FEBS Letters 380 (1996) 194–197 FEBS 16642

Co-expression of human protein disulphide isomerase (PDI) can increase the yield of an antibody Fab′ fragment expressed in *Escherichia coli*

David P. Humphreys[a], Neil Weir[b], Alastair Lawson[b], Andrew Mountain[b], Peter A. Lund[a,*]

[a]*School of Biological Sciences, University of Birmingham, Edgbaston, Birmingham B15 2TT, UK*
[b]*Celltech Therapeutics Ltd., 216 Bath Road, Slough, Berkshire SLI 4EN, UK*

Received 3 January 1996

Abstract Secretion to the periplasm of *Escherichia coli* enables production of many eukaryotic extracellular proteins in a soluble form. The complex disulphide bond arrangement of such proteins is probably a major factor in determining the low yield of correctly folded product observed in many cases. Here we show that co-expression of human protein disulphide isomerase increased the yield of a monoclonal antibody Fab′ fragment in the periplasm of *E. coli*.

Key words: Protein disulphide isomerase; Antibody fragment; *E. coli* periplasm; Protein folding

1. Introduction

Four components involved in disulphide bond formation in the periplasm of *E. coli* have been characterised: DsbA [1,2], DsbB [3,4], DsbC [5], and DsbD [6]. DsbA and DsbC act predominantly to oxidise proteins in vivo, although DsbC may have a greater ability to act as a disulphide isomerase than DsbA. DsbB is thought to re-oxidise molecules of DsbA that have become reduced during its function, whilst DsbD is thought to act as a reductase [6,7]. PDI is the best characterised protein of a growing family of related proteins involved in disulphide bond formation in the ER lumen of eukaryotes that also includes ERp72 (CaBP2), CaBP1, and ERp61 [8–11]. PDI and DsbA are structurally related, both being members of the 'thioredoxin fold' family of proteins [12].

Various fragments of antibodies have been expressed in soluble and active form by secretion to the periplasm of *E. coli*. The effects on in vivo folding for such fragments of the co-expression of PDI, DsbA, and peptidyl-prolyl-*cis–trans*-isomerase (PPIase) have been reported. No significant effects on antibody yield were observed [13]. PDI has however been shown to be associated with immunoglobulins in vivo, and involved in their folding in vitro [14,15]. Here we show that human PDI can have a significant effect on the yield of a Fab′ fragment expressed in *E. coli*, in an assembled and soluble form, and that this enhancement may be substrate (Fab) specific.

*Corresponding author. Fax: (44) (121) 414-6557.
E-mail: p.a.lund@bham.ac.uk

Abbreviations: Carb, carbenicillin; Cm, chloramphenicol; Fab′, antibody fragment with hinge containing the variable and first constant domains of both heavy and light chain; FCS, foetal calf serum (heat inactivated); IAA, 3-β-indole acrylic acid; IPTG, isopropyl β-D-thiogalactopyranoside; PBS, phosphate buffered saline; PDI, protein disulphide isomerase; PPIase, peptidyl-prolyl-*cis–trans*-isomerase; TMB, 3,3′,5,5′-tetramethylbenzidine; Tween-20, polyoxyethylenesorbitan monolaurate.

2. Materials and Methods

2.1. Reagents

Carb, Cm, FCS, IAA, IPTG, TMB, Tween-20, and H_2O_2 were from Sigma and were of the highest purity available. Casein was from BDH. Bradford reagent was from Bio-rad. ELISA plates were 96 well maxi-sorp Nunc plates. Dulbecco's modified Eagle medium (with sodium pyruvate, without L-glutamine) was from Gibco-BRL. The anti-human heavy chain antibody used in the assembly ELISA was a mouse monoclonal HP6045 purified from ATCC cell line ref. no. CRL 1757. This recognises the C_H1 domain of all subclasses of human immunoglobulin. The anti human light chain conjugated to horseradish peroxidase used was GD12, purchased from The Binding Site, Birmingham, UK. The revealing antibody used in FACS analysis was a polyclonal phycoerythrin conjugated goat anti-human kappa from Southern Biotechnology Assoc. Inc., Birmingham, Alabama, USA.

2.2. Bacterial strains, plasmids, and cell lines

W3110, wild type *E. coli*, ATCC 27325; pDPH5, PDI expression plasmid, *trp* promoter, colE1 origin, Amp^R, [16]; pCT54, Control plasmid for pDPH5, *trp* promoter, colE1 origin, Amp^R, [17]; pAW6, A33γ1 Fab′ expression plasmid, *tac* promoter, pACYC184 origin, Cm^R, [18]; pMRR60, A33γ4 Fab′ expression plasmid, *tac* promoter, pACYC184 origin, Cm^R, [18]; SW1222, Human colon cancer cell line, with surface expressed A33 antibody antigen, [19].

2.3. E. coli growth conditions and periplasm extraction

Luria-broth containing Cm at 50 $\mu g \cdot ml^{-1}$, Carb at 250 $\mu g \cdot ml^{-1}$ and IAA at various concentrations (0–80 $\mu g \cdot ml^{-1}$) shaken at 250 rpm, 30°C was used throughout. 200 ml of L-broth in 2 litre baffled flasks were inoculated with cells concentrated by centrifugation to give an initial OD_{600}=0.1. After growth to an OD_{600} of 0.5, a pre-induction sample equivalent to 1 ml at OD_{600}=9 was taken before inducing Fab expression by addition of IPTG to 0.2 mM. Samples were centrifuged for 10 min at 4500 rpm and cell pellets were stored on ice until resuspension in periplasmic extraction buffer (100 mM Tris-Cl, 10 mM EDTA pH 7.4) to a final volume of 300 µl. Cell suspensions were shaken overnight at 250 rpm, 30°C in 1.5 ml Eppendorf tubes and then centrifuged at 13,000 rpm in a microfuge. The supernatant was removed to a clean tube as the periplasmic fraction, and stored at −20°C until required.

2.4. Analysis of Fab′ concentration by assembly ELISA

ELISA plates were coated overnight at 4°C with HP6045 at 2 $\mu g \cdot ml^{-1}$ in PBS. After washing 4× with dH_2O, serial 1/2 dilutions of samples and standards were performed on the plate in 100 µl of sample/conjugate buffer (100 mM Tris-Cl pH 7, 100 mM NaCl, casein 0.2% (w/v), Tween-20 0.0002% (v/v)), and the plate shaken at 250 rpm, room temperature for 1 h. After washing 4× with dH_2O, 100 µl of the revealing antibody GD12 was added, diluted 1/1000 in sample/conjugate buffer and the plate shaken at 250 rpm, room temperature for 1 h. After washing 4× with dH_2O, 100 µl of TMB substrate was added (0.1 M sodium acetate/citrate pH 6, 100 $\mu g \cdot ml^{-1}$ TMB, H_2O_2 0.01% (v/v)), and the A_{630} recorded using an automated plate reader. The concentration of Fab′ in the periplasmic extracts were

SSDI S0014-5793(96)00028-2

calculated by comparison with purified Fab' standards of the appropriate isotype.

2.5. Culture of SW1222, and analysis of Fab' antigen binding by FACS analysis

SW1222 was maintained by sub-culturing in Dulbecco's modified Eagle medium (with L-glutamine and 10% FCS added), at 37°C, 5% CO_2. Adherent cells were removed from flasks by treatment with trypsin/EDTA (Gibco-BRL) for 5 min at 37°C, 5% CO_2. Cells were washed twice, by centrifugation at 250×*g*, and re-suspension in equivalent culture volumes of medium. 100 μl of cell suspension containing 1×10^5 SW1222 cells was incubated at 4°C for 1 h with 100 μl of 8× concentrated/dialysed periplasmic extract that had been serially diluted in 'FACS buffer' (10% (w/v) BSA, 10% (w/v) FCS, 0.1% (w/v) sodium azide in PBS). The cells were washed with 'FACS buffer' before re-suspension in 200 μl 'FACS buffer' containing the phycoerythrin conjugated goat anti-human kappa revealing antibody at 1/1000 dilution. After incubation at 4°C for 1 h, the cells were washed as described above, and resuspended in 200 μl 'FACS buffer'. The fluorescent label bound to the cells was measured using a FACScan (Becton Dickinson).

8× concentrated/dialysed periplasmic extract was prepared by extracting 1 ml of 150 $OD_{600}\cdot ml^{-1}$ bacterial cell pellet at 60 $OD_{600}\cdot ml^{-1}$ (2-fold concentrated) in periplasmic extraction buffer, as described above, dialysing overnight at 4°C in PBS using a 10 kDa molecular weight cut-off, then concentrating a further 4-fold using an Amicon ultra-filtration unit with a 5 kDa molecular weight cut-off membrane. The concentration of Fab' in these samples was determined by assembly ELISA.

2.6. Protein concentration

Periplasmic protein concentration was determined by the Bradford method, using BSA as a standard.

3. Results

3.1. Effect of the co-expression of periplasmic human PDI on the yield of A33γ4 Fab'

The PDI vector pDPH5 contains an OmpA-PDI gene fusion under the control of the *trp* promoter. This results in the efficient expression of mature PDI to the periplasm of *E. coli*

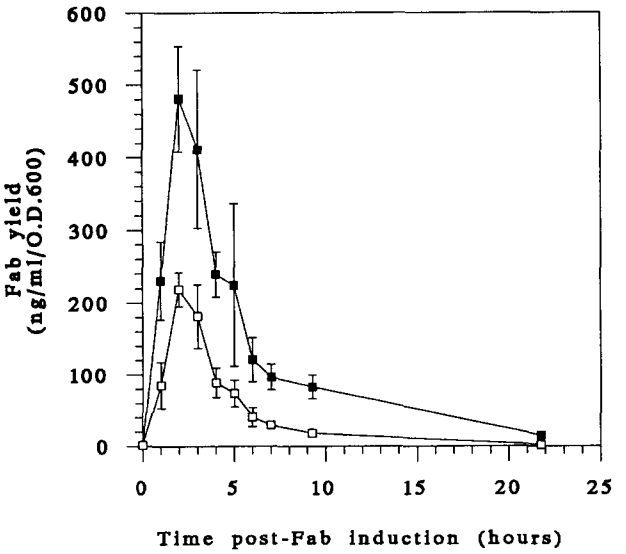

Fig. 1. Effect of PDI on the yield of A33γ4 Fab. Closed squares (■) show the yield of A33γ4 in periplasmic extracts prepared from a strain bearing the PDI expression plasmid pDPH5, whilst the open squares (□) show the yield from the control strain bearing pCT54 both grown in the presence of 40 $\mu g\cdot ml^{-1}$ of IAA. Yields are $ng\cdot ml^{-1}\cdot OD_{600}^{-1}$, and the lines are the mean of three independent experiments.

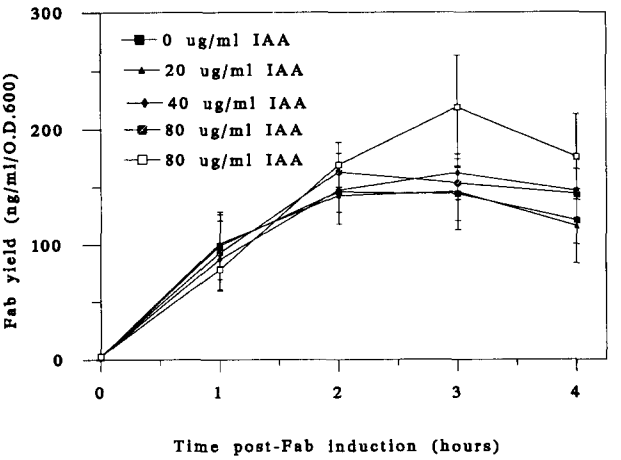

Fig. 2. Effect of PDI on the yield of A33γ1 Fab. Filled symbols show the yield of A33γ1 in periplasmic extracts prepared from a strain bearing the PDI expression plasmid pDPH5 induced at 0, 20, 40, and 80 $\mu g\cdot ml^{-1}$ IAA, whilst the open squares (□) show the yield from the control strain bearing pCT54 grown in the presence of 80 $\mu g\cdot ml^{-1}$ IAA. Yields are $ng\cdot ml^{-1}\cdot OD_{600}^{-1}$, and the lines are the mean of five independent experiments.

when the cells are grown in the presence of the inducer IAA, as described previously [16]. This plasmid is compatible with those encoding the Fab's, enabling use of strains bearing both plasmids to investigate the effect of PDI on the folding of Fab's in the *E. coli* periplasm.

Assembly ELISA was used as the method of assessing the concentration of Fab' in periplasmic extracts. The antibody bound to the wells of the ELISA plate recognises only heavy chain, whilst the revealing antibody recognises only light chain. Therefore the absorbance measured gives an estimate of correctly folded and assembled Fab'.

The effect of the co-expression of PDI on the yield of the γ4 humanised A33 Fab' is shown in Fig. 1. When the expression of PDI was induced by including IAA at 40 $\mu g\cdot ml^{-1}$, the amount of A33 recovered was increased, to almost twice that seen in the control strain bearing pCT54 (plasmid lacking OmpA-PDI) as its second plasmid. Inclusion of the PDI plasmid under un-induced conditions (no IAA) gave an increase in the concentration of correctly assembled A33 Fab' approximately between the two curves shown in Fig. 1 (data not shown). We have shown previously that pDPH5 produces biologically active PDI under these conditions, although much less PDI than under inducing conditions [16]. Slowing of bacterial growth rate can increase the yield of proteins expressed in *E. coli* [20]. However, the increase in yield in the PDI expressing strain was not due to decreased growth rate, as indicated by culture density (data not shown).

The yield of A33 and other Fab's in the periplasm increases to a peak a few hours after induction of Fab' expression, and then rapidly decreases thereafter. This decrease probably due to the toxic effects of Fab' expression as this period is also associated with decreased cell viability, and leakage of periplasmic proteins into fermentor growth media (N.W., unpublished data).

3.2. Effect of the co-expression of periplasmic human PDI on the yield of A33γ1 Fab'

The effect of PDI co-expression on the yield of soluble, assembled Fab' was next evaluated for a different humanised

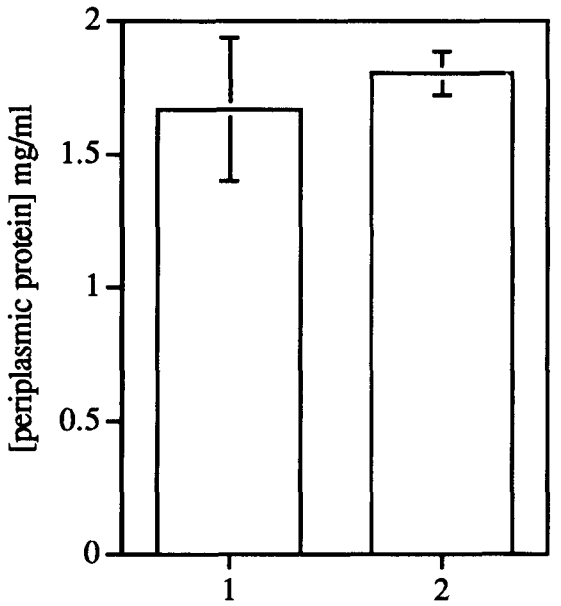

Fig. 3. Effect of PDI on the periplasmic extraction procedure. Columns 1 and 2 show the total protein concentration of periplasmic extracts made from strains bearing the A33γ4 Fab′ plasmid pMRR60 and the PDI expression plasmid pDPH5, or the control plasmid pCT54, respectively. Both were grown in the presence of 40 $\mu g \cdot ml^{-1}$ of IAA, and the results are the mean of three independent experiments. Cultures were harvested 2.5 h post-Fab induction.

variant of A33 Fab′, with a few amino acid substitutions in the variable domains and with a C_H1 domain of γ1 isotype. PDI expression was induced with 0, 20, 40, and 80 $\mu g \cdot ml^{-1}$ IAA, but no increase in yield was observed, as shown in Fig. 2. Indeed the peak yield of Fab′ observed in the PDI co-expressing strain was lower than that for the strain carrying the control plasmid, although these differences were not statistically significant.

3.3. PDI dependent increase in A33γ4 Fab′ is not an artifact of the periplasmic extraction procedure

That the PDI dependent increase in Fab′ yield was not due to differential leakiness, or more efficient periplasmic extraction of strains expressing PDI, is shown in Fig. 3. If PDI expression affected the integrity of the cell or the extraction procedure, a corresponding increase in general periplasmic protein concentration after extraction would result. The total protein measured would not be biased directly by the presence of Fab′ or PDI. Periplasmic extracts from cultures harvested 2.5 h post-Fab′ induction, that were grown identically to those in Fig. 1 and gave similar PDI dependent increases in Fab′ yield were assayed for total protein concentration, using the Bradford method. There is no significant difference in total periplasmic protein concentration between the strain expressing PDI to that without (compare columns 1 and 2, Fig. 3), indicating that PDI alone does not increase the 'leakiness' of the W3110 *E. coli* strain.

3.4. Correct folding and assembly of A33γ4 Fab′ as demonstrated by antigen binding

Fab′ concentration and assembly was determined by ELISA. In order to confirm that A33γ4 Fab′ in periplasmic extracts was functional and correctly folded as well as assembled, the binding of Fab′ to cells bearing the uncharacterised A33 antigen was measured by FACS analysis. Bound Fab′ was revealed with an anti-light chain antibody, in order that any weakly bound heavy chain was not measured — heavy chains of some antibodies are known to have a weak antigen binding capacity (A.L., unpublished observation). Fig. 4 shows that A33γ4 Fab′ from strains both with and without PDI bind to the SW1222 cells. More total antigen binding capacity was detected from the strain co-expressing PDI, confirming the result seen using assembly ELISA. No binding was observed by a periplasmic extract containing a control Fab′ at similar concentrations.

4. Discussion

Previous studies showed that co-expression of PDI, DsbA or PPIase did not result in an increase in the yield of soluble, assembled IgA Fab in the *E. coli* periplasm. In contrast, we demonstrate here that in the case of a γ4 humanised murine Fab′, co-expression of PDI can increase the yield of Fab′ produced in *E. coli*. The increase in yield shows some dependence on the concentration of PDI as higher levels of induction of PDI, which are known to increase the levels of accumulated periplasmic PDI [16], result in greater yields of soluble assembled Fab′.

The increase in A33γ4 Fab yield was shown not to be due to any PDI induced differences in periplasmic 'leakiness' or differential extraction of proteins. The Fab′ in crude periplasmic extracts was also shown to bind to antigen expressed on the surface of SW1222 cell line. This demonstrated that the Fab′ measured in the assembly ELISA is correctly folded, since incorrect tertiary structure would disrupt the antigen binding pocket of the Fab′. Fab′ in periplasmic extracts from the two strains (with/without PDI) has similar antigen binding affinity, as shown by the closely lying lines in Fig. 4. Western blots of reducing and non-reducing SDS-PAGE gels indicated that the heavy and light chains of assembled periplasmic A33γ4 Fab′ were covalently associated (data not shown).

In addition to increasing the specific yield of soluble γ4 Fab′, PDI co-expression also increased the duration over which this higher specific yield is maintained. It is likely

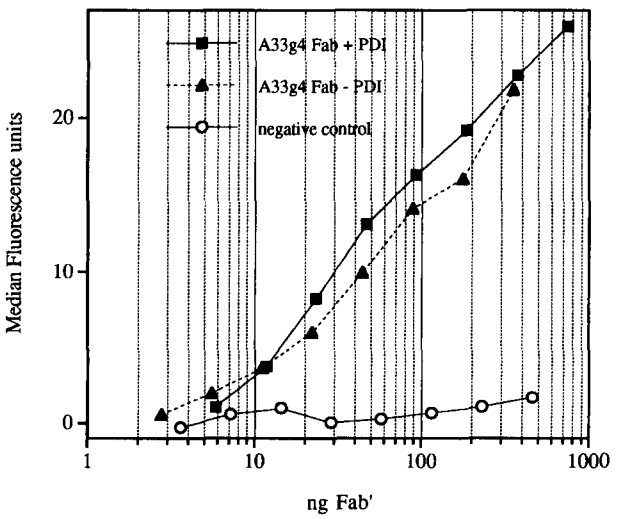

Fig. 4. Binding of A33γ4 Fab′ in periplasmic extracts to SW1222 colon cancer cells.

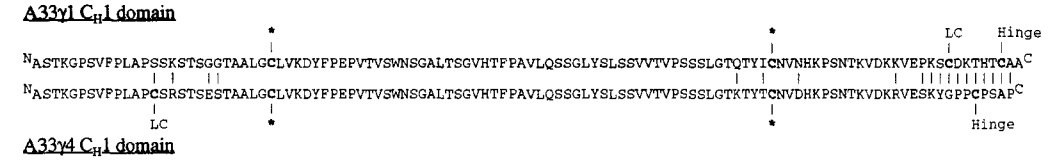

Fig. 5. Amino acid sequence and disulphide bonding patterns of the C_H1 domains of A33γ1 and A33γ4 Fabs. N.B. '*' denotes Cys residues forming intradomain disulphide bond, 'LC' denotes Cys forming disulphide bond to light chain, and 'Hinge' denotes free thiol in the hinge region. Dissimilar amino acids are shown by a vertical dash. A33γ1 is encoded by pAW6 and is OmpA-vH_2-C_H1γ1 and OmpA-vL_4-hu kappaC. A33γ4 is encoded by pMRR60 and is OmpA-vH_1-C_H1γ4 and OmpA-vL_2-hu kappaC.

that the toxic effects of Fab′ expression, which lead to decreased cell viability after induction, are caused by incorrectly folded Fab′. Co-expression of PDI may reduce toxicity by reducing the proportion of incorrectly folded Fab′. This feature could be particularly useful for production in fermentors, where it can be very difficult to judge accurately the optimal time to harvest the cells.

Co-expression of PDI was found not to improve the yield of soluble assembled Fab′ for the γ1 humanised version of the same antibody. The two humanised variants have identical light chains, and although they differ by a few residues in their variable domains, none of these differences concerned cysteines or prolines, and most are conservative residue changes. It is likely that the differential effect of PDI co-expression is mediated through effects on folding of the two different C_H1 hinge regions. The amino acid sequences of these two regions are shown in Fig. 5. Although crystallographic studies suggest γ1 and γ4 antibodies have intra-chain and inter-chain disulphide bonds in very similar conformational locations, the position of the cysteines involved in the inter-chain bond in the primary sequence is very different. For the γ1 variant, the two cysteines concerned in the intra-domain disulphide would be translocated prior to those involved in the light-heavy and heavy-heavy interchain disulphides. For the γ4 variant, the cysteine involved in the light-heavy interchain disulphide would be translocated in advance of the cysteines destined to form the intra-domain disulphide. Unlike the γ1 variant therefore, the γ4 variant has the opportunity to form an inappropriate intra-domain disulphide before translocation of the second cysteine which should participate in formation of the appropriate intra-domain disulphide. A33 γ4 Fab′ heavy chain with this inappropriate disulphide may require the isomerase activity of PDI before progressing along its correct folding pathway.

This interpretation is consistent with the observation that, in identical growth and induction conditions and using the identical expression vector and host strain, the yield of soluble assembled Fab′ in the absence of co-expressed PDI is substantially greater for the γ1 Fab′ than for the γ4 Fab′ (data not shown). This interpretation is consistent also with the view that PDI only assists folding of proteins for which spontaneous folding is restricted by some intrinsic structural feature conferred by the primary sequence. In the only previous report of similar studies [12] co-expression of human PDI did not give an increase in the yield of soluble assembled Fab′. It may be that this Fab′, like the A33γ1 Fab′, had no opportunity to form an inappropriate disulphide. Failure to achieve adequate expression of PDI would be an alternative explanation, since our studies indicate high level PDI expression is required to give substantial yield improvement for A33γ4 Fab′. The two plasmid system, used in our studies allowed the level and timing of PDI and Fab′ expression to be controlled independently and PDI was accumulated to high level in the periplasm before induction of Fab′ expression. PDI is very abundant in the ER lumen, so facilitation of Fab′ folding may require high concentration of PDI in the periplasm before translocation of the Fab′ substrate.

Acknowledgements: We thank Mukesh Sehdev for his helpful advice with shake flask culture and ELISAs, Andrew Chapman and Mari Spitali for help with antigen binding studies, and Dee Attwal for providing Fab′ sequences. This work was funded by BBSRC and Celltech Ltd.

References

[1] Bardwell, J.C.A., McGovern, K. and Beckwith, J. (1991) Cell 67, 581–589.
[2] Kamitani, S., Akiyama, Y. and Ito, K. (1992) EMBO J. 11, 57–62.
[3] Bardwell, J.C.A., Lee, J.O., Jander, G., Martin, N., Belin, D. and Beckwith, J. (1993) Proc. Natl. Acad. Sci. USA 90, 1038–1042.
[4] Missiakas, D., Georgopoulos, C. and Raina, S. (1993) Proc. Natl. Acad. Sci. USA 90, 7084–7088.
[5] Missiakas, D., Georgopoulos, C. and Raina, S. (1994) EMBO J. 13, 2013–2020.
[6] Missiakas, D., Schwager, F. and Raina, S. (1995) EMBO J. 14, 3415–3424.
[7] Bardwell, J.C.A. (1994) Mol. Microbiol. 14, 199–205.
[8] Freedman, R.B. (1989) Cell 57, 1069–1072.
[9] Mazzarella, R.A., Srinivasan, M., Haugejorden, S.M. and Green, M. (1990) J. Biol. Chem. 265, 1094–1101.
[10] Fullekrug, J., Sonnichsen, B., Wunsch, U., Arseven, K., Nguyen Van, P., Soling, H.D. and Mieskes, G. (1994) J. Cell Sci. 107, 2719–2727.
[11] Srivastava, S.P., Fuchs, J.A. and Holtzman, J.L. (1993) Biochem. Biophys. Res. Commun. 193, 971–978.
[12] Creighton, T.E. and Freedman, R.B. (1993) Curr. Biol. 3, 790–793.
[13] Knappik, A., Krebber, C. and Pluckthun, A. (1993) Bio/Technology 11, 77–83.
[14] Roth, R.A. and Pierce, S.B. (1987) Biochemistry 26, 4179–4182.
[15] Lilie, H., McLaughlin, S., Freedman, R. and Buchner, J. (1994) J. Biol. Chem. 269, 14290–14296.
[16] Humphreys, D.P., Weir, N., Mountain, A. and Lund, P.A. (1995) in press.
[17] Emtage, J.S., Angal, S., Doel, M.T., Harris, T.J.R., Jenkins, B., Lilley, G. and Lowe, T.J.R. (1983) Proc. Natl. Acad. Sci. USA 80, 3671–3675.
[18] Yarranton, G.T. and Mountain, A. (1992) in: Protein Engineering — a Practical Approach (Rees, A.R., Starnberg, M.J.E. and Wetzel, R. eds.) IRL Press, Oxford, pp. 303–326.
[19] Leibovitz, A., Stinson, J.C., McCombs III, W.B., McCoy, C.E., Mazur, K.C. and Mabry, N.D. (1976) Cancer Res. 36, 4562–4569.
[20] Cabilly, S. (1989) Gene 85, 553–557.