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Improved folding of apo-retinol-binding protein in the periplasm of *Escherichia coli*: positive influences of *dsb*C coexpression and of an amino acid exchange in the vitamin A binding site

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The *in vivo* folding of the serum retinol-binding protein (RBP), a representative of the lipocalin structural family, is known to be complex. In order to gain insight into the essential steps along its folding pathway the heterologous production of the functional protein in *Escherichia coli* was investigated. Simultaneous overexpression of the bacterial *dsb*C gene, which codes for a periplasmic thiol-disulphide oxidoreductase, prevented the formation of soluble RBP variants with non-native disulphide bonds that were otherwise observed. Although the coexpression of *dsb*C had furthermore a stabilizing effect on the cell viability, the relative yield of the solubly produced RBP was not much better. In an attempt to enhance its folding efficiency, a favourable point mutation in the inner part of the retinol-binding pocket was predicted. Replacement of the polar Gln117 with an Ile side chain seemed not only to relieve the unfavourable energetics of the carboxamide group in the environment of predominantly non-polar residues but also to fill an adjacent cavity in the hydrophobic core. Indeed, this single substitution reproducibly resulted in a more than threefold increase in the amount of functional recombinant RBP. Ligand binding experiments showed that the affinity of this mutant for retinol was slightly enhanced. Kinetic measurements revealed that this was due to a higher association rate whereas the dissociation of the complex with retinol was essentially unaffected. Although the question remained why nature did not select this obviously beneficial mutation, our results demonstrate that the folding pathway of a lipocalin can be optimized by protein engineering.

Keywords: protein-disulphide isomerase/disulphide bond isomers/ligand binding/molecular modelling/bacterial expression

Introduction

The mammalian serum retinol-binding protein (RBP) transports vitamin A from the liver, the site of its storage, to a variety of target tissues. RBP is a small monomeric protein of 183 amino acids and possesses a central eight-stranded anti-parallel β-barrel, which forms the binding pocket for the hydrophobic and chemically sensitive ligand. Due to its medical importance and to its interesting structural properties—in fact, it was the first structurally characterized member of the so-called lipocalin family of proteins (Pervaiz and Brew, 1987)—RBP has been the subject of many biochemical and crystallographic studies (Soprano and Blaner, 1994; Newcomer *et al.*, 1984; Cowan *et al.*, 1990; Zanotti *et al.*, 1993). Although RBP folds into a single stable domain that exhibits a reversible two-state unfolding/refolding transition (Müller and Skerra, 1993) its folding mechanism *in vivo* appears to be complex.

In the hepatocyte, nascent pre-RBP is translocated into the lumen of the endoplasmic reticulum, where it is charged with retinol and from which it becomes finally secreted into the extracellular fluid (Blomhoff *et al.*, 1990). Initially it was speculated whether complexation of the ligand induces a change in conformation that is crucial for the secretion of the holo-protein (Ronne *et al.*, 1983). Recently, however, it was shown that folding of the processed apo-protein is distinct from the uptake of retinol, which finally triggers intracellular transport and secretion of the holo-protein in a subtle manner (Kaji and Lodish, 1993a). During the folding of apo-RBP the formation of its three disulphide bonds constitutes an important step. Different disulphide-bonded forms of the newly synthesized protein could be distinguished when the intracellular folding and unfolding of RBP in the presence of a reducing agent was studied (Kaji and Lodish, 1993a) and a role for the eukaryotic protein-disulphide isomerase (PDI) was suggested in this context (Kaji and Lodish, 1993b).

These findings were complemented by our own observations when we first established the functional secretion of recombinant apo-RBP in *E.coli* (Müller and Skerra, 1993). Although it was possible to isolate stably folded RBP, with ligand-binding properties indistinguishable from those of the natural protein, from the bacterial periplasm the formation of protein isomers with apparently non-native disulphide bonds was noted as well. This unusual phenomenon—compared, for example, with the bacterial secretion of recombinant antibody fragments (Skerra and Plückthun, 1991)—was suppressed by overproducing the disulphide-oxidoreductase DsbA of *E.coli* using a second, compatible expression plasmid (Müller and Skerra, 1993). In addition, however, the bacterial culture medium had to be supplemented with the thiol-reductant *N*-acetylcysteine. Under these conditions soluble apo-RBP with a homogeneous disulphide bond pattern was obtained.

Nevertheless, the fraction of recombinant protein that could be isolated from the periplasmic protein extract was—in comparison with the amount of spheroplast-associated, aggregated protein—still low with yields of approximately 0.12 mg/l·OD_{550} of the bacterial culture (Müller and Skerra, 1993). Therefore, we set out to study the folding efficiency of apo-RBP by introducing amino acid substitutions. In the present contribution we report a single side chain replacement within the retinol-binding pocket that leads to a significantly higher yield of correctly folded protein. As an improvement to the bacterial production system we describe the overexpression of the *E.coli dsb*C gene (Missiakas *et al.*, 1994; Shevchick *et al.*, 1994) from the same vector as RBP, which prevents the formation of non-physiological disulphide bond isomers without the need for the addition of a reductant.

Materials and methods

Vector construction

For the construction of pASK75-dsbC the structural gene encoding the DsbC protein, including the signal peptide and

the translational initiation site, was amplified from *E.coli* DNA and inserted into pASK75 (Skerra, 1994). Chromosomal DNA was prepared from the K-12 strain JM83 (Yanisch-Perron *et al.*, 1985) and subjected to a polymerase chain reaction with the primers 5′-GTC TGA AAG CTT CGG GAA GAT TTA TGA AGA AAG G (the *Hin*dIII site is underlined) and 5′-ACG CGA ATT ATT TAC CGC TGG TC in the presence of *Pfu* DNA polymerase, according to a published protocol (Skerra, 1992). The resulting single PCR product was purified on an agarose gel and cut with *Hin*dIII, so that a DNA fragment with one sticky end and one blunt end was obtained. pASK75 was cut with *Hin*dIII, followed by filling-in the 5′-protrusion with Klenow polymerase. After a second cut with *Xba*I the large DNA fragment was isolated. The two fragments, plus the small fragment of pASK75 that was obtained by cutting fresh plasmid with *Xba*I and *Hin*dIII, were combined in a three-fragment ligation, resulting in the directional insertion of the *dsb*C gene into the singular *Hin*dIII restriction site at the downstream end of the multiple cloning region on pASK75. As a consequence, one *Hin*dIII site was retained as part of the polylinker whereas the *Hin*dIII site was omitted downstream of the *dsb*C gene. Thus, another structural gene can be inserted by means of the multiple cloning region in front of *dsb*C, just as with pASK75. When the plasmid construction was checked by DNA sequencing an additional proline codon compared with the original *xpr*A sequence (Missiakas *et al.*, 1994; Shevchick *et al.*, 1994) was detected adjacent to the codon for Gln197 (in the mature sequence), as had also been noted by Zapun *et al.* (1995).

For the preparation of the RBP mutants the structural gene encoding RBP/H_3(A) was excised via *Xba*I and *Hin*dIII from the *lac* promoter-based expression plasmid (Müller and Skerra, 1994) and inserted into the likewise cut pASK75. Site-directed mutagenesis was performed after production of single-stranded DNA from this plasmid (Geisselsoder *et al.*, 1987). The oligodeoxynucleotides 5′-GCG GCA GGA GTA AAT CAC GGC GTA GGT GTC and 5′-ATA GTC CGT GTC GAT AAT CCA CCA GTC ATC GTT TCC were used for introducing the amino acid substitutions Gln117→Ile and His104→Trp, respectively. For coexpression with *dsb*C the genes were finally transferred, again via *Xba*I/*Hin*dIII, into pASK75-dsbC described above.

Recombinant protein production

Production of the RBP variants was carried out in 2l cultures of *E.coli* JM83 harbouring the appropriate derivative of pASK75 or pASK75-dsbC (see above). The cells were grown in a shaking flask at 22°C in LB medium (Sambrook *et al.*, 1989) containing 100 mg/l ampicillin up to an OD_{550} of 0.5. Expression was then induced by the addition of 200 µg/l anhydrotetracycline (ACROS; dissolved to 2 mg/ml in dimethyl formamide). After induction for 3 h, or overnight, the cells were collected by centrifugation. For periplasmic cell fractionation the cells were resuspended in 20 ml 0.5 M sucrose, 40 mM sodium phosphate pH 7.5, 1 mM EDTA and, after addition of 20 µg/ml lysozyme, incubated on ice for 30 min (Müller and Skerra, 1993). Due to a reduced lytic effect in the case of the mutant I^{117}, 100 µg/ml lysozyme had to be added to the cell fractionation buffer in order to achieve quantitative release of the soluble protein. The spheroplasts were removed by repeated centrifugation and the supernatant was dialysed against 100 mM Tris–HCl pH 8.0. The efficiency of the periplasmic fractionation was confirmed by measuring the activity of β-lactamase in the extract (Faraci and Pratt, 1985) and comparing it with that in a French press lysate.

Protein purification and characterization

The RBP variants were purified from the periplasmic protein fraction via the *Strep*-tag by streptavidin affinity chromatography (Schmidt and Skerra, 1994). After equilibration of 4 ml Sepharose, to which 5 mg/ml of an engineered version of streptavidin (Voss and Skerra, 1997) had been coupled, with 100 mM Tris–HCl pH 8.0 the dialysed periplasmic protein solution (~30 ml) was applied (flow rate 40 ml/h). The column was washed until the A_{280} of the flow-through diminished to the baseline. Pure recombinant RBP was then eluted with 2.5 mM desthiobiotin (Sigma) dissolved in the chromatography buffer. The purified protein was dialysed three times against KSH buffer (50 mM K_2SO_4, 20 mM HEPES pH 7.5). The concentrations of the RBP mutants were determined using molar absorption coefficients of 41 300 M^{-1} cm^{-1} for RBP/H_3(A), 39 050 M^{-1} cm^{-1} for the mutant I^{117}, and 47 270 M^{-1} cm^{-1} for the double mutant I^{117}/W^{104}, which were calculated and corrected according to Gill and von Hippel (1989). 15% SDS–PAGE was performed using the buffer system of Fling and Gregerson (1986), followed by staining with Coomassie brilliant blue.

Fluorescence titration

Fluorescence measurements were carried out in a Perkin Elmer LS50 fluorimeter using a 1×1 cm^2 Hellma quartz cuvette thermostatically controlled to 22°C and equipped with a small magnetic stirrer. Retinol fluorescence was excited at 334±2.5 nm and detected at 455±2.5 nm. Two ml of a 1 µM solution of the purified RBP variant in KSH buffer was titrated in steps of 1, 2 or 5 µl with a 200 µM solution of retinol in ethanol. The fresh stock solution was gravimetrically prepared and checked by absorption measurement at 325 nm using an extinction coefficient of 46 000 M^{-1} cm^{-1} (Cogan *et al.*, 1976). After adding an aliquot of the ligand solution the mixture was stirred for 5 min before fluorescence was detected (averaged over 2 s). The fluorescence intensities were fitted by non-linear least squares regression according to the theory of bimolecular complex formation, i.e. under the assumption of a 1:1 stoichiometry, and then normalized to 100%. For the fluorescence titration with retinoic acid the quenching of the protein's Trp and Tyr emission was followed with λ_{Ex} = 295±2.5 nm and λ_{Em} = 340±2.5 nm as previously described (Müller and Skerra, 1993).

Retinol binding kinetics

The kinetics of retinol binding to RBP was determined by measuring the increase in the ligand fluorescence using the same instrument and buffer conditions as above. The reaction was initiated by simultaneously pipetting 1 ml of a 1 µM protein solution and 1 ml of either 2, 1 or 0.5 µM retinol (diluted from a 200 µM stock solution in ethanol) in KSH buffer into the quartz cuvette. The change in fluorescence was detected every 0.1 s during 5 min with continuous stirring. The fluorescence increase was then evaluated according to the general kinetic equation for reversible ligand binding

$$\frac{d[PL]}{dt} = k_{on}[P][L] - k_{off}[P \cdot L] \quad (1)$$

where P denotes the protein, L the ligand, and $P \cdot L$ their complex. Since unbound retinol is quenched in an aqueous buffer and protein shows no fluorescence upon excitation at

334 nm, the observed fluorescence intensity is directly related to the concentration of the complex

$$F = \phi\,[P{\cdot}L] \tag{2}$$

where F denotes the fluorescence intensity and ϕ the specific fluorescence coefficient. Using the known dissociation constant K_d from the equilibrium fluorescence titration experiment for substituting k_{off}

$$k_{off} = k_{on}\,K_d \tag{3}$$

and the balance equations, in which the index t denotes total concentration

$$[P]_t = [P] + [P{\cdot}L] \tag{4}$$

$$[L]_t = [L] + [P{\cdot}L] \tag{5}$$

the following differential equation is obtained:

$$\frac{dF}{dt} = \frac{k_{on}}{\phi}\;F^2 - k_{on}\,([P]_t + [L]_t + K_d)F + k_{on}\phi[P]_t\,[L]_t \tag{6}$$

Separation of the variables t and F leads to an integral of the type

$$\int \frac{1}{ax^2 + bx + c}\,dx$$

with the generic solution

$$f(x) = \frac{1}{\sqrt{b^2 - 4ac}}\,\ln\left(\frac{2ax + b - \sqrt{b^2 - 4ac}}{2ax + b + \sqrt{b^2 - 4ac}}\right) - C$$

Thus, the fluorescence intensity can be expressed as

$$F = -\frac{\phi}{2}\left\{\sqrt{S^2 - 4[P]_t\,[L]_t}\left(1 + \frac{2}{e^{k_{on}(t + C)\sqrt{S^2 - 4[P]_t\,[L]_t}} - 1}\right) - S\right\} \tag{7}$$

whereby C denotes the integration constant and $S = [P]_t + [L]_t + K_d$. Equation (7) was used in a non-linear least squares fit of the experimental data (with k_{on}, C and ϕ as the free parameters) using standard mathematical tools from the program KaleidaGraph (Abelbeck Software) that was run on a Macintosh computer.

Results

Design of the RBP mutants

As a model for investigating the *in vivo* folding of bacterially expressed RBP we chose a previously constructed variant of the porcine protein, termed RBP/H_3(A), which carried a Zn(II)-binding site on the β-barrel surface and the *Strep*-tag at the C-terminus of the polypeptide chain (Müller and Skerra, 1994). This protein was shown before to exert the same folding stability and ligand affinity for retinoic acid as wild-type RBP (Müller and Skerra, 1993, 1994). The central feature of RBP is its deep and extremely hydrophobic binding pocket, which is almost perfectly complementary to the aliphatic part of several retinoid compounds. Whereas all-*trans* retinol (vitamin A) constitutes the physiological ligand, retinoic acid and other derivatives with differing substituents at C^1 are complexed equally well (Cogan *et al.*, 1976). At least the lower part of the pocket in RBP seems to be structurally rigid and no significant conformational changes were, for example, detected between the crystal structures of the human apo- and holo-protein (Zanotti *et al.*, 1993). Notably, the ligand pocket was seen filled with seven ordered solvent molecules in the absence of retinol.

Closer inspection of the RBP structure revealed, however, one deviation from the almost ideal mould-like shape with respect to the lipophilic retinoid moiety. A polar Gln side chain, which becomes buried at the bottom of the binding pocket upon complex formation, participates in a contact with the β-ionone ring (Figure 1). This Gln117 residue is surrounded by the hydrophobic side chains of Tyr133, Phe135, Phe137 and His104, which also contact the β-ionone ring of the ligand. Consequently, it was anticipated that the carboxamide group of Gln117 should be energetically disfavoured in this environment. In addition, a cavity was detected in the hydrophobic core of RBP directly underneath Gln117 (Figure 1a). Visual inspection indicated that a branched aliphatic side chain might fit much better into the structure without compromising RBP's ligand binding capability. In a molecular modelling study an Ile side chain was thus introduced instead of Gln117, with the preferred side chain rotamer angles $\chi_1 = -174.8°$ and $\chi_2 = -60.0°$ (as revealed with the WHAT IF program package; Vriend, 1990). The Ile side chain appeared to be ideally situated at this position since no structural clashes with surrounding side chains or the bound ligand became obvious and its ethyl moiety almost filled the cavity in the hydrophobic core (Figure 1b).

As a control for the ligand-binding properties of this RBP mutant (see below) an additional substitution was planned. His104 was replaced by the much bigger Trp side chain so that a significant part of the volume that is normally accessible to the β-ionone ring of the ligand became occupied (Figure 1c). The coding sequences for both RBP mutants, I^{117} and I^{117}/W^{104}, were prepared by site-directed mutagenesis (see Materials and methods) and used for subsequent expression in *E.coli*.

Expression strategy and effect of DsbC on the folding of RBP

In previous studies the formation of non-natural disulphide isomers of RBP in the periplasm of *E.coli* was suppressed by the overproduction of the DsbA protein (Müller and Skerra, 1993). DsbA was the first known thiol-disulphide oxidoreductase resident in the periplasmic space of *E.coli* (Bardwell *et al.*, 1991; Kamitani *et al.*, 1992). Another *E.coli* thiol-disulphide oxidoreductase, DsbC, was subsequently discovered (Missiakas *et al.*, 1994; Shevchick *et al.*, 1994). In order to investigate the influence of this protein and also to avoid working with a second plasmid, as for the coexpression of *dsb*A (Müller and Skerra, 1993), a new expression system for RBP was developed based on the vector pASK75-dsbC (Figure 2). This plasmid was constructed from the generic cloning and expression vector pASK75, on which recombinant gene expression was tightly controlled by the chemically inducible *tet*A promoter (Skerra, 1994). For the construction of pASK75-dsbC the *dsb*C structural gene was cloned immediately downstream of the multiple cloning region (for details see Materials and methods) such that upon insertion of a foreign gene—like that of RBP—a dicistronic operon was created (Figure 2). Thus, transcriptional coupling under control of the *tet* promoter/operator was ensured.

The effect of *dsb*C coexpression on the bacterial production of RBP was investigated by comparing host cells which carried either the pASK75-RBP plasmid or pASK75-RBP/dsbC. The results are shown in Figure 3 for the RBP/H_3(A) variant I^{117}/W^{104}. About twice the amount of soluble recombinant protein

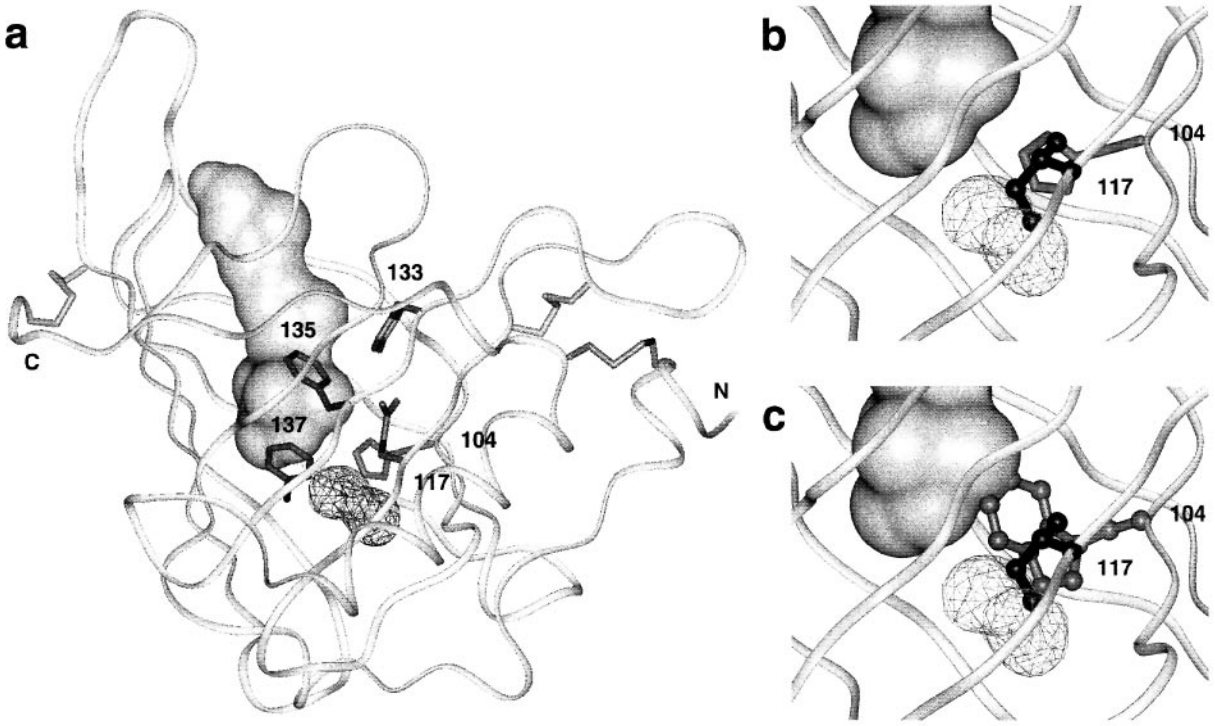

Fig. 1. Structural model for the replacement of Gln117 by Ile and of His104 by Trp in RBP. (**a**) Three-dimensional structure of human RBP (PDB entry 1RBP; Cowan *et al.*, 1990). The characteristic β-barrel of the retinol-binding protein is shown in a ribbon representation whereby the polypeptide termini are marked with N and C. The three structural disulphide bridges are depicted as sticks and the bound retinol is shown with a solid Connolly surface (generated with Insight II software; Biosym/MSI). The polar Gln117 residue and the surrounding hydrophobic amino acids Tyr133, Phe135, Phe137 and His104 are labelled. The adjacent cavity in the hydrophobic core of the protein is shown below the retinol with an inverse Connolly surface in a grid representation. (**b**) In the mutant I^{117} the newly introduced Ile side chain occupies the cavity with its ethyl moiety and retains a non-polar contact with the bound retinol. (**c**) The introduction of Trp104 results in a structural overlap with the ligand.

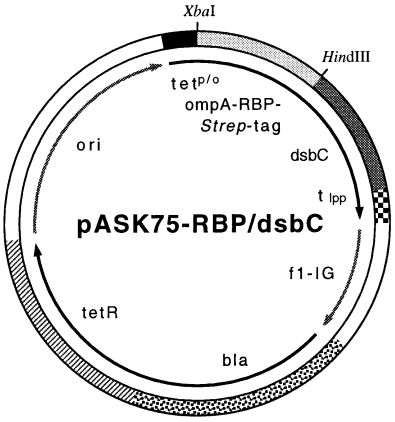

Fig. 2. Expression vector pASK75-RBP/dsbC for the simultaneous secretion of the retinol-binding protein, RBP, fused with the OmpA leader sequence and the C-terminal *Strep*-tag, and the disulphide isomerase DsbC. *ori*, origin of plasmid replication; $tet^{p/o}$, tetracycline promoter/operator; t_{lpp}, lipoprotein terminator; f1-IG, intergenic region of filamentous phage f1; *bla*, β-lactamase-encoding gene; *tet*R, tetracycline repressor gene.

per OD_{550} was isolated from the periplasmic cell fraction by means of the *Strep*-tag and streptavidin affinity chromatography (Schmidt and Skerra, 1994) after 3 h induction in the presence of DsbC. Furthermore, during SDS–PAGE of the non-reduced protein sample several bands became visible in the case of the ordinary expression plasmid, pASK75-RBP, whereas a homogeneous product was seen when *dsb*C had been coexpressed (Figure 3). Finally, the cells showed improved growth after induction of gene expression and were less prone to lysis (cf. the periplasmic fractions with and without overexpressed *dsb*C in Figure 3), especially when induction was carried out overnight (see Figure 4).

Thus the DsbC protein had a similar positive influence on the correct disulphide bond formation of RBP as it was seen with DsbA before (Müller and Skerra, 1993), with the advantage that no cofactors had to be added to the culture medium. Due to the higher cell densities that were obtained with the present expression system, the total yield of correctly folded protein could be increased up to fivefold.

Comparison of the yields and ligand-binding properties of the RBP mutants

Using the pASK75-RBP/dsbC expression plasmid, wild-type RBP [i.e., $RBP/H_3(A)$] and the mutants I^{117} and I^{117}/W^{104} were produced under identical experimental conditions. Pure protein, devoid of disulphide isomers, was isolated in each case via the *Strep*-tag as above (Figure 4). Compared with the moderate yields of the wild-type protein, as described before (Müller and Skerra, 1994), the I^{117} mutant was obtained in a more than three-fold higher amount (Table I). The substitution of Gln117 by Ile thus enhanced the folding efficiency of this protein and, at the same time, the toxic effects of the recombinant protein on the host cells were reduced. This became apparent from the higher concentration of lysozyme that was needed for the quantitative release of the mutants I^{117} and I^{117}/W^{104} from the bacterial periplasm (see Materials and methods). In the case of the I^{117}/W^{104} double mutant the yield of soluble protein was essentially the same (Table I).

In order to assess the ligand-binding properties of the I^{117}

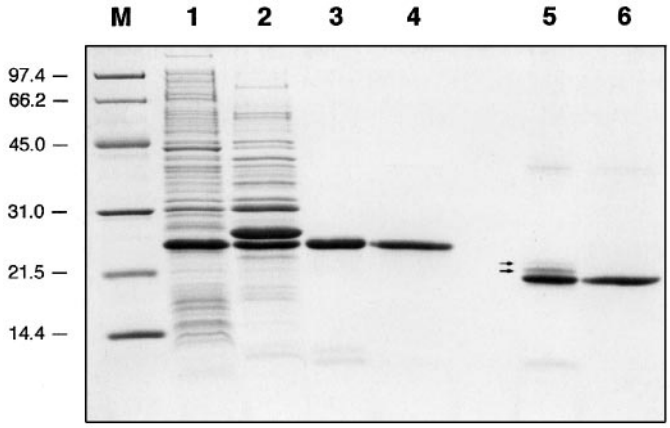

Fig. 3. Effect of DsbC on the disulphide bond formation in the RBP mutant I^{117}/W^{104} as revealed by SDS–PAGE. *Escherichia coli* JM83 transformed with pASK75-RBP (lanes 1, 3 and 5) or pASK75-RBP/dsbC (lanes 2, 4 and 6) was induced for 3 h at 22°C and periplasmic protein extracts were prepared (lanes 1 and 2). After purification via the *Strep*-tag equivalent amounts of the RBP mutant were applied under reducing (lanes 3 and 4) and non-reducing conditions (lanes 5 and 6). At least two non-native disulphide isomers with decreased electrophoretic mobility were resolved (lane 5) and are indicated by arrows. This pattern was reproducible but differed slightly among the RBP mutants. The strong extra band which is visible in lane 2 corresponds to the DsbC protein. Molecular sizes of the marker bands (M) are given in kDa.

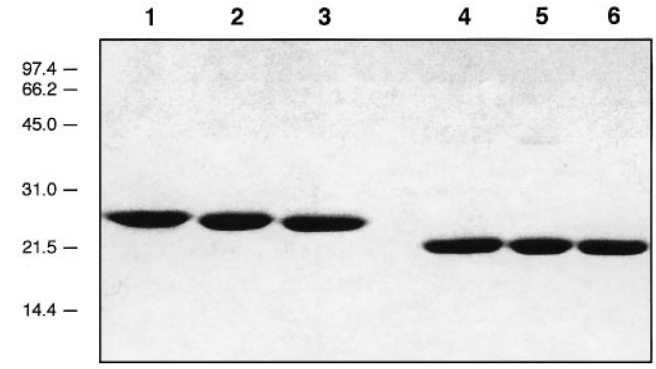

Fig. 4. Analysis of the different purified RBP mutants by SDS–PAGE after overnight expression in the presence of overproduced DsbC. Equivalent amounts of RBP/H_3(A) (lanes 1 and 4) and of the mutants I^{117} (lanes 2 and 5) and I^{117}/W^{104} (lanes 3 and 6) were applied under reducing (lanes 1–3) and non-reducing conditions (lanes 4–6). Molecular sizes are given in kDa.

mutant a fluorescence titration with retinol was performed (Figure 5). The burial of retinol in the protein's binding pocket effects intense fluorescence at 455 nm when the conjugated double bond system is excited, whereas it is almost completely quenched if the molecule is surrounded by water. As a result, a slightly stronger binding of the ligand was detected for the I^{117} mutant in comparison with the wild-type protein. In contrast, the affinity for retinol was significantly diminished in the I^{117}/W^{104} double mutant (Figure 5) as it had to be expected from the structural considerations above. Fluorescence titration experiments with retinoic acid, which had served as a reference ligand in previous studies (Müller and Skerra, 1993, 1994), yielded similar results (Table II).

Finally, we investigated whether the mutation Gln117→Ile had an effect on the binding kinetics for vitamin A. The emerging ligand fluorescence that accompanies complex formation was measured after rapid mixing of two solutions containing the apo-protein and retinol, respectively. By fitting the

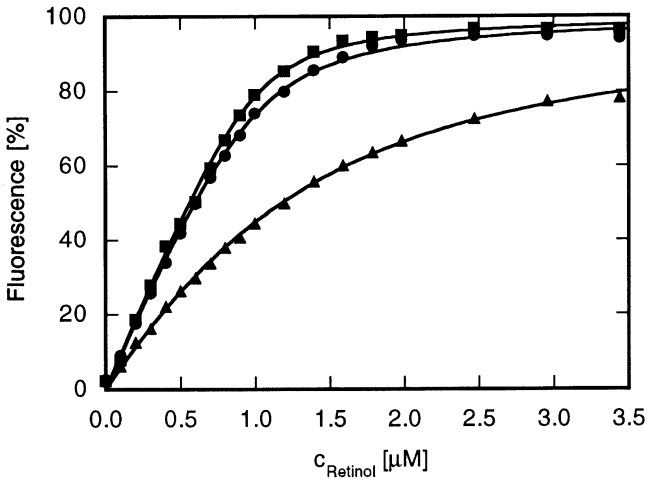

Fig. 5. Determination of ligand affinities for the RBP mutants in fluorescence titration experiments with retinol. Purified samples (Figure 4) of RBP/H_3(A) (circles) and of the mutants I^{117} (squares) and I^{117}/W^{104} (triangles) were each applied at a concentration of 1 µM. Aliquots of a retinol solution were added and the fluorescence intensity was measured (λ_{Ex} = 334 nm; λ_{Em} = 455 nm). The curves represent non-linear least squares fits for bimolecular complex formation. For the calculated K_d values see Table II.

Table I. Yields of the recombinant RBP variants that were produced in *E.coli* for 3 h at 22°C with simultaneous overexpression of *dsb*C

Protein	Yield [µg/($l_{culture}$×OD_{550})][a]
RBP/H_3(A)	282±61
I^{117} mutant	880±26
I^{117}/W^{104} mutant	842±34

[a]Determined after purification from the periplasmic protein fraction via the *Strep*-tag using the absorption coefficients given in Materials and methods. The values represent mean and standard deviations from three independent experiments.

Table II. Thermodynamic and kinetic parameters at 22°C for the binding of retinol to the RBP mutants (see Figures 5 and 6)

Protein	K_d [nM]	k_{on} [mM^{-1} s^{-1}][a]	k_{off} [10^{-3} s^{-1}][b]	K_d for retinoic acid [nM]
RBP/H_3(A)	94±9	12.7±4.0	1.19±0.04	194±23
I^{117} mutant	58±7	19.6±5.3	1.14±0.04	120±13
I^{117}/W^{104} mutant	675±32			594±26

[a]Mean and standard deviations of the k_{on} values that were obtained from the three experiments shown in Figure 6.
[b]Calculated from the K_d and k_{on} values in the preceding columns.

time-resolved fluorescence intensities to Equation (7) the kinetic constants for association, k_{on}, and for dissociation of the complex, k_{off}, were determined for RBP/H_3(A) and for the I^{117} mutant (Figure 6). It was found that the mutant exhibited a somewhat faster binding of the ligand whereas the dissociation rate was roughly the same as for the wild-type protein (Table II).

Discussion

The formation of non-native disulphide isomers during the bacterial secretion of recombinant RBP has been noted since this expression system was established (Müller and Skerra,

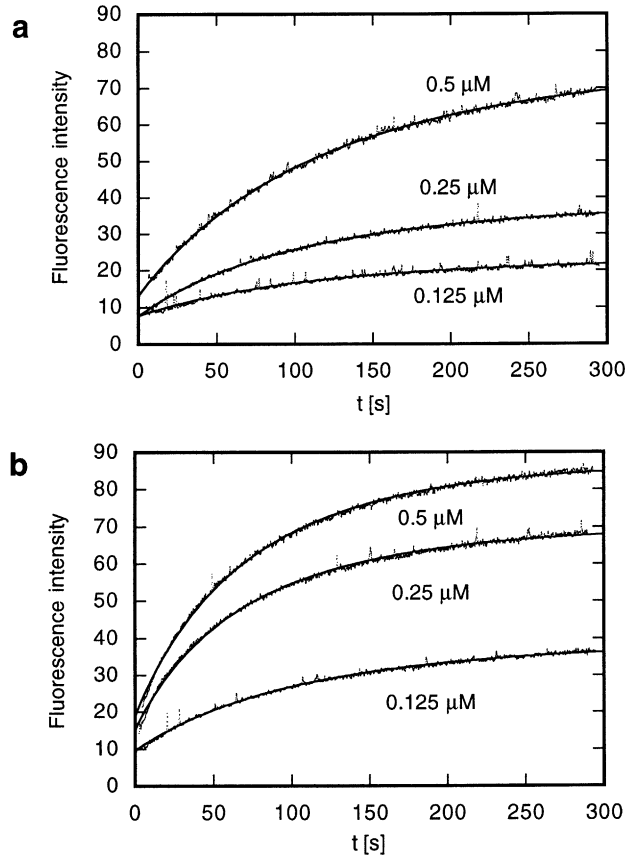

Fig. 6. Determination of the retinol binding kinetics for (**a**) RBP/H_3(A) and (**b**) the mutant I^{117}. The fluorescence increase (λ_{Ex} = 334 nm; λ_{Em} = 455 nm; arbitrary units) was measured in three experiments with differing concentrations of retinol as indicated. The final protein concentration in the assay was 0.5 μM in each case. The data were subjected to non-linear least squares fits according to Equation (7). The resulting k_{on} and k_{off} values are listed in Table II.

1993). A structural explanation for this unusual phenomenon might be deduced from the exposed location of two of the three disulphide bonds (Figure 1). These crosslinks fix the loose N- and C-terminal ends of the polypeptide chain, which are not part of secondary structure elements, to the rigid core of the protein. Due to the obvious flexibility of these segments it may be envisaged that an interchanged disulphide connectivity can be retained even after the proper folding of the central part of RBP. Such wrong crosslinks are likely to correspond just to minute differences in the free energy of folding so that the thermodynamic driving force towards the correct disulphide bond pattern could be low. Consequently, the action of a catalyst might be needed in order to finish the isomerization process. A previous study of the disulphide bond formation and reduction in RBP after expression in its natural host cell did already point at a corresponding function for the PDI (Kaji and Lodish, 1993b).

In the past years two soluble proteins have been identified in the periplasm of *E.coli* that exert thiol-disulphide oxidoreductase activity: DsbA and DsbC (Bardwell, 1994). After the initial discovery of DsbA (Bardwell *et al.*, 1991; Kamitani *et al.*, 1992) it was certainly reasonable to speculate about its role as a bacterial PDI. But it soon became clear during subsequent investigations that its physiological function rather lies in a strong oxidant that ensures rapid formation of disulphide bonds in freshly secreted periplasmic proteins. DsbC, on the other hand, was shown to be less oxidizing and to catalyse protein–disulphide rearrangements during the refolding of bovine pancreatic trypsin inhibitor *in vitro* (Zapun *et al.*, 1995). Further evidence for a role of DsbC in disulphide isomerization was accumulated from experiments *in vivo* (Rietsch *et al.*, 1996).

Our present finding that RBP is produced in *E.coli* with a homogeneous and probably native disulphide bond pattern in the presence of the overproduced enzyme supports the notion that DsbC can act as a bacterial PDI. In contrast, as long as DsbA had been used instead of DsbC it was necessary to add a thiol compound to the culture medium. By this manipulation the thiol/disulphide redox equilibrium was shifted such that DsbA could probably reduce some of the wrong disulphide bonds and thus promote their isomerization. However, a high concentration of DsbC in the periplasma seems to be needed in order to effect quantitative disulphide isomerization for RBP. Similar experiences were previously made with the eukaryotic PDI when expressed in *E.coli* in a study on the functional secretion of a recombinant antibody fragment (Humphreys *et al.*, 1996).

Despite the assistance of the DsbC protein the yield of solubly secreted wild-type RBP was not significantly affected and remained in the range of 10% of the total recombinant protein (data not shown). Consequently, there must be a step earlier in the pathway which decides about the correct folding of the protein versus aggregation of the polypeptide chains. A similar mechanism had been discussed for the limited efficiency during the assembly of recombinant antibody fragments in the periplasm of *E.coli* (Skerra and Plückthun, 1991). Meanwhile, a mutagenesis study was described for the F_v fragment of the antibody McPC603 which aimed at elucidating structural determinants that may be limiting for crucial folding steps (Knappik and Plückthun, 1995). Amino acid substitutions were identified that increased the yield of the functional F_v fragment by a factor of 3 and it was claimed that mutations which influence the aggregation of proteins during folding would be most often located in turns, at least in β-barrel proteins.

Remarkably, in the case of RBP, which exhibits an almost ideal β-barrel architecture, a side chain replacement in the core of the structure was here shown to significantly improve the folding efficiency. Apart from the location, however, some parallels with the situation for antibody fragments exist. For example, the thermodynamic folding stability of the I^{117} mutant remained essentially unchanged (Schmidt and Skerra, manuscript in preparation) and secretion of the mutant caused a less toxic effect on the host cells compared with the wild-type protein.

The hydrophilic Gln117 side chain forms part of the binding pocket for the hydrophobic ligand and its exchange by the apolar Ile resulted in an increased affinity for retinol. If, as a control, the neighbouring His104 residue was furthermore replaced by the bulky Trp the ligand affinity dropped significantly, as expected. Negative effects of the Gln117→Ile exchange were not apparent. In particular, the dissociation rate of the complex was unchanged so that the release of vitamin A from RBP to target cells should remain unaffected by this mutation (Noy and Xu, 1990). In the hepatocytes, on the other hand, a quicker uptake of retinol would seem possible. Consequently, the question arises why this mutation had not been selected by nature during the evolution of this physiologically important protein. In contrast, Gln117 is a

conserved residue, which is found throughout the RBPs of higher vertebrates such as man, pig, cow, mouse, rat and rabbit (SWISSPROT database entries P02753, P27485, P18902, Q00724, P04916 and P06912).

Interestingly, in the structurally related cellular retinol-binding protein (Winter *et al.*, 1993) a Gln residue is also buried in the interior and participates in the binding of the ligand. In this case, however, the orientation of retinol is inverted. The Gln side chain interacts with the polar hydroxyl group and its replacement by Arg was shown to result in a diminished affinity (Stump *et al.*, 1991). It could thus be speculated that the Gln117 residue in RBP constitutes an evolutionary remnant from ancient times when vitamin A might have been bound to the β-barrel in a different manner. Once again this example demonstrates that nature has not necessarily optimized all of its molecules so that there is plenty of room for the improvement of their stability or function by protein engineering.

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