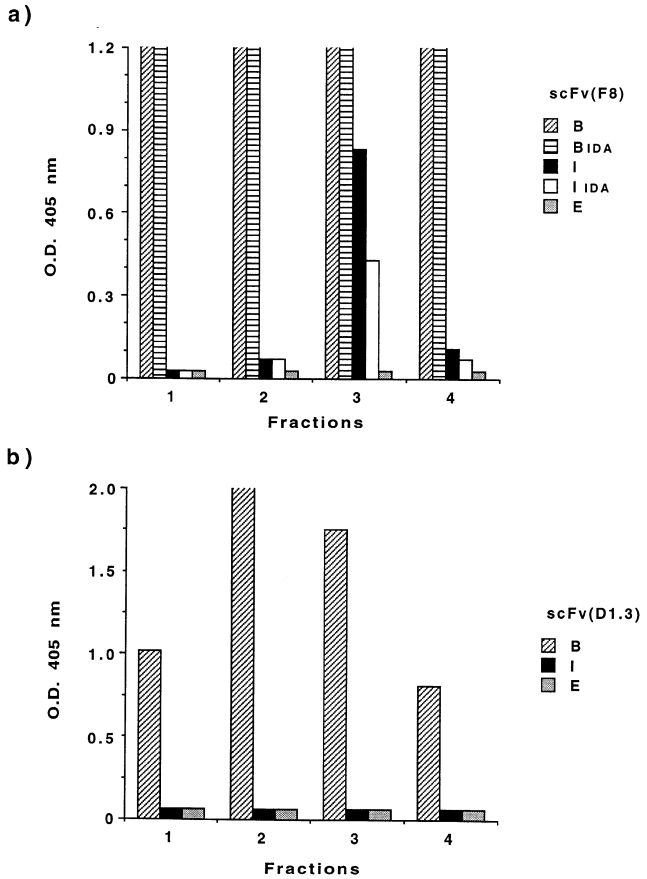

Fig. 2. Binding activity of scFv(F8) and scFv(D1.3) expressed in bacteria. (a) ELISA of various subcellular fractions of bacteria harboring the secretory (B) or the intracellular (I) scFv(F8) construct. Extracts were obtained both in the presence (B IDA, I IDA) and in the absence (B, I) of IDA. (b) ELISA of various subcellular fractions of bacteria bearing the secretory (B) or the intracellular (I) scFv(D1.3) construct. ELISA values were recorded 15 min after colorimetric reaction development and normalized to 10 mL of initial culture. 1 and 2, periplasmic fractions; 3 and 4, cytoplasmic fractions. (E): untransformed control bacteria.

14321033, 1999, 2, Downloaded from https://febs.onlinelibrary.wiley.com/doi/10.1046/j.1432-1327.1999.00443.x by Universidad De Valladolid, Wiley Online Library on [21/01/2025]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

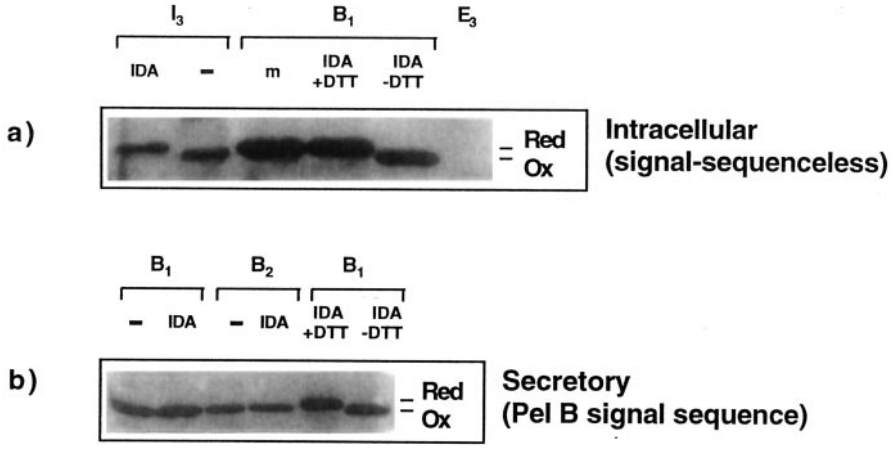

Fig. 3. Redox state of cysteines in scFv(F8) expressed in bacteria. (a) Intracellular scFv(F8) construct. ScFv(F8) was extracted from the cytoplasm (fraction 3, see Experimental procedures) of bacteria expressing the signal-sequenceless scFv(F8) construct both in the presence (I_3 IDA) and in the absence (I_3) of IDA. (b) Secretory scFv(F8) construct. ScFv(F8) was extracted from the periplasm (fractions 1 and 2) of bacteria expressing the secretory scFv(F8) construct both in the presence (B_1 IDA, B_2 IDA) and in the absence (B_1, B_2) of IDA. After immunoprecipitation, scFv(F8) was analyzed by SDS/PAGE under nonreducing conditions and detected by Western blot. B_1 IDA/+DTT, carboxamidomethylated marker (see Experimental procedures); B_1 IDA/−DTT, noncarboxamidomethylated oxidized control; E_3, cytoplasmic fraction of bacteria harboring no construct; B_1 m, B_1 analyzed by SDS/PAGE in the presence of 2-mercaptoethanol in the sample preparation buffer (reduced control). Bars on the right indicate the gel shift.

of I-bacteria in the presence of IDA (I_3 IDA) migrated more slowly than that extracted in the absence of IDA (I_3) on nonreducing SDS/PAGE (Fig. 3a). The migration of the IDA-treated scFv(F8) molecules (I_3 IDA) corresponded to that of the carboxamidomethylated marker (B_1 IDA/+DTT) and the reduced control (B_1 m), while the migration of the untreated scFv(F8) molecules (I_3) corresponded to that of the noncarboxamidomethylated control (B_1 IDA/−DTT) (Fig. 3a). These results indicate that the scFv(F8) molecules targeted to the cytoplasm of bacteria possess free sulfhydryl groups, which react with IDA. ScFv(F8) extracted in the presence of IDA from the periplasm of the B-bacteria (B_1 IDA, B_2 IDA) migrated similarly to both the oxidized control (Ox) and the scFv(F8) extracted in the absence of IDA (B_1, B_2) (Fig. 3b), showing that IDA does not interfere with the mobility of the oxidized scFv(F8).

The scFv(F8) molecules extracted from the cytoplasm of the I-bacteria in the presence of IDA are able to recognize the virus in ELISA (Fig. 2a). However, a decrease of the corresponding ELISA values was observed, similarly to the IDA-treated plant extracts, possibly due to a reaction of IDA with protein groups other than thiols.

ScFv(F8) half-life in plant cells

High levels of scFv(F8) accumulation in the cytoplasm of transgenic plants was observed [9]. To evaluate if this derives from an elevated stability, the turnover rate of this molecule was determined. Cell suspensions and protoplasts prepared from transgenic plants were pulse-labeled for 2 h and chased for different time intervals. Aliquots containing the same number of living cells or protoplasts were sampled at each time point and analyzed for the presence of labeled scFv(F8) molecules. The amount of the labeled scFv(F8) remained constant for at least 16 h in both cell suspensions (Fig. 4a) and protoplasts (Fig. 4b). These results indicate slow turnover of the scFv(F8) expressed in the plant cell.

GdmCl denaturation equilibria

Incubation of scFv(F8) for 3 h at 20 °C at increasing GdmCl concentrations resulted in a progressive change of the intrinsic fluorescence emission intensity and a red-shift of the maximal emission wavelength. No further changes occurred after this incubation time. At pH 7.2 and at pH 9.0, in the presence and in the absence of 2 mM DTT, at GdmCl concentrations higher than 3 M only 50% of the intrinsic fluorescence emission intensity of the native protein were measured and the maximum fluorescence emission wavelength was shifted from 350 to 358 nm. No aggregation of particles detectable by right-angle light scattering at 480 nm were observed at any denaturant concentration. Determination of the red-shift of the intrinsic fluorescence emission was obtained by calculation of the intensity-averaged emission wavelength $\bar{\lambda}$, an integral measurement negligibly influenced by the noise, which reflects changes in the shape and the position of the emission spectrum. Figure 5 shows the plots

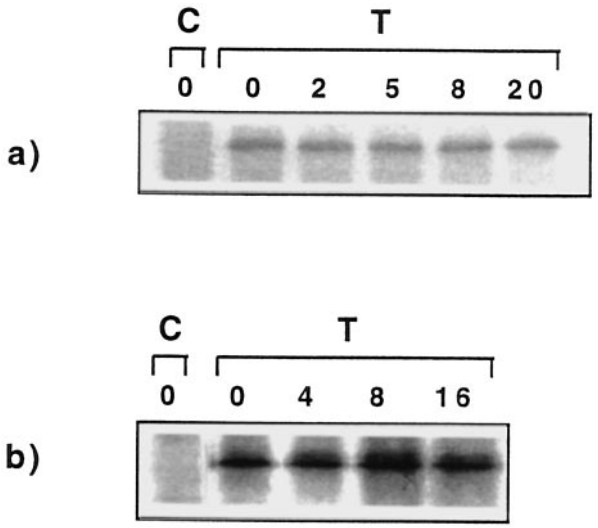

Fig. 4. Half-life of scFv(F8) in plant cells. Cell suspensions (a) and protoplasts (b) from transgenic (T) and untransformed control (C) plants were pulse-labeled and chased for various time intervals. ScFv(F8) was extracted from the same amount of alive cells at each time point, immunoprecipitated and detected by fluorography. Numbers indicate the chase period (hours).

14321033, 1999, 2, Downloaded from https://febs.onlinelibrary.wiley.com/doi/10.1046/j.1432-1327.1999.00443.x by Universidad De Valladolid, Wiley Online Library on [21/01/2025]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

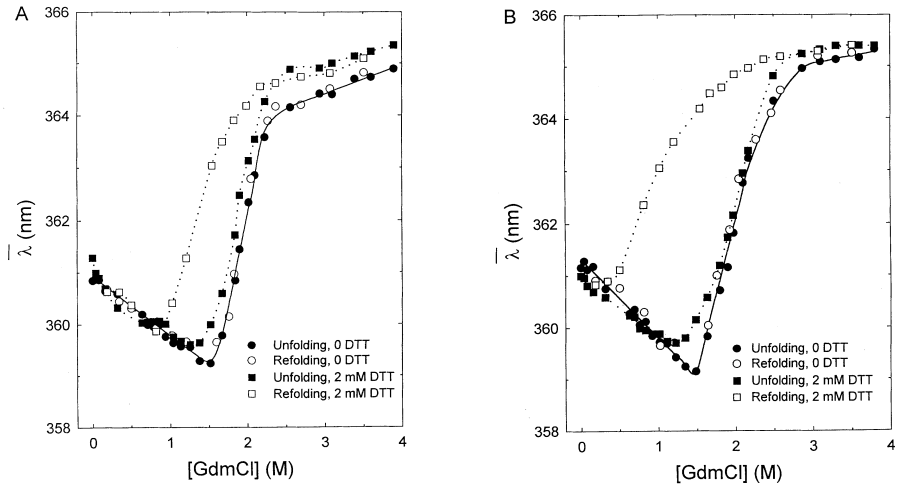

Fig. 5. GdmCl-induced unfolding/refolding transition of scFv(F8). Experiments were performed at 20 °C at pH 7.2 (A) and pH 9.0 (B). Unfolding and refolding experiments were carried out in the presence and in the absence of 2 mM DTT. Continuous lines result from nonlinear regression to Eqn (3) (see Experimental procedures). Reversibility points were not included in the nonlinear regression analysis. Dotted lines are present to guide the eye of the reader and do not represent the fitting of the data. Fluorescence changes are reported as intensity-averaged emission wavelength ($\bar{\lambda}$) obtained by Eqn (1) (see Experimental procedures). Spectra were recorded after 3 h incubation at 20 °C at the indicated GdmCl concentrations.

of $\bar{\lambda}$ as a function of the GdmCl concentration at pH 7.2 (Fig. 5A) and at pH 9.0 (Fig. 5B). The sigmoidal denaturation curves shown in Fig. 5 indicate that the unfolding of scFv(F8) apparently follows a two-state mechanism from the native to the unfolded state without any detectable intermediate. The unfolding profiles at pH 7.2 and pH 9.0 were not significantly affected by the presence of DTT (Fig. 5). The unfolding process was reversible and hysteresis in the refolding was observed in the presence of DTT (Fig. 5). No further changes were observed by increasing the refolding time from 3 h to 24 h. The equilibrium transition curves in the absence of DTT were analyzed according to a linear extrapolation model [29] and the parameters obtained are reported in Table 2. The hysteresis observed in the refolding in the presence of DTT prevented the determination of thermodynamic parameters. Samples refolded in the presence or in the absence of DTT were tested for functionality by ELISA. Figure 6 demonstrates that refolding is accompanied by regain of antigen-binding activity independently of the presence of DTT.

Characterization of refolded scFv(F8)

The characterization of scFv(F8) refolded under reducing conditions was performed after unfolding at pH 9.0 in the presence of 20 mM DTT, as described in Experimental procedures. The refolded protein revealed the same intrinsic fluorescence properties, the same far-UV CD spectrum (data not

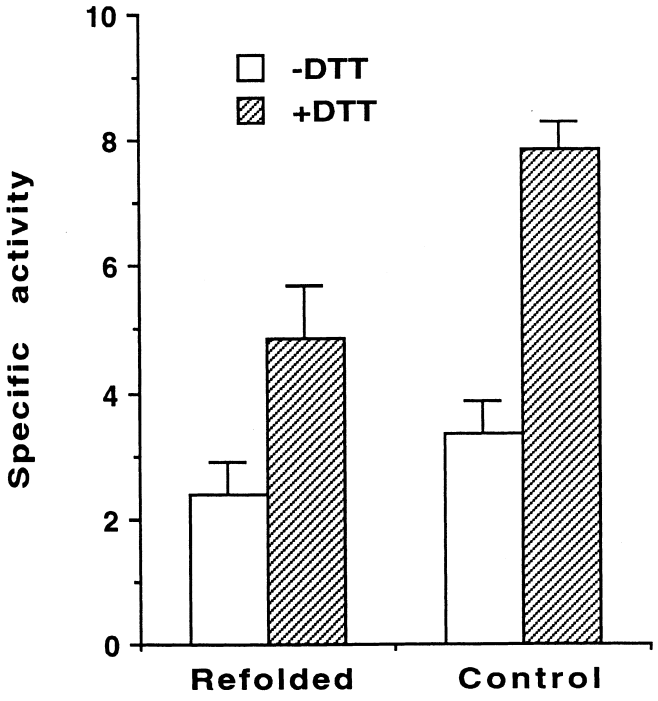

Fig. 6. Binding activity of the refolded scFv(F8). ELISA of the refolded scFv(F8) in the presence and in the absence of DTT. Native scFv(F8) was used as a control under the same binding conditions (0.23 M GdmCl, 20 mM Tris/HCl, pH 9.0, 0.15 M NaCl). Specific activity was expressed as absorbance at 405 nm per pg of scFv(F8).

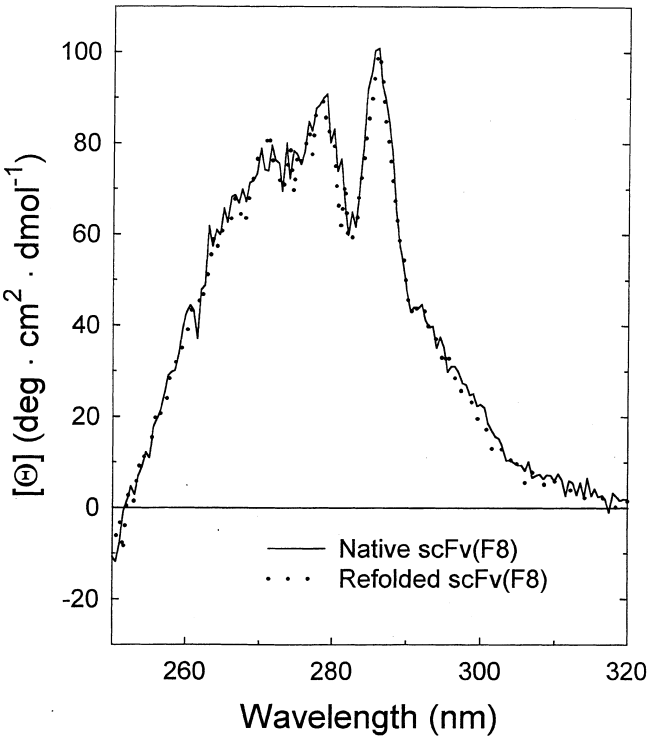

Fig. 7. Near-UV CD spectra of native and refolded scFv(F8). Spectra of the native (continuous line) and of the refolded under reducing conditions at pH 9.0 (dotted line) scFv(F8) were recorded at 20 °C in NaCl/P_i at 1.2 mg·mL^{-1} protein concentration in a 1.0-cm quartz cuvette.

14321033, 1999, 2, Downloaded from https://febs.onlinelibrary.wiley.com/doi/10.1046/j.1432-1327.1999.00443.x by Universidad De Valladolid, Wiley Online Library on [21/01/2025]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

Table 2. Analysis of GdmCl-induced equilibrium unfolding of scFv(F8). The standard errors of the data are shown in parentheses. The buffers used were: at pH 7.2 NaCl/P_i and at pH 9.0 20 mM Tris/HCl, 0.15 M NaCl.

pH	ΔG^{H_2O} ($J \cdot mol^{-1}$)[a]	m ($J \cdot mol^{-1} M^{-1}$)[a]	$[GdmCl]_{0.5}$ (M)[b]
7.2	33.9 (± 0.8)	18.0 (± 0.4)	1.89 (± 0.05)
9.0	25.9 (± 0.8)	13.4 (± 0.4)	1.95 (± 0.05)

[a] Values were obtained by nonlinear regression fitting of the data according to Eqn (3). [b] Values were obtained according to Eqn (4).

shown) and the same near-UV CD spectrum as the native scFv(F8). In particular, the near-UV CD spectra of the native and refolded scFv(F8) shown in Fig. 7 present the same sharp definition of the 286-nm band of the tryptophan accompanied by an identical ellipticity in all the other spectral regions typical of the contributions from the aromatic residues. The refolded protein was also assayed for functionality by ELISA (data not shown). The results demonstrated that structure recovery of scFv(F8) is accompanied by function recovery independently of the presence of DTT.

The uncharged quencher acrylamide was used to probe the dynamic properties of the native scFv(F8) in comparison with the protein refolded in a reducing environment. Effective acrylamide quenching constants obtained from modified Stern–Vollmer plots [27] were 20.0 and 19.4 M^{-1} for the refolded and the native scFv(F8), respectively. A quantitative interpretation is not possible because the protein is a heterogeneously emitting system, but this result indicates that the refolded protein has the same accessibility of fluorophores and the same global dynamics as the native state.

DISCUSSION

The anti-viral scFv(F8) antibody has the intrinsic capacity to fold functionally in the plant cytoplasm and to interfere with viral replication [9]. It is also expressed in a functional and soluble form in the bacterial cytoplasm. The gel-shift assay demonstrates that the signal-sequenceless scFv(F8) molecules accumulating in transgenic plants and bacteria have free sulfhydryl groups, similar to the data reported for the anti-$p21^{ras}$ [30]. This result confirms cytoplasmic targeting, excluding the possibility of mistranslocation of scFv(F8) to the oxidative secretory pathway [31]. The IDA-extracted scFv(F8) molecules form a unique band in nonreducing SDS/PAGE, indicating that all the scFv(F8) molecules accumulate in an identical redox state in the plant cytoplasm. On the basis of our experimental results we cannot distinguish whether just one or both cysteines in each variable domain are derivatized, but both disulfide bonds are probably reduced, as the *in-situ* carboxamidomethylated scFv(F8) migrates similarly to the reduced controls. Even if it is unlikely that after reaction with IDA both cysteine residues can be accepted within the compact 'pin' structure [32] of variable antibody domains, the conformational changes induced by this reaction do not hamper the scFv(F8) solubility and antigen-binding activity.

The reduced form of the scFv(F8) is able to recognize and bind the viral antigen in ELISA. This is in agreement with the *in-vivo* activity of the scFv(F8) [9] and the *in-vitro* denaturation/renaturation experiments. The unfolding transitions obtained in the presence and in the absence of the reducing agent are similar. The native and the unfolded states are not influenced by the presence of DTT as indicated by the similar static amplitude measured for the unfolding process in the absence and presence of the reducing agent. The hysteresis observed in the presence of DTT in the equilibrium refolding may be due to the formation of refolding intermediate(s) that are poorly stable at denaturant concentrations where scFv(F8) is in the native state. The recovery of the native structure can then occur because of the increased stabilization of the refolding molecules at decreasing denaturant concentrations.

Cytoplasmic antibodies are generally unstable, exhibiting shorter half-lives and lower accumulation levels than their secretory counterparts [5,7,10,30,33]. Conversely, substantial amounts of scFv(F8) accumulated in the cytoplasm of transgenic plants, with a half-life *in vivo* longer than 16 h. This value exceeds that reported for other scFvs ectopically expressed in eukaryotic cells [34]. This antibody has also remarkable stability *in-vitro*, as demonstrated by the values of GdmCl transition which are similar to that reported for the stable ABPC48-Cys mutant [19] and higher than those reported for other scFvs [35,36]. In particular, the free energy change for unfolding of scFv(F8) does not differ from that of the hu4D5–8 scFv fragment which has been successfully used to obtain cysteine-free scFv [37].

To our knowledge, the scFv(F8) is one of the few antibody fragments that are expressed in both a soluble and functional form in the reducing environment of the bacterial and plant cytoplasm. In the case of the anti-carcinoembryonic antigen Fab fragment that is also functionally expressed in the bacterial cytoplasm [4], no data are given on the *in-vivo* redox state and the *in-vivo* folding. The anti-(phytochrome A) scFv antibody is expressed in low amounts in the plant cytoplasm while it forms inclusion bodies in the bacterial periplasm [10,33]. Improved expression of an antibody fragment in the plant cytoplasm was observed when the endoplasmic reticulum retention signal KDEL was added [38]. However, no data were given on the antibody function under reducing conditions. The ABPC48 scFv antibody, naturally lacking a cysteine residue, accumulates in inclusion bodies when expressed in the bacterial cytoplasm [19], while stable frameworks for cytoplasmic expression have recently been obtained by molecular evolution starting from this antibody [20]. A similar strategy was adopted to obtain high expression levels of an antibody fragment in the bacterial cytoplasm [39]. This strengthens evidence that folded and functional antibody fragments can accumulate in a reducing environment.

The natural scaffold provided by the anti-viral scFv(F8) fulfills, without complex engineering, most of the requisites for the design of an ideal antibody framework that is functional under reducing conditions and may be used for 'designer' libraries and intracellular immunization. The analysis of the factors influencing the solubility and functionality of scFv(F8) in a reducing environment might very well lead to advances in the study of immunoglobulin folding and stability.

ACKNOWLEDGEMENTS

This work was partially supported by a grant (FAIR1-CT95-0905) from the European Commission (to E.B.) and the Italian Ministero dell'Università e della Ricerca Scientifica e Tecnologica (MURST). A fellowship was awarded (to F.J.A.) by the European Commission.

REFERENCES

1. Cattaneo, A. & Biocca, S. (1997) *Intracellular Antibodies: Developments and Application*. Springer, New York, Berlin.
2. Stieger, M., Neuhaus, G., Momma, T., Schell, J. & Kreuzaler, F. (1991) Self assembly of immunoglobulins in the cytoplasm of the alga *Acetabularia mediterranea*. *Plant Sci.* **73**, 181–190.

14321033, 1999, 2, Downloaded from https://febs.onlinelibrary.wiley.com/doi/10.1046/j.1432-1327.1999.00443.x by Universidad De Valladolid, Wiley Online Library on [21/01/2025]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

3. Carlson, J.R. (1988) A new means of inducibly inactivating a cellular protein. *Mol. Cell. Biol.* **8**, 2638–2646.
4. Cabilly, S. (1989) Growth at sub-optimal temperatures allows the production of functional, antigen-binding Fab fragments in *Escherichia coli*. *Gene* **85**, 553–557.
5. Biocca, S., Neuberger, M.S. & Cattaneo, A. (1990) Expression and targeting of intracellular antibodies in mammalian cells. *EMBO J.* **9**, 101–108.
6. Hiatt, A., Cafferkey, R. & Bowdish, K. (1989) Production of antibodies in transgenic plants. *Nature* **342**, 76–78.
7. Bowdish, K., Tang, Y., Hicks, J.B. & Hilvert, D. (1991) Yeast expression of a catalytic antibody with chorismate mutase activity. *J. Biol. Chem.* **266**, 11901–11908.
8. Biocca, S., Pierandrei-Amaldi, P., Campioni, N. & Cattaneo, A. (1994) Intracellular immunization with cytosolic recombinant antibodies. *Bio/Technol.* **12**, 396–399.
9. Tavladoraki, P., Benvenuto, E., Trinca, S., De Martinis, D., Cattaneo, A. & Galeffi, P. (1993) Transgenic plants expressing a functional single-chain Fv antibody are specifically protected from virus attack. *Nature* **366**, 469–472.
10. Owen, M., Gandecha, A., Cockburn, B. & Whitelam, G. (1992) Synthesis of a functional anti-phytochrome single-chain Fv protein in transgenic tobacco. *Bio/Technol.* **10**, 790–794.
11. Duan, L., Bagasra, O., Laughlin, M.A., Oakes, J.W. & Pomerantz, R.J. (1994) Potent inhibition of human immunodeficiency virus type 1 replication by an intracellular anti-Rev single-chain antibody. *Proc. Natl Acad. Sci. USA* **91**, 5075–5079.
12. Mhashilkar, A.M., Bagley, J., Chen, S.Y., Szilvay, A.M., Helland, D.G. & Marasco, W.A. (1995) Inhibition of HIV-1 Tat-mediated LTR transactivation and HIV-1 infection by anti-Tat single chain intrabodies. *EMBO J.* **14**, 1542–1551.
13. Fiedler, U. & Conrad, U. (1995) High-level production and long-term storage of engineered antibodies in transgenic tobacco seeds. *Bio/Technol.* **13**, 1090–1093.
14. Schouten, A., Roosien, J., van Engelen, F.A., de Jong, G.A.M.I., Borst-Vrenssen, A.W.M., Zilverentant, J.F., Bosch, D., Stiekema, W.J., Gommers, F.J., Schots, A. & Bakker, J. (1996) The C-terminal KDEL sequence increases the expression level of a single chain antibody designed to be targeted to both the cytosol and the secretory pathway in transgenic tobacco. *Plant Mol. Biol.* **30**, 781–793.
15. Bruyns, A.M., DeJaeger, G., DeNeve, M., De Wilde, C., Van Montagu, M. & Depicker, A. (1996) Bacterial and plant-produced scFv proteins have similar antigen-binding properties. *FEBS Lett.* **386**, 5–10.
16. Glockshuber, R., Schmidt, T. & 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.
17. Steipe, B., Schiller, B., Plückthun, A. & Steinbacher, S. (1994) Sequence statistics reliably predict stabilizing mutations in a protein domain. *J. Mol. Biol.* **240**, 188–192.
18. Knappik, A. & Plückthun, A. (1995) Engineered turns of a recombinant antibody improve its *in vivo* folding. *Protein Engineering* **8**, 81–89.
19. Proba, K., Honegger, A. & Plückthun, A. (1997) A natural antibody missing a cysteine in VH: consequences for thermodynamic stability and folding. *J. Mol. Biol.* **265**, 161–172.
20. Proba, K., Wörn, A. & Plückthun, A. (1998) Antibody scFv fragments without disulfide bonds made by molecular evolution. *J. Mol. Biol.* **275**, 245–253.
21. Tavladoraki, P., Franconi, R., Bradbury, A., Cattaneo, A. & Benvenuto, E. (1995) The expression of 'single chain' antibodies in transgenic plants. In *Antibody Engineering* (Borrebaeck, C. A. K., ed.), pp. 363–376. Oxford University Press, New York and Oxford.
22. Amit, A.G., Mariuzza, R.A., Phillips, S.E.V. & Poljak, R.J. (1986) Three-dimensional structure of an antigen-antibody complex at 2.8Å resolution. *Science* **233**, 747–753.
23. Derman, A.I., Prinz, W.A., Belin, D. & Beckwith, J. (1993) Mutations that allow disulfide bond formation in the cytoplasm of *Escherichia coli*. *Science* **262**, 1744–1747.
24. Evan, G.I., Lewis, G.K., Ramsay, G. & Bishop, J.M. (1985) Isolation of monoclonal antibodies specific for human *c-myc* proto-oncogene product. *Mol. Cell. Biol.* **5**, 3610–3616.
25. Zhu, X.-Y. & Negrutiu, I. (1991) Isolation and culture of protoplasts. In *A Laboratory Guide for Cellular and Molecular Plant Biology* (Negrutiu, I. & Gharti-Chhertri, G., eds), pp. 18–27. Birkhäuser, Basel, Boston and Berlin.
26. Eftink, M.R. & Ghiron, C.A. (1981) Fluorescence quenching studies with proteins. *Anal. Biochem.* **114**, 199–227.
27. Lehrer, S.S. (1971) Solute perturbation of protein fluorescence. The quenching of the tryptophylfluorescence of model compounds and of lysozyme by iodide ion. *Biochemistry* **10**, 3254–3263.
28. Royer, C.A., Mann, C.J. & Matthews, C.R. (1993) Resolution of the fluorescence equilibrium unfolding profile of trpaporepressor using single tryptophan mutants. *Protein Sci.* **2**, 1844–1852.
29. Santoro, M.M. & Bolen, D.W. (1988) Unfolding free energy changes determined by the linear extrapolation method. 1. Unfolding of phenylmethanesulfonyl alpha-chymotrypsin using different denaturants. *Biochemistry* **27**, 8063–8068.
30. Biocca, S., Ruberti, F., Tafani, M., Pierandrei-Amaldi, P. & Cattaneo, A. (1995) Redox state of single chain Fv fragments targeted to the endoplasmic reticulum, cytosol and mitochondria. *Bio/Technol.* **13**, 1110–1115.
31. Muesch, A., Hartmann, E., Rohde, K., Rubartelli, A., Sitia, R. & Rapoport, T.A. (1990) A novel pathway for secretory proteins? *Trends Biochem. Sci.* **15**, 86–88.
32. Lesk, A.M. & Chothia, C. (1982) Evolution of proteins formed by beta-sheets. II. The core of the immunoglobulin domains. *J. Mol. Biol.* **160**, 325–342.
33. Firek, S., Draper, J., Owen, M.R.L., Gandecha, A., Cockburn, B. & Whitelam, G.C. (1993). Secretion of a functional single-chain Fv protein in transgenic tobacco plants and cell suspension cultures. *Plant Mol. Biol.* **23**, 861–870.
34. Marasco, W.A., Haseltine, W.A. & Chen, S.Y. (1993) Design, intracellular expression, and activity of a human anti-human immunodeficiency virus type 1 gp120 single-chain antibody. *Proc. Natl Acad. Sci. USA* **90**, 7889–7893.
35. Pantoliano, M.W., Bird, R.E., Johnson, S., Asel, E.D., Dodd, S.W., Wood, J.F. & e Hardman, K.D. (1991). Conformational stability and ligand-binding affinity of single-chain Fv immunoglobulin fragments expressed in *Escherichia coli. Biochemistry* **30**, 10117–10125.
36. Bedzyk, W.D., Weidner, K.M., Denzin, L.K., Johnson, L.S., Hardman, K.D., Pantoliano, M.W., Asel, E.D. & Voss, E.W. Jr (1990) Immunological and structural characterization of a high affinity anti-fluorescein single-chain antibody. *J. Biol. Chem.* **265**, 18615–18620.
37. Wörn, A. & Plückthun, A. (1998) An intrinsically stable antibody scFv fragment can tolerate the loss of both disulfide bonds and fold correctly. *FEBS Lett.* **427**, 357–361.
38. Schouten, A., Roosien, J., de Boer, J.M., Wilmink, A., Rosso, M.N., Bosch, D., Stiekema, W.J., Gommers, F.J., Bakker, J. & Schots, A. (1997) Improving scFv antibody expression levels in the plant cytosol. *FEBS Lett.* **415**, 235–241.
39. Martineau, P., Jones, P. & Winter, G. (1998) Expression of an antibody fragment at high levels in the bacterial cytoplasm. *J. Mol. Biol.* **280**, 117–127.

14321033, 1999, 2, Downloaded from https://febs.onlinelibrary.wiley.com/doi/10.1046/j.1432-1327.1999.00443.x by Universidad De Valladolid, Wiley Online Library on [21/01/2025]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License