Influence of the conserved disulphide bond, exposed to the putative binding pocket, on the structure and function of the immunoglobulin-like molecular chaperone Caf1M of *Yersinia pestis*

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The *Yersinia pestis* protein Caf1M is a typical representative of a subfamily of periplasmic molecular chaperones with characteristic structural and functional features, one of which is the location of two conserved cysteine residues close to the putative binding pocket. We show that these residues form a disulphide bond, the reduction and alkylation of which significantly increases the dissociation constant of the Caf1M–Caf1 (where Caf 1 is a polypeptide subunit of the capsule) complex [from a K_d of $(4.77 \pm 0.50) \times 10^{-9}$ M for the intact protein to one of $(3.68 \pm 0.68) \times 10^{-8}$ M for the modified protein]. The importance of the disulphide bond for the formation of functional Caf1M *in vivo* was demonstrated using an *Escherichia coli dsbA* mutant carrying the *Y. pestis f1* operon. In accordance with the CD and fluorescence measurements, the disulphide bond is not important for maintenance of the overall structure of the Caf1M molecule, but would appear to affect the fine structural properties of the subunit binding site. A three-dimensional model of the Caf1M– Caf1 complex was designed based on the published crystal structure of PapD (a chaperone required for Pap pili assembly) complexed with a peptide corresponding to the C-terminus of the papG subunit. In the model the disulphide bond is in close proximity to the invariant Caf1M Arg-23 and Lys-142 residues that are assumed to anchor the C-terminal group of the subunit. The importance of this characteristic disulphide bond for the orchestration of the binding site and subunit binding, as well as for the folding of the protein *in vivo*, is likely to be a common feature of this subfamily of Caf1M-like chaperones. A possible model for the role of the disulphide bond in Caf1 assembly is discussed.

INTRODUCTION

The biogenesis of a number of virulence-associated surface structures of pathogenic Gram-negative bacteria follows a common pathway involving periplasmic molecular chaperones. These chaperones specifically recognize and bind to subunits of surface structures (pili, fimbriae, polypeptide capsule), prevent non-productive aggregation of the subunits in the periplasm and probably aid in folding of the subunit, following translocation of the subunits across the inner membrane. The molecular chaperone then delivers the subunit to an outer-membrane molecular usher protein which is responsible for translocation of the subunit through the outer membrane, correct assembly of ordered surface structures and anchorage of some structures to the cell surface [1–3].

Expression of the capsular Caf1 subunit of *Yersinia pestis* [4] is mediated by the product of the *caf1M* gene [5], which shares sequence similarity with a superfamily of molecular chaperones that includes the well characterized PapD protein of uropathogenic *Escherichia coli* [6]. The three-dimensional structure of PapD has been solved by X-ray crystallography [7,8]. The protein consists of two immunoglobulin-like domains oriented towards one another, giving the molecule an overall 'boomerang' shape with a cleft between the two domains. It has been proposed that

PapD is a prototype member of the superfamily of periplasmic chaperones, all of which are predicted to have a similar immunoglobulin-like topology [9]. Based on this assumption, the steric structure of the *Y. pestis* chaperone Caf1M was reconstructed by computer modelling using the atomic co-ordinates for PapD [10]. In our previous analysis of the primary structure alignment of 17 of the periplasmic chaperones, we discovered that Caf1M belongs to a subfamily of seven chaperones [10]. This subfamily is characterized by possession of (i) two cysteine residues, one located in the F1 β-strand and the other in the G1 β-strand, with the potential of forming a disulphide bond exposed to the putative subunit binding pocket, and (ii) an accessory sequence encompassed by the two cysteine residues [10]. We also noted that a characteristic functional feature of the Caf1M-like subfamily is the chaperoning of virulence-associated surface organelles with a simple composition; for example, the *Y. pestis* capsule consists of a single subunit, Caf1. In a recent publication, Hung and colleagues [11] have extended this subfamily (termed FGL subfamily, for F1–G1 long loop) by the addition of two more chaperones, and also noted the functional subdivision of the periplasmic chaperones into the FGS subfamily, which form rod-like structures, and the FGL subfamily, which form non-pilus organelles with an atypical morphology. The PapD chaperone possesses a quite different disulphide bond, located at

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Abbreviations used: Caf1M, chaperone involved in *Yersinia pestis* capsule assembly; Caf1, polypeptide subunit of capsule; PapD, chaperone required for Pap pili assembly; DsbA and DsbC, *Escherichia coli* disulphide-bond isomerases; Hsp25, small heat shock-protein of 25 kDa.
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a distance from the binding site between the last two β-strands of the C-terminal domain. Using a strain lacking the disulphide bond isomerase DsbA, it has been shown that formation of this bond is essential for the correct folding of PapD *in vivo*, but that the disulphide bond is not required for folding of PapD *in vitro* or for pilus subunit binding [12]. It is reasonable to predict that, in the case of the Caf1M-like subfamily of proteins, the conservative disulphide bond which is exposed to the putative binding pocket will be important not only for the correct folding of the chaperone but also for interaction with the respective chaperoned subunit. In the present study, we investigate the influence of this disulphide bond on both the structure and function of the Caf1M chaperone molecule.

EXPERIMENTAL

Bacterial strains, plasmids and culture conditions

Bacterial strains used were *E. coli* HB101, DH5α and the two isogenic strains JCB570 and JCB571 (*dsbA*::*kan*) [13], which were kindly provided by J. Bardwell (Department of Biology, University of Michigan, Ann Arbor, MI, U.S.A.). Cultures carrying the appropriate plasmid, as indicated, were routinely grown at 37 °C in LB broth containing ampicillin (100 μg/ml) and kanamycin (40 μg/ml) as required. The cosmid pFS2 (carrying the entire *f1* operon) [4] and plasmids p12R (carrying a major fragment of the *f1* operon) [3], pTCA1 (encoding Caf1M under *ptrc* control) [14] and pFM1 (encoding Caf1M and the Caf1 subunit under *ptrc* control) [14] have been described. For overexpression of Caf1 subunit alone, a 596 bp *Bam*HI fragment encompassing the majority of the *caf1M* gene (from nucleotide 242 to the end) was deleted from pFM1 to yield pFM1S (encoding only the Caf1 subunit under *ptrc* control). *E. coli* cultures carrying pTCA1, pFM1 or pFM1S were maintained in the presence of 0.6 % glucose and were induced with 1 mM isopropyl β-D-thiogalactoside.

Purification of Caf1M

Caf1M was isolated from induced *E. coli* DH5α/pTCA1 cells by osmotic shock and purified to homogeneity using MonoQ and Superose 12 H/R chromotography as previously described [14].

Production and purification of recombinant Caf1 protein

E. coli HB101/p12R was cultured in Hottinger broth [15] at 37 °C for 18–20 h on a shaker at 120 rev./min. The cells were removed by centrifugation at 10000 *g* for 25 min at 4 °C. The supernatant was concentrated 20-fold on a Millipore concentrator, urea was added to 5 M final concentration and the mixture was then incubated for 30 min at 37 °C in a water bath. Caf1 was purified to homogeneity using urea-containing 4 % agarose (Olaine) equilibrated with 0.02 M ammonium acetate, pH 8.0. The first peak, which contained Caf1 protein, was desalted on an Ultragel AcA-54 column equilibrated with 0.02 M ammonium acetate, pH 8.0. The homogeneity and purity of Caf1 were checked by SDS/PAGE and by the two-dimensional immunoelectrophoresis technique described by Laurell [16] using commercial poly- and mono-specific antisera (Antiplague Institute 'Microb' Institute, Saratov, Russia) obtained from mice immunized with vaccine strain EV of *Y. pestis* and with purified Caf1 antigen derived from this strain respectively.

Detection of SH groups on Caf1M with biotin–maleimide

Serial dilutions of the protein samples were applied in 1 ml aliquots to pre-cut (2 cm × 3.5 cm) nitrocellulose strips. The blotted proteins were then treated for 1 h with biotin–maleimide (6 mg/ml in PBS) at 20 °C, rinsed with PBS and incubated with 2 % BSA (in PBS) for 1 h, further rinsed with PBS, incubated with avidin–peroxidase complex for 1 h at 37 °C, rinsed well with PBS and incubated with peroxidase substrate solution, as described [17]. For prior reduction of S–S bonds, strips were treated for 1 h with 1 % β-mercaptoethanol and extensively rinsed with PBS prior to treatment with biotin–maleimide and avidin–peroxidase complex as above.

Reduction and alkylation of purified Caf1M

Native, purified Caf1M (2 mg/ml in 20 mM Tris/HCl, pH 7.2) was reduced with 1 % β-mercaptoethanol for 1 h at 20 °C and incubated for 2 h on ice in the presence 300 mM iodoacetamide. The reagents were removed by gel filtration on a PD-10 column in 20 mM phosphate buffer, pH 7.2.

Measurement of dissociation constants of Caf1M binding to Caf1 by ELISA

Binding constant determinations were carried out essentially as described by Murray and Brown [18], with some modifications. Plastic microtitre plates (96-well) were coated with purified Caf1 (2 μg/ml in 20 mM Tris/HCl, pH 7.2) for 18 h at 4 °C. After blocking the remaining plastic sites with 20 mM Tris/HCl, pH 7.2, containing 150 mM NaCl and 0.5 % BSA (blocking buffer), wells were sequentially incubated for 1 h each with 100 μl portions of (i) blocking buffer containing increasing concentrations of intact or reduced and alkylated Caf1M (2–2000 ng/ ml), (ii) rabbit anti-Caf1M serum (1:1000) and (iii) anti-(rabbit IgG)–peroxidase conjugate. Between successive incubations the wells were rinsed five times with blocking buffer. The assays were read on a Titertek® Multiskan (Flow) after incubation with 0.4 mM 3,3′5,5′-tetramethylbenzidine in 100 mM sodium acetate buffer, pH 5.5, containing 0.004 % (w/w) H_2O_2. The concentration of bound antigen was determined by reference to a standard curve, and this value was subtracted from the total concentration of Caf1M added to obtain the concentration of free Caf1M. The K_d value was subsequently obtained by linear regression analysis of bound/free versus bound plots. To determine the concentration of bound antigen, a standard curve was established in wells of the same plate. Decreasing amounts of intact or reduced and alkylated Caf1M were titrated into empty wells of plates by 2-fold serial dilutions and then wells were incubated with blocking buffer, rabbit anti-Caf1M serum and anti-(rabbit IgG) conjugated to peroxidase, as described above. A plot of absorbance value obtained in the original plate against Caf1M concentration (20–1000 ng/ml, which represented the range over which all Caf1M protein was bound) yielded a standard curve which was used to obtain the concentration of bound Caf1M antigen in affinity constant determination assays.

Isolation of periplasmic fractions from *E. coli* JCB570 and JCB571 (*dsbA*::*kan*) strains

Cultures of 10 ml, inoculated at a 1:50 dilution from an overnight culture, were incubated at 37 °C with shaking at 250 rev./min to the mid-exponential phase of growth (A_{600} 0.4–0.5) and then induced for 2 h. Cells harvested from 1.5 ml aliquots were resuspended in 100 μl of 20 % (w/v) sucrose, 20 mM Tris/HCl, pH 8.0, and 5 mM EDTA at 20 °C, incubated for 10 min and recovered by centrifugation at room temperature. This supernatant ('sucrose fraction') occasionally contained low levels of induced protein. The pelleted cells were resuspended in 100 μl of ice-cold 10 mM $MgCl_2$, incubated for 10 min on ice and recovered

by centrifugation ('cells minus periplasm'); the supernatant contained the 'periplasmic fraction'. Cells were resuspended in 100 μl of deionized water and samples (10 μl) were analysed by SDS/PAGE [19].

CD spectroscopy

Spectra were recorded on a J-500A dichrograph (Jasco) with a 1 mm temperature-controlled cell. The concentrations of Caf1M samples were measured on a Shimadzu UV-2100 spectrophotometer using an $A_{280, 1\,cm}^{0.1\%}$ of 1.4 ± 0.2 mg$^{-1} \cdot$cm^{-1}. Samples were studied in 50 mM phosphate buffer, pH 7.2. The program Contin [20] was used for secondary structure computations.

Fluorescence spectroscopy

Spectra were recorded on an MPF-44A spectrofluorimeter (Perkin–Elmer) with a 3 mm temperature-controlled cell.

Molecular modelling

The three-dimensional model of the Caf1M–Caf1 complex was constructed using the molecular modelling software packages SYBYL (Tripos Associates, St. Louis, MO, U.S.A.) on an Evans & Sutherland ESV 30 Workstation and QUANTA (Molecular Simulations, Waltham, MA, U.S.A.) on a Silicon Graphics Iris Crimson Workstation. Corey–Pauling–Koltun models were constructed using the molecular modelling software package Chem-X (Chemical Design, Oxford, U.K.) on a Hewlett–Packard Vectra Workstation.

Other procedures

All DNA manipulations were performed using standard procedures [19]. SDS/PAGE was performed according to the Laemmli procedure using 15 % acrylamide gels under reducing conditions [19]. Gels were stained with Coomassie Brilliant Blue. Molecular mass markers were from Pharmacia (LMW Calibration kit for stained gels). Protein concentrations were determined with the Bio-Rad protein assay kit, using BSA as standard.

RESULTS

Detection of disulphide linkages in Caf1M

The periplasmic molecular chaperone Caf1M of *Y. pestis* contains two cysteine residues per molecule [5], positioned at a distance that would permit formation of a disulphide bond [10]. As free SH groups of cysteine residues, in contrast with disulphide bonds, interact specifically with maleimide conjugates [17], we used blotting with biotin–maleimide to monitor for the presence of free SH groups in the Caf1M molecule. Figure 1(a) shows the direct staining of blotted proteins with biotin–maleimide and avidin–peroxidase complexes and the sensitivity of the system. Of the control proteins, only those with free cysteine residues were labelled; for example, both chymotrypsinogen (the cysteines of which are all involved in disulphide linkages) and Caf1 protein of *Y. pestis* (from which cysteines are absent) remained unlabelled at all concentrations tested, while ovalbumin and BSA, which have been reported to have an average of only 3 and 0.7 free SH groups per molecule respectively [17], were labelled. Under these conditions the native Caf1M protein was not labelled. However, following reduction with 1 % β-mercaptoethanol, Caf1M could be readily labelled using biotin–maleimide (Figure 1b), providing good evidence that the cysteines of the Caf1M protein form a

disulphide linkage. Chymotrypsinogen that was not labelled by direct biotin–maleimide treatment was also labelled following reduction, thereby confirming published data that all five cysteine pairs participate in disulphide linkages [17].

Influence of the disulphide bond on the binding properties of Caf1M

Figure 1(c) shows Scatchard plots of the binding of native and reduced Caf1M to the immobilized Caf1 polymer. The binding of Caf1M with an intact disulphide bond is characterized by a lower K_d value [$(4.77 \pm 0.50) \times 10^{-9}$ M] than is that of the reduced and alkylated Caf1M protein [$(3.2 \pm 0.68) \times 10^{-8}$ M]. The re-oxidation of non-alkylated reduced Caf1M restores the characteristic affinity constant.

Importance of the disulphide bond for Caf1M function *in vivo*

The influence of the disulphide bond on Caf1M function *in vivo* was investigated using *E. coli* JCB571 (*dsbA*::*kan*), a strain that is deficient in the periplasmic disulphide bond isomerase DsbA. Capsule production was dramatically decreased in *E. coli* JCB571 carrying the pFS2 cosmid in comparison with that in the parent strain (*E. coli* JCB570) carrying the same cosmid, as indicated by a much lower rate of agglutination (> 10 min) for JCB571/pFS2 with anti-Caf1 serum compared with the immediate immuno-agglutination of JCB570/pFS2 cells. The lower level of subunit present in *E. coli* JCB571/pFS2 compared with *E. coli* JCB570/pFS2 cells was also evident in SDS/PAGE analyses of whole cells (Figure 2a). Culture supernatant samples (containing Caf1 subunit released into the medium) from *E. coli* JCB571/pFS2 also contained only very low levels of Caf1 subunit compared with similar fractions from *E. coli* JCB570/pFS2 (results not shown).

As the Caf1 subunit contains no cysteine residues [4], an effect on the Caf1M chaperone was the most likely explanation for the observed decrease in subunit assembly. High levels of periplasmic chaperone–subunit complex accumulate in *E. coli* strains expressing only Caf1M and Caf1 (but no outer-membrane usher Caf1A) from the plasmid pFM1 [14] (Figure 2c, lane 1). When the Caf1 subunit was produced alone, i.e. in the absence of Caf1M chaperone, in *E. coli* JCB570/pFM1S, it was evidently degraded. Periplasmic levels of subunit in this strain were less than 5 % of those in *E. coli* JCB570/pFM1 (Figure 2b). The subunit was also rapidly degraded in *E. coli* JCB571 (*dsbA*::*kan*) carrying plasmid pFM1, which encodes both Caf1 subunit and chaperone (Figure 2c; compare lanes 2, 4 and 6 with lanes 1, 3 and 5 respectively). This can be attributed to the absence of functional Caf1M chaperone. The structure of Caf1M was evidently affected in the *dsbA* mutant, resulting in degradation of the chaperone itself as well as of the Caf1 subunit.

Influence of the disulphide bond on the conformational properties of the Caf1M molecule

CD measurements

Figure 3(a) shows a comparison of the CD spectrum of the intact Caf1M chaperone with the spectra of the Fab and pFc′ fragments of human myeloma IgG1 and of the murine small heat-shock protein of 25 kDa (Hsp25). The CD spectra reveal a high content of β-structure and a very low content of α-helix for all proteins analysed (Table 1), in accordance with the X-ray analysis data for immunoglobulins [21], with the PapD-like model of the three-dimensional structure of Caf1M [10], and with the predicted immunoglobulin-like fold of small heat-shock proteins [22]. However, there are some significant differences. For example, the
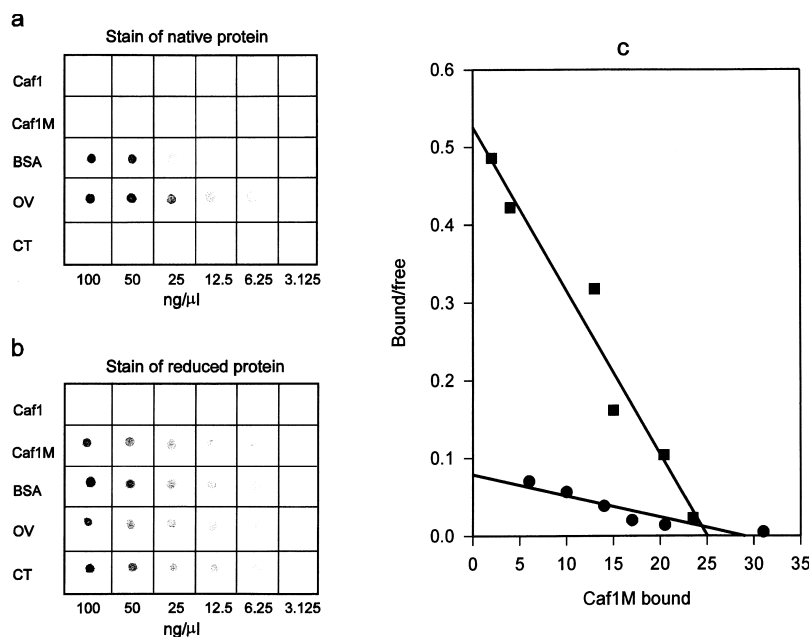
Figure 1   Selective staining of protein thiols and disulphides using biotin–maleimide (a, b), and Scatchard analysis of the binding of Caf1M to immobilized Caf1 (c)

(**a**) Direct staining of the intact blotted proteins Caf1, Caf1M, BSA, ovalbumin (OV) and chymotrypsinogen (CT) at the concentrations indicated. (**b**) Staining of reduced proteins. (**c**) ■, Intact Caf1M; ●, reduced and alkylated Caf1M. The amount of Caf M1 bound (*x* axis) is given in units of $10^{10} \times$ M.
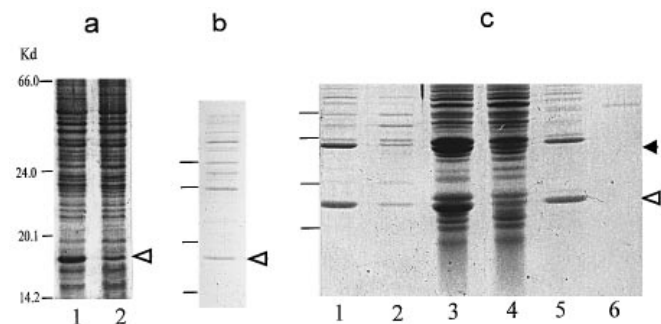

Figure 2   SDS/PAGE analysis of Caf1 in *E. coli dsbA::kan*

(**a**) Whole cells from exponentially growing cultures of *E. coli* JCB570 (lane 1) and JCB571 *dsbA::kan* (lane 2) carrying cosmid pFS2 (Kd = kDa). (**b**) Periplasmic fraction of *E. coli* JCB570 carrying plasmid pFM1S. Very little subunit was detected in the sucrose fraction or in shocked cells from this strain. (**c**) Periplasmic fractions (lanes 1 and 2), cells minus periplasm (lanes 3 and 4) and sucrose fraction (lanes 5 and 6) from *E. coli* JCB570 (lanes 1, 3 and 5) and JCB571 *dsbA::kan* (lanes 2, 4 and 6) carrying plasmid pFM1. Closed arrowhead, Caf1M chaperone; open arrowhead, Caf1 subunit (identified by immunoblotting).

spectrum of Caf1M has a minimum of ellipticity at 210–212 nm and a maximum at 228 nm, whereas that of murine Hsp25 has a minimum of ellipticity at 217 nm and no maxima within the range observed. The most obvious explanation for such differences in the region from 225 to 235 nm of the CD spectra of highly β-structured proteins might be the different contributions of -SH and -S–S- groups in an asymmetrical environment. Following reduction of the disulphide bond and alkylation of the cysteine residues, the CD spectrum of Caf1M was changed at the maximum of ellipticity at 228 nm as well as at the minimum of ellipticity at 210–212 nm (Figure 3b). The latter changes probably

reflect perturbation of the peptide bonds in the β-structural segments nearest the disulphide bridge. The CD spectra of intact and of reduced and alkylated Caf1M in the near-UV region have practically the same amplitude, with a small change at 308 nm in the spectrum of the reduced chaperone (Figure 4). This latter change is characteristic of a specific tryptophan interaction, while the absence of a general decrease in spectral intensity indicates no changes in the dynamics or loosening of packing of the molecules. The change in the spectra at the lower wavelength is probably associated with the change in the sulphur chromophore on reduction.

The effects of different temperatures on the CD spectrum of Caf1M are shown in Figure 5(a). The spectrum did not change significantly within the range 20–55 °C, but in the range 55–70 °C a highly co-operative transition with a transition temperature of 63.5 ± 1 °C was detected (Figure 5b). Thus, according to the CD data, heat denaturation of Caf1M takes place between 55 and 70 °C. The melting of reduced and alkylated Caf1M was characterized by practically the same transition temperature (63 ± 1 °C) (Figure 5b, curve 2), indicating the absence of significant changes in the overall structure of the Caf1M molecule. The amplitude of the change in ellipticity at 228 nm in the case of reduced and alkylated Caf1M, however, was lower. This may be due to different sensitivities of -SH and -S–S- groups to the changes in asymmetry of the microenvironment on thermal denaturation.

Fluorescence measurements

Figure 6 shows the temperature-dependence of emission at 338 nm, which indicates the state of tryptophan residues in the intact and the reduced and alkylated Caf1M molecules. In the range 25–60 °C, both samples are characterized by a linear temperature-dependence of tryptophan emission, typical of a solution of free tryptophan or of exposed tryptophan residues
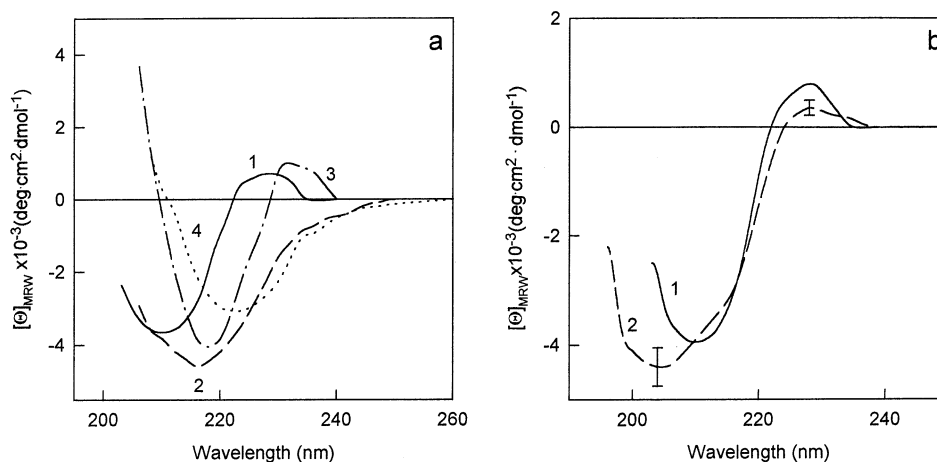
Figure 3   CD spectra of Caf1M

(**a**) Comparison of the CD spectra of Caf1M (curve 1), murine Hsp25 (curve 2), and the Fab (curve 3) and pFc′ (curve 4) fragments of human IgG1. (**b**) Comparison of the CD spectra of the intact (curve 1) and reduced and alkylated (curve 2) Caf1M protein.

Table 1   Estimation of secondary structure from CD spectra

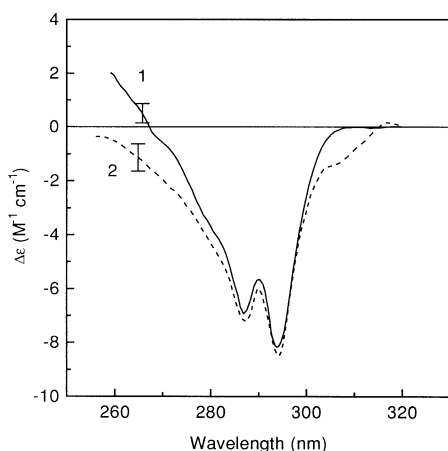| Protein	Content (%)		
	α-Helix	β-Sheet	Remainder
Caf1M	3	50	47
Hsp25	8	41	51
Fab	0	82	18
pFc′	4	36	60

Figure 4   Comparison of the CD spectra in the near-UV region of intact (curve 1) and reduced and alkylated (curve 2) Caf1M protein

[23]. In the case of reduced and alkylated Caf1M, this dependence is higher. Evidence from thermal unfolding suggests no change in enthalpy on reduction of the protein (Figure 5). Therefore, in agreement with the near-UV CD spectra (Figure 4), the observed changes in tryptophan fluorescence are likely to be very local, and might be interpreted in terms of changes in the micro-environment of Trp-99 due to rotation of the Cys-101 side chain on reduction of Caf1M (see Figure 7b). In the range 60–70 °C,

the linear temperature-dependence of tryptophan emission is disturbed as a result of heat denaturation [the temperature dependence of $\mathrm{dRF}/\mathrm{d}T$ (where RF is relative fluorescence) would be very similar to the temperature-dependence of ellipticity shown in Figure 5b]. In the denatured state, the temperature-dependence of the tryptophan emission of both samples is practically the same, and higher than that in the native state.

Molecular modelling of the Caf1M–Caf1 complex

The steric structure of Caf1M has been constructed, based on a statistically significant primary structural similarity between Caf1M and PapD protein, using the known atomic co-ordinates obtained by X-ray crystallography of PapD [10]. In the Caf1M structure, Cys-101 in the F1 β-strand and Cys-140 in the G1 β-strand are exposed to solvent and are located at a distance compatible with formation of a disulphide bond, as experimentally proven above. The additional sequence located between these two cysteine residues, in the model, forms two additional β-strands (in comparison with PapD) at one edge of the β-bilayer structure of the N-terminal domain. This additional sequence does not conceal the invariant Arg-23 and Lys-142 residues (numbering in the Caf1M sequence) from contact with the solvent and other molecules. The steric structure of the envelope Caf1 protein has also been reconstructed [24], taking into account structural similarities between Caf1 and interleukins-1α, -β and -ra, and by using the known atomic co-ordinates for human interleukin-1β obtained by X-ray crystallography. In support of this, it has been demonstrated [14] that human interleukin-1β and Caf1 compete for a common or overlapping binding site on the Caf1A outer-membrane molecular usher protein [25]. Site-directed mutagenesis has been used to demonstrate that the invariant residues Arg-8 and Lys-112 in the PapD sequence (corresponding to Arg-23 and Lys-142 in Caf1M) are essential for the interactions between PapD and the subunits of surface structures [26]. From the crystal data of a PapD–PapG C-terminal fragment complex, it was shown that Arg-8 and Lys-112 in PapD form hydrogen bonds with the C-terminus of the PapG subunit, which then extends along the G1 β-strand towards the F1–G1 loop [26]. Identity between the C-terminal sequences of PapG and Caf1 subunits has been noted [11,26].
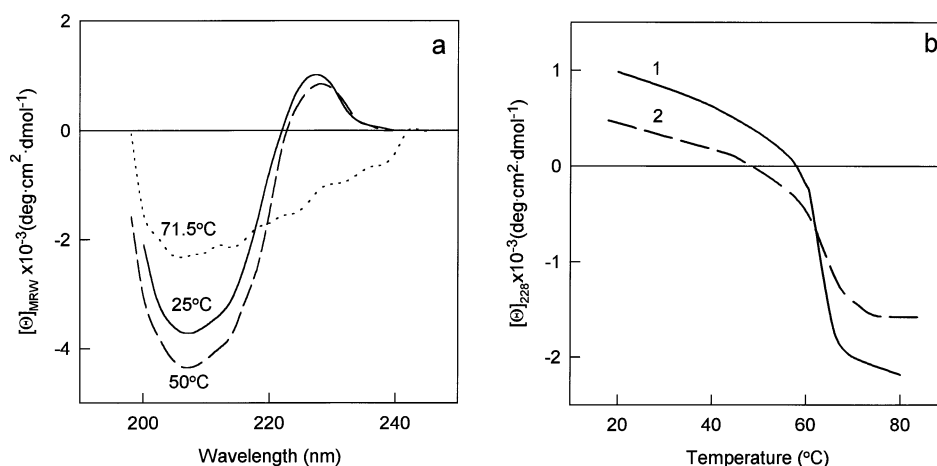
Figure 5   Temperature-dependence of the Caf1M protein CD spectrum

(**a**) CD spectra of intact Caf1M protein at different temperatures. (**b**) Temperature-dependence of ellipticity at 228 nm for the intact (curve 1) and reduced and alkylated (curve 2) Caf1M protein.
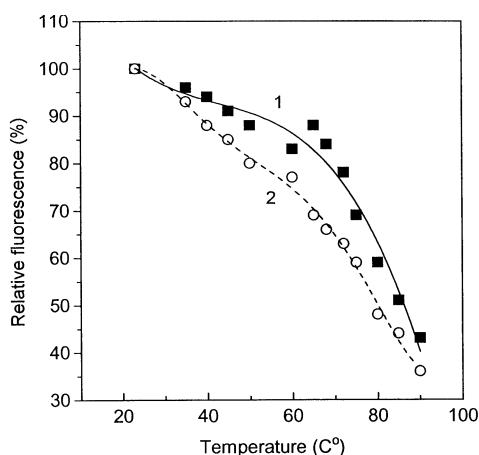

Figure 6   Temperature-dependence of relative fluorescence at 338 nm for the intact (○) and reduced and alkylated (■) Caf1M protein

The samples were studied in 50 mM phosphate buffer, pH 7.2.

Based on these data, we have attempted to reconstruct the Caf1M–Caf1 complex. The C-terminal group (Gln-149) of Caf1 was positioned to permit hydrogen-bond formation with Arg-23 and Lys-142 of Caf1M. The C-terminal sequence of Caf1 was then extended along the G1 β-strand of Caf1M towards the F1–G1 loop. The resulting steric structure of the Caf1M–Caf1 complex obtained is shown in Figure 7 (upper panel). In this reconstruction of the complex, the disulphide bond between Cys-101 and Cys-140 is located very close to Gln-149 of Caf1, which forms hydrogen bonds with the invariant Arg-23 and Lys-142 of Caf1M. Only part of the additional sequence forming the proposed FG''1 β-strand of Caf1M [10] is in contact with the Caf1 subunit, whereas other parts are exposed to solvent. The N-terminal sequence of the Caf1 subunit in the complex interacts with the C-terminal domain of Caf1M.

DISCUSSION

Possession of a conserved pair of cysteine residues close to the proposed binding site is a characteristic property of a relatively

large subfamily of specific periplasmic chaperones, including *Y. pestis* Caf1M [10,11]. The *in vitro* data presented here demonstrate that the two cysteine residues do indeed form a disulphide bond in native Caf1M, and that there is a valid influence of this disulphide bond on the binding of the capsular subunit Caf1 by the Caf1M molecular chaperone, although the change in K_d is not dramatic [from $(4.77 \pm 0.50) \times 10^{-9}$ M for the intact protein to $(3.68 \pm 0.68) \times 10^{-8}$ M for the modified protein]. In accordance with the CD and fluorescence measurements, the disulphide bond is not important for maintenance of the overall structure of the Caf1M molecule, but does affect the orchestration of the fine structure of the binding site. Most importantly, Cys-140 in the Caf1M sequence lies within the extrapolated G1 β-strand and adjacent to the invariant residue Lys-142 which, by analogy with the PapD–PapG peptide complex [26], would be involved in β-zipper binding and anchoring of the C-terminal group of the Caf1 subunit respectively (for details, see Figure 7). Support for a β-zipper mechanism of subunit binding of Caf1 by Caf1M was recently obtained by Hung and co-workers [11], who demonstrated binding to PapD of a synthetic peptide (H$_2$N-AGKYTDAVTVTVSNQ-CO$_2$H) corresponding to the C-terminus of Caf1.

Unlike most of the specific periplasmic chaperones, PapD contains a disulphide bond far from the binding site and close to the C-terminus of the protein. In contrast with the studies described here with Caf1M, no effect on binding of the pilin subunit was observed following *in vitro* reduction of the PapD disulphide bond [12]. Jacob-Dubuisson and co-workers [12], however, found that the PapD chaperone was unable to fold into a native conformation capable of binding pilin subunits *in vivo* in the absence of the disulphide bond isomerase DsbA, and concluded that DsbA functions as a chaperone prior to catalysing the formation of a disulphide bond. The disulphide bond in Caf1M has only a localized effect on the structure of the folded protein (see above); however, in a strain lacking DsbA, only low levels of Caf1M were detected. A dual chaperone/oxidant function of DsbA would readily explain this loss of Caf1M in *E. coli* JCB571 (*dsbA::kan*), as a misfolded or longer-lived folding intermediate would be likely to be more susceptible to degradation by periplasmic proteases. Thus, in the case of Caf1M, DsbA can control not only folding of this periplasmic protein through its chaperone-like activity but also the binding properties of Caf1M through its oxidative activity (Scheme 1).
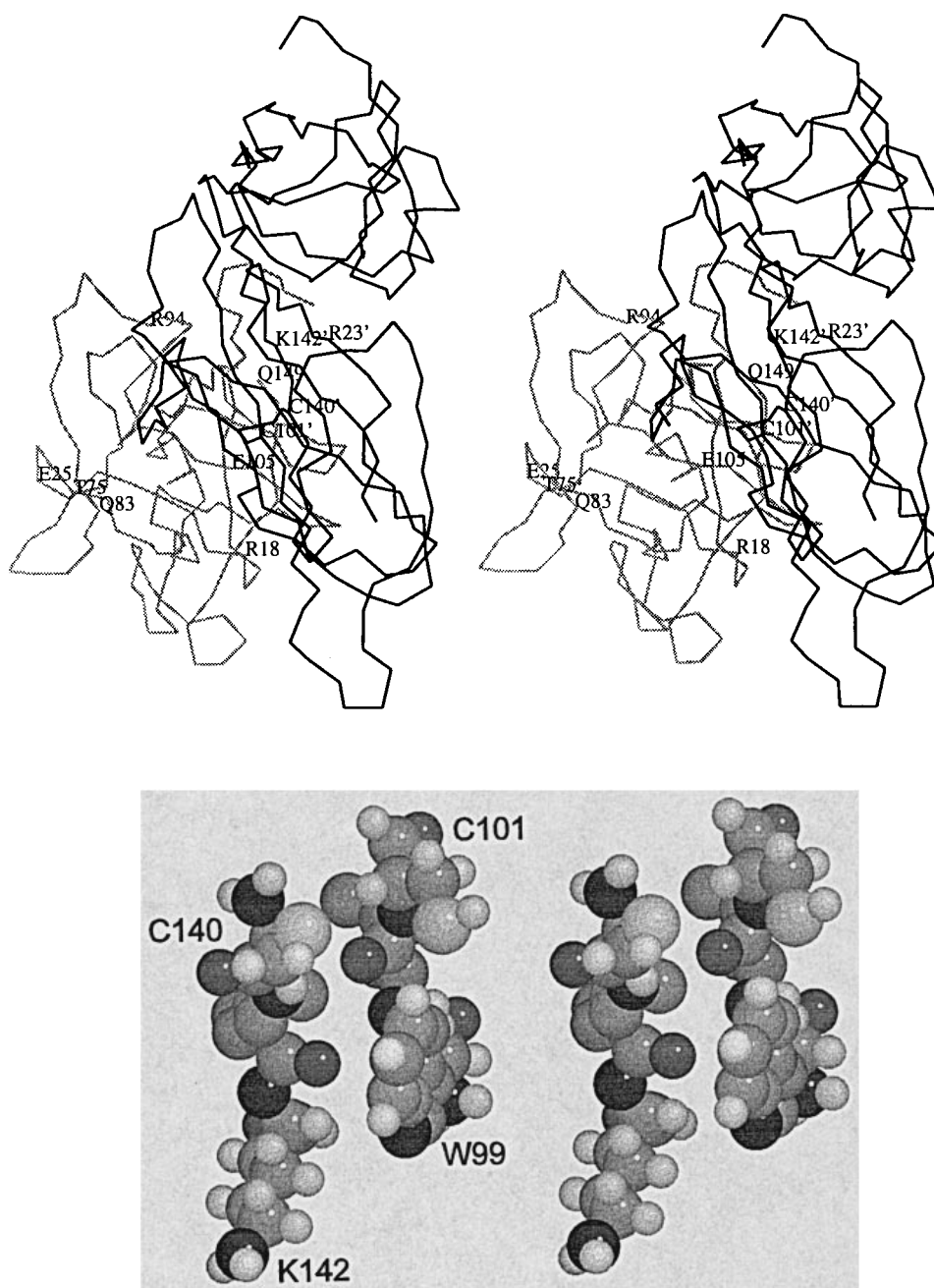
Figure 7   (a) Stereo view of the three-dimensional structure of the Caf1M–Caf1 complex, and (b) Corey–Pauling–Koltun model of part of the putative binding pocket of Caf1M

(**a**) The backbone of the Caf1M molecular chaperone is shown by the black line. In the structure of Caf1M, the positions of conserved Cys-101′ and Cys140′ are shown, as well as those of the invariant residues Arg-23′ and Lys-142′. In the structure of Caf1 the position of the C-terminal residue Gln-149 is shown, as well as those of Arg-18, Glu-25, Arg-94 and Glu-105. In addition, the positions of Thr-75 and Gln-83 are shown in the structure of Caf1, indicating the main B-cell epitope accessible to antibodies in polymeric Caf1 [24]. In (**b**), the side chains of Cys-101, Cys-140, Trp-99 and Lys-142 are shown.

It is quite probable that the conserved cysteine residues in the other members of the Caf1M-like subfamily of chaperones similarly form a disulphide bond that is important for the fine structure of the binding site. In each of these chaperones, the disulphide bond would then encompass a poorly conserved sequence which is generally longer than the same region in the rest of the superfamily of chaperones (10–18 amino acids longer

than this region in PapD) [10]. Since in the model of Caf1M this 'accessory' sequence is proximal to the binding site, current investigations are addressing the significance of this region for the affinity and specificity of binding of the Caf1M-like subfamily of chaperones. The results from CD and fluorescence spectroscopy suggest that the role of the disulphide bond is not simply to stabilize this longer loop. In view of the decreased
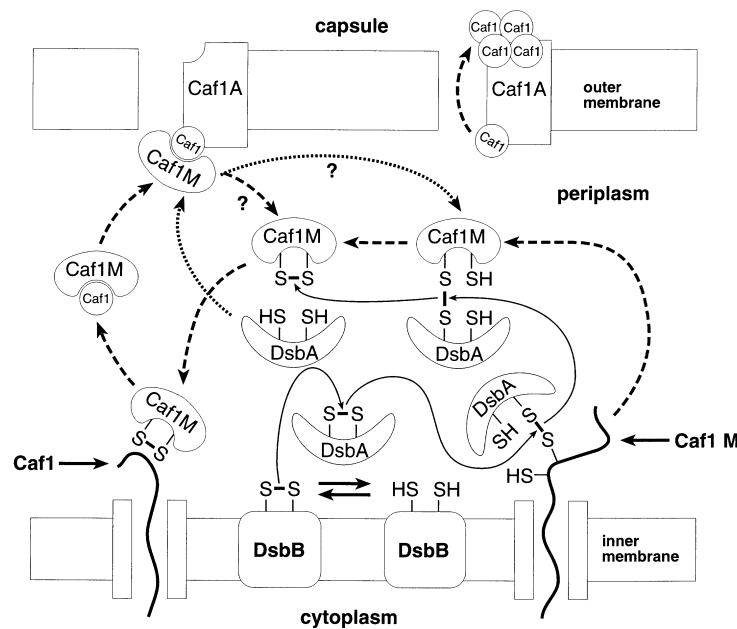
Scheme 1   Role of Dsb proteins in *Y. pestis* F1 capsule formation in *E. coli*

The direction of disulphide transfer is shown by the solid arrows, and changes in Caf1M by the dashed arrows. The molecular chaperone Caf1M and the capsular subunit Caf1 are secreted into the periplasm in an unfolded reduced state. The DsbA protein binds the unfolded chain of Caf1M, and then rapidly transfers its disulphide to the folding protein. Reduced DsbA is re-oxidized by DsbB, an integral membrane protein [12]. The folded and oxidized Caf1M binds the unfolded chain of Caf1, helping in the folding and preventing non-productive aggregation in the periplasm. The Caf1–Caf1M complex interacts with the outer-membrane usher Caf1A. Caf1M is released as the Caf1 subunits assemble at the cell surface to form the F1 capsule. The stimulation for the dissociation of Caf1M is unknown, but one hypothesis involves the reduction of Caf1M by, for example, DsbA or DsbC (as indicated by dotted arrow), with formation of a competitive Caf1M–DsbA(C) complex, resulting in the release of the reduced form of Caf1M which could then re-enter the cycle following re-oxidation.

affinity of reduced Caf1M for the Caf1 subunit and the proximity of the disulphide bond to the binding site, another, more hypothetical but nonetheless intriguing, possible role of the disulphide bond may be envisaged in a reduction/re-oxidation cycle relating to release of the Caf1 subunit on interaction with the outer-membrane usher protein (Scheme 1). Should such a cycle occur, one has to address the question of the identity of the agent involved in the reduction of Caf1M and the role (if any) of the DsbA, DsbB and DsbC [27] proteins in this cycle, as well as mechanistic differences with regard to Caf1M and PapD in the assembly of the *Y. pestis* capsule and Pap pili respectively.

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