General Mutagenesis/Gene Expression Procedure for the Construction of Variant Immunoglobulin Domains in *Escherichia coli*

Production of the Bence-Jones Protein REI_v via Fusion to β -Lactamase

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A novel mutagenesis/gene expression and protein purification scheme was established for ready construction and purification of variant immunoglobulin domains in *Escherichia coli*. This procedure, which has been applied to the production of the V_k domain of the Bence-Jones protein REI and structural variants of it, rests on the synthesis of chimeric proteins with β -lactamase as the amino-terminal fusion partner. The β -lactamase not only guides the fusion protein to the periplasmic space, but also allows affinity chromatography on phenylboronate-Sepharose as an efficient and general purification procedure, independent of hypervariable loop structure. The REI_v protein was released from the purified fusion protein by site-specific proteolytic cleavage. After a second passage through the same affinity column, up to 2 mg of pure REI_v was obtained starting from one liter of bacterial liquid culture. A scheme of oligonucleotide-directed mutagenesis was introduced for replacement of DNA stretches encoding hypervariable loops. It exploits a colony color genetic screen and can be applied to any DNA sequence replacement. Mutations can be constructed by simple co-transformation with single-stranded template DNA and mutagenic oligonucleotide.

Keywords: affinity chromatography; immunoglobulin; β -lactamase; protein secretion; IgA protease

During recent years, a number of strategies have emerged for engineering antibodies and antibody fragments (for a review, see Winter & Milstein, 1991). With the ease of manipulating antibody genes, e.g. for the construction of antibody fragments with catalytic activity (Baldwin & Schultz, 1989), production of single-chain antibody fragments (Bird *et al.*, 1988; Huston *et al.*, 1988;

Glockshuber *et al.*, 1990) or generation of combinatorial libraries of the immunoglobulin repertoire *via* PCR|| cloning (Ward *et al.*, 1989; Huse *et al.*, 1989), *Escherichia coli* became an attractive expression host for the production of variant immunoglobulins.

For production of engineered antibody fragments in *E. coli*, numerous methods have been devised, which differ mainly in the cellular locale of the protein produced, i.e. cytoplasm or periplasmic space (for a review, see Wetzel, 1988). Generally, cytoplasmic localization of newly synthesized

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|| Abbreviations used: PCR, polymerase chain reaction; CDR, complementarity-determining region; bp, base-pair(s); IPTG, isopropyl- β -D-thiogalactopyranoside; X-P, 5-bromo-4-chloro-3-indolyl-phosphate.

immunoglobulin Fab or Fv fragments in large quantities was accompanied by deposition of the proteins within the cell as insoluble inclusion bodies (Cabilly *et al.*, 1984; Boss *et al.*, 1984; Field *et al.*, 1990). However, reconstitution often led to only a small fraction of correctly folded protein. Alternative strategies have been established, that mimic the folding and assembly pathway of antibodies in eukaryotic cells by simultaneous expression of both immunoglobulin chains in the same *E. coli* cell and concomitant secretion into the periplasmic space, where protein folding, disulfide bond formation as well as heterodimer association was shown to occur correctly (Better *et al.*, 1988; Skerra & Plückthun, 1988).

With the ease of introducing site-specific mutations within genes cloned in *E. coli* (for a review, see Kramer & Fritz, 1990), generation and analysis of a broad immunoglobulin mutant spectrum is a powerful tool to gain closer insight into the influence of certain residues on antigen binding (Roberts *et al.*, 1987), protein stability (Glockshuber *et al.*, 1990) and loop conformation (Chothia *et al.*, 1989). We are particularly interested in elucidating the structural role of key conserved residues within immunoglobulin complementarity determining regions (CDRs). Comparative studies of known antibody structures indicate that these residues mainly determine hypervariable loop conformations (Chothia & Lesk, 1987; Chothia *et al.*, 1989). For example, a particular CDR1 loop conformation was deduced to be due to interaction of V_K L1 loop residue 29 with framework residues 2, 25, 33 and 71 (Chothia & Lesk, 1987; Chothia *et al.*, 1989). We have embarked on an attempt to understand the nature of these interactions and their influence on loop conformation and protein stability by systematically varying such key conserved residues by means of directed mutagenesis. As a start, we decided to produce a set of variants of the V_K domain of the Bence-Jones protein REI (REI_V; Palm, 1970; Epp *et al.*, 1975) carrying different residues at position 29 of CDR1.

For the facile generation and purification of these REI_V-derived variant proteins the mutagenesis/expression vector pHKREI has been constructed (Fig. 1(a)). It contains a tripartite fusion gene consisting of the complete coding sequence for pBR322-derived β -lactamase (Sutcliffe, 1978), a synthetic gene encoding the variable domain REI_V of the Bence-Jones protein REI (*rei*; Fig. 1(b)) and the coding region for mature alkaline phosphatase from *E. coli* (Chang *et al.*, 1986). All three genes are fused in frame. Presence of the β -lactamase signal sequence directs the fusion protein, synthesized under control of the *lac* promoter/operator region, to the periplasmic space. The REI_V coding region is preceded by a 30 bp linker region coding for the recognition sequence for IgA protease from *Neisseria gonorrhoeae* (Pohlner *et al.*, 1987). The *phoA* gene segment lacks its promoter, signal sequence-encoding region and the first five codons of its coding sequence. An amber stop codon has been

introduced between the *rei* and the *phoA* gene leading to production of a tripartite β -lactamase-REI_V-alkaline phosphatase fusion protein in an amber suppressor host. Expression in a non-suppressor host yields only the bipartite β -lactamase-REI_V fusion protein.

Upon induction with IPTG, an additional protein of expected apparent molecular weight (β -lactamase-REI_V fusion protein: M_r 41,800) was found in the periplasmic extract of a non-suppressor *E. coli* K-12 host (WK-6) carrying pHKREI (Fig. 2(a)). In immunoblots, this protein was recognized by an anti- β -lactamase serum (data not shown). Cells harboring pHKREI were able to grow, even without addition of IPTG, on agar plates containing ampicillin (100 μ g/ml). This indicates that β -lactamase is enzymatically active when fused to the REI_V domain and that the fusion protein is secreted into the periplasmic space, which is the usual cellular locale of pBR322-encoded β -lactamase (Koshland & Botstein, 1980).

The β -lactamase-REI_V fusion protein was purified from periplasmic extracts exploiting the affinity of the β -lactamase moiety to boric acid and phenylboronic acid (Kiener & Waley, 1978; Beesley *et al.*, 1983). The fusion protein could be purified to near homogeneity (Fig. 2(b)) using a phenylboronate-Sepharose affinity column with hydrophobic spacer arms (Cartwright & Waley, 1984). Using *E. coli* B strain BL21 (Studier & Moffat, 1986), usually 6 mg of fusion protein per liter of liquid culture have been obtained. Amounts of fusion protein found in the periplasmic space were lower for WK-6 than for BL21. A possible explanation is the absence of both the intracellularly located La protease (Waxman & Goldberg, 1982) and the outer membrane protease OmpT (Baneyx & Georgiou, 1990) from *E. coli* B cells. With a slightly modified purification procedure (Hennecke *et al.*, 1991) a fusion protein with β -lactamase as amino-terminal and a single-chain Fv fragment (Clackson *et al.*, 1991) of antibody NQ10.12.5. (Berek *et al.*, 1985) as carboxy-terminal fusion partner could be highly enriched, which exhibited binding to the NQ10.12.5 hapten 2-phenyloxazol-5-one (data not shown).

Release of the REI_V moiety from the purified fusion protein was achieved by site-specific proteolytic cleavage of the Pro-Thr peptide bond within the sequence Pro-Pro-Thr-Pro, the recognition site of IgA protease from *N. gonorrhoeae* (Pohlner *et al.*, 1987). This sequence had been introduced into the β -lactamase-REI_V fusion protein directly preceding the REI_V amino terminus (see Fig. 1(a)). IgA protease cleavage was performed with a molar ratio of substrate to enzyme of 150:1. After 24 hours of incubation at 37°C, cleavage was almost complete (Fig. 3). In contrast, the site-specific protease factor Xa (Nagai & Thøgersen, 1984) failed to cleave a β -lactamase-REI_V fusion protein containing, instead, the recognition sequence Ile-Glu-Gly-Arg. This may be due to different demands of the two proteolytic enzymes with respect to accessibility of their respective cleavage site.

Figure 1. Physical and genetic map of expression vector pHKREI and nucleotide sequence of the synthetic *rei* gene. (a) Expression vector pHKREI, which has been constructed by several rounds of cloning and oligonucleotide directed mutagenesis using standard techniques (Maniatis *et al.*, 1982; Kunkel *et al.*, 1987; Stanssens *et al.*, 1989). It contains an *f1* phage-derived origin of replication

The REI_v protein released by IgA protease contains two additional amino-terminal residues (Thr-Pro) and is therefore designated REI_vTP. The cleavage mixture, containing IgA protease, REI_vTP, β -lactamase and uncleaved β -lactamase-REI_v fusion protein, was applied directly to the phenylboronate-Sepharose affinity column. The liberated REI_vTP moiety was found exclusively in the flow-through, while the β -lactamase-REI_v fusion protein and the β -lactamase moiety of the fusion were retained (Fig. 3). Fortunately, IgA protease also binds to the phenylboronate-Sepharose affinity column. After passage through the second affinity column, 2 mg of pure REI_vTP was obtained starting from one liter of bacterial liquid culture.

In gel filtration experiments using a Sephadex G75 column, REI_v protein isolated from genuine myeloma Bence-Jones protein REI exhibits an apparent M_r of about 24,000 indicating dimerization as expected from the crystal structure (Epp *et al.*, 1975). The same apparent molecular weight was found for REI_vTP isolated as described above (data not shown). Since it is reasonable to assume dimerization to require native structure of the monomer, we take this finding as an indication of correct folding of REI_vTP. Furthermore, REI_vTP and genuine REI_v exhibited similar transition curves upon reversible urea denaturation indicating a conformational stability of both proteins of the order of 21 kJ/mol (data not shown).

In phasmid vector pHKREI the β -lactamase-*rei* fusion gene is separated from the *E. coli* gene *phoA*, encoding alkaline phosphatase by an amber stop codon. In the amber suppressor strain BMH71-18 (Kramer *et al.*, 1984) a tripartite fusion protein consisting of β -lactamase, REI_v and alkaline phos-

(*f1*), a *ColE1* plasmid replication origin derived from pBR322 (*colE1*), a gene encoding chloramphenicol acetyl transferase (*cat*) and a tripartite fusion gene encoding β -lactamase (*bla*; ranging from nucleotide pairs 2546 to 1689 according to GenEMBL accession no. VB0040), REI_v (*rei*; (b)) and alkaline phosphatase (*phoA*; ranging from nucleotide pairs 361 to 1695 according to GenEMBL accession no. X04586). The *bla* and *rei* genes are connected by a synthetic 30 bp adaptor encoding the recognition sequence of IgA protease from *N. gonorrhoeae* (Pohlner *et al.*, 1987). The recognition sequence for IgA protease is shaded, the point of cleavage is indicated by an arrow. The fusion gene is expressed under *lac* promoter control (Messing & Vieira, 1982). (b) Nucleotide sequence of the synthetic *rei* gene and corresponding REI_v protein sequence. The DNA sequence was deduced from the protein sequence (Palm & Hilschmann, 1973). The redundancy of the genetic code was exploited to introduce a number of restriction sites. Otherwise, the most frequently occurring *E. coli* codons were chosen. The gene was assembled in phasmid vector pMa5-8 (Stanssens *et al.*, 1989) by stepwise cloning of complementary oligonucleotide pairs. Relevant restriction enzyme sites are indicated. The DNA stretches encoding the IgA protease recognition sequence and the hypervariable loop regions are displayed in shaded boxes.

Figure 2. (a) Production of β -lactamase-REI_V fusion protein analyzed by SDS/polyacrylamide gel electrophoresis (15% gel; Coomassie staining). Lane 1, fraction of periplasmic proteins of *E. coli* WK-6 harboring phasmid pHKREI; lane 2, fraction of periplasmic proteins of an IPTG-induced *E. coli* WK-6 harboring pHKREI. The position of the β -lactamase-REI_V fusion protein is indicated by an arrow. M, molecular mass markers with masses indicated ($\text{Da} \times 10^{-3}$). *E. coli* WK-6 [$\Delta(\text{lac-pro})$, *galE*, *rpsL*, *F'lacI^a Z Δ M15*, *proA⁺B⁺*] (source W. Kramer) harboring pHKREI was grown overnight in 1 l of rich medium at 30°C with chloramphenicol (25 mg/l) and IPTG (1 mM) added. Periplasmic extracts were prepared by an adaptation of the procedure described by Neu & Heppel (1965). Cells were collected by centrifugation and resuspended in 200 ml of 20% (w/v) sucrose, 100 mM-EDTA, 200 mM-Tris-HCl (pH 9.0). The suspension was gently stirred for 30 min. Cells were pelleted and shocked by resuspending in 200 ml of

Figure 3. Cleavage of bipartite β -lactamase-REI_V fusion protein and affinity purification of REI_VTP. Lane 1, β -lactamase-REI_V fusion protein, affinity-purified as described for Fig. 2(b); lane 2, after IgA protease cleavage; lane 3, flow-through of phenylboronate-Sepharose affinity column; lane 4, after elution with buffer B (see the legend to Fig. 2(b)); M, molecular mass markers with masses indicated ($\text{Da} \times 10^{-3}$). IgA protease cleavage was performed with a molar ratio of enzyme to substrate of 1:150 in 50 mM-ammonium acetate (pH 7.0) at 37°C for 24 h. The sample was reapplied to the same affinity column used for the purification of the fusion protein (see the legend to Fig. 2(b)), pre-equilibrated with 50 mM-ammonium acetate (pH 7.0). The flow-through containing unbound REI_VTP protein (M_r 12,100) was collected. Bound material was eluted with buffer B (see the legend to Fig. 2(b)).

10 mM-Tris-HCl (pH 8.8). They were stirred again for 30 min at 4°C and recentrifuged. Periplasmic protein was precipitated from the supernatant by addition of ammonium sulfate to 90% saturation. After centrifugation, the protein precipitate was dissolved in 20 ml of 0.5 M-NaCl, 20 mM-triethanolamine-HCl (pH 7.0). (b) Analysis of various stages in the purification of β -lactamase-REI_V fusion protein (SDS/15% polyacrylamide gel; Coomassie staining). Lanes 1 and 4, total periplasmic extract of induced *E. coli* BL21 (lane 1) or WK-6 (lane 4), respectively, harboring pHKREI; lanes 2 and 5, fraction of unbound proteins after passage through phenylboronate-Sepharose affinity column; lanes 3 and 6, affinity-purified β -lactamase-REI_V fusion protein after column elution with 0.5 M-sodium borate, 0.5 M-NaCl, pH 7.0; M, molecular mass markers with masses indicated ($\text{Da} \times 10^{-3}$). Affinity chromatography was performed on phenylboronate-Sepharose with a hydrophobic spacer arm (Cartwright & Waley, 1984). The column (10 ml bed volume) was equilibrated with buffer A (20 mM-triethanolamine-HCl, 0.5 M-NaCl, pH 7.0). The sample was loaded at 4°C with a flow rate of 40 ml/h and washed with 5 column volumes of buffer A. Fractions eluted with buffer B (0.5 M-sodium borate, 0.5 M-NaCl, pH 7.0) were combined and dialyzed against 50 mM-ammonium acetate (pH 7.0) at 4°C.

Figure 4. A diagram showing the oligonucleotide-directed mutagenesis procedure employed for the construction of REI_v derivatives with codon substitutions at position 29 of CDR1. The situation at the DNA stretch of the *rei* gene encompassing the CDR1 coding region is shown above the phasmid. The nucleotide sequence of the lower strand corresponds to the mutagenic oligonucleotide. Likewise, in pHKREI-ΔCDR2 and pHKREI-ΔCDR3 the CDR2 or CDR3-encoding regions, respectively, were removed by directed mutagenesis using the mutagenic oligonucleotides MUΔCDR2 (5'-pGGAACACCATACGTAGATCAG-3') or MUΔCDR3 (5'-pCCGAAAGTATACGTACTGCTG-3'). For the generation of variant *rei* genes *via* transformation of a mismatch repair-deficient host strain with single-stranded DNA and mutagenic oligonucleotide, 10 pmol of the respective phosphorylated oligonucleotide was hybridized to 100 ng of single-stranded DNA of pHKREI-ΔCDR1 prepared from *E. coli* strain BMH71-18. Hybridization conditions were 150 mM-sodium chloride, 15 mM-sodium citrate in a final volume of 20 μl. After incubation at 100°C for 2 min and at 65°C for 5 min, the mixture was used to transform *E. coli* strain BMH71-18/*mutS* (Kramer *et al.*, 1984). The transformation mixture was plated onto indicator plates containing DIFCO Antibiotic Medium 3 (17.5 g/l), Bacto-Agar (15 g/l), chloramphenicol (25 mg/l), IPTG (238 mg/l) and X-P disodium salt (60 mg/l). To allow complete marker segregation, phasmid DNA was isolated from blue colonies and used for retransformation of *E. coli* BMH71-18.

phatase is synthesized and secreted. This results in a blue colony phenotype on indicator plates containing the chromogenic substrate 5-bromo-4-chloro-3-indolyl-phosphate (X-P; Sarthy *et al.*, 1981). Alkaline phosphatase was shown to be enzymatically active only when secreted into the periplasmic space (Michaelis *et al.*, 1983). For that reason measurement of alkaline phosphatase activity provides a simple and quantitative means to determine the efficiency of protein synthesis plus secretion (Hoffman & Wright, 1985; Manoil & Beckwith, 1986).

To facilitate oligonucleotide-directed mutagenesis within the CDR-encoding DNA regions of the *rei* gene, three derivatives of vector pHKREI were constructed, each containing a deletion within the DNA segment encoding CDR1, CDR2, or CDR3, respectively (Fig. 4). In addition to removing several codons, each deletion causes a frameshift such that formation of the tripartite fusion protein is precluded even in the amber suppressor host. A structurally altered CDR can now be introduced with restoration of the reading frame simply by insertion mutagenesis with a synthetic oligonucleotide. Thus, successful mutation constructs are scored as blue colonies after transformation of a host strain carrying an amber suppressor. Here we demonstrate the procedure as used for substitutions of CDR1 residue Ile29 (Fig. 4). With the mutagenesis protocol described by Kunkel *et al.* (1987) routinely 20% blue colonies were found on indicator plates containing X-P. Alternatively, mutant genes

were generated by plain cotransfection of the DNA mismatch repair deficient strain BMH71-18/*mutS* (Kramer *et al.*, 1984) with the pHKREI-ΔCDR1 single-stranded DNA and the mutagenic oligonucleotide (Fig. 4). With this procedure, a further simplification of the *mix-heat-transfect* protocol (Fritz *et al.*, 1988), around 1 to 2% blue colonies were obtained. Reintroduction of the CDR1-encoding DNA stretch together with the desired substitution of codon 29 was confirmed in each mutagenesis experiment by nucleotide sequence analysis (Ansorge *et al.*, 1987). A number of REI_v variants, containing e.g. Leu, Val, Ala, Ser or His at the CDR1 position 29 have been isolated and purified to homogeneity using the purification scheme described above. A detailed analysis of the conformational stabilities of these REI_v derivatives will be published elsewhere.

In summary, a novel expression/purification scheme for the production of immunoglobulin light chain variable domains and single-chain Fv fragments in *E. coli* was established, which rests on fusion to β-lactamase. We exploited the fact that *E. coli* β-lactamase is a periplasmic enzyme to guide the bipartite fusion protein consisting of β-lactamase and the immunoglobulin variable domain REI_v and the tripartite fusion protein consisting of β-lactamase, REI_v and alkaline phosphatase into the periplasmic space. In a number of instances, the secretion of heterologous proteins into the periplasmic space of *E. coli* has proven to be helpful for correct protein folding (Michaelis *et al.*, 1983;

Hsiung *et al.*, 1986). This is particularly important for cases like immunoglobulins, in which disulfide bonds must be formed, a process requiring an oxidizing environment as provided by the periplasmic space (Skerra & Plückthun, 1988).

For the purification of immunoglobulins, their respective hapten-binding properties can be exploited in affinity chromatography schemes (Skerra & Plückthun, 1988; Riechmann *et al.*, 1988). Obviously, for the isolation of immunoglobulins modified in such a way, that their hapten binding properties are changed (e.g. by mutations in their hypervariable regions), a general purification procedure must be based on principles other than hapten binding. We have solved this problem by employing β -lactamase as a specific affinity handle. Similarly, an oligohistidine extension of the immunoglobulin has been used (Skerra *et al.*, 1991). After purification by chromatography on phenylboronate-Sepharose (Cartwright & Waley, 1984), the β -lactamase moiety can be removed by site-specific enzymatic proteolysis and subsequent affinity chromatography using the same purification scheme.

For the generation of specific CDR modifications, a mutagenesis and screening procedure was established, in which successful mutation constructions are scored as blue colonies. Starting from three, or for single-chain Fv fragments six, different constructions of the immunoglobulin gene carrying deletions in the respective CDR-encoding DNA stretches, the desired mutation(s) can be introduced simply by insertion mutagenesis with a synthetic oligonucleotide; the latter brings in the sequence of the desired loop replacement. By means of an alternating phenotypic detection (colorless/blue colony phenotype) of clones carrying the desired mutation, multiple replacements can be performed in consecutive rounds of oligonucleotide-directed mutagenesis (J. Pschorr & G. Kleemann, unpublished results). Since the genetic detection scheme and the procedures of protein production/purification introduced here are of a general nature, we propose that they will be applicable to the generation of framework mutants and also to the engineering of proteins other than immunoglobulins.

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