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Crystallization of a pentapeptide-repeat protein by reductive cyclic pentylation of free amines with glutaraldehyde

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The pentapeptide-repeat protein *Efs*Qnr from *Enterococcus faecalis* protects DNA gyrase from inhibition by fluoroquinolones. *Efs*Qnr was cloned and purified to homogeneity, but failed to produce diffraction-quality crystals in initial crystallization screens. Treatment of *Efs*Qnr with glutaraldehyde and the strong reducing agent borane–dimethylamine resulted in a derivatized protein which produced crystals that diffracted to 1.6 Å resolution; their structure was subsequently determined by single-wavelength anomalous dispersion. Analysis of the derivatized protein using Fourier transform ion cyclotron resonance mass spectrometry indicated a mass increase of 68 Da per free amino group. Electron-density maps about a limited number of structurally ordered lysines indicated that the modification was a cyclic pentylation of free amines, producing piperidine groups.

1. Introduction

Crystallization remains a major bottleneck in the determination of the structure of proteins by X-ray methods. A number of methods are increasingly being utilized to increase the probability that a given protein will form diffraction-quality crystals (see Derewenda, 2004*b*, and references therein). Surface-exposed residues play an inordinately large role in the formation of a stable crystal lattice, as these are the residues which are most accessible for the formation of intermolecular contacts (McElroy *et al.*, 1992; D'Arcy *et al.*, 1999). Since surface residues are typically the least conserved residues amongst orthologous proteins, a highly successful first approach is to attempt the crystallization of species variants of the protein of interest (Kendrew *et al.*, 1954; Campbell *et al.*, 1972). This essentially shuffles the properties of the entire surface, resulting in a fresh screening of crystallization space without altering the general information held in the core structures of the protein. Secondly, the molecular constructs can be altered by, for example, changing the type or position of affinity tags or removing flexible regions of the structure (Gao *et al.*, 2005; Carson *et al.*, 2007; Graslund *et al.*, 2008). However, even then proteins may remain recalcitrant to crystallization and/or one may wish not to change species or constructs if a large body of biophysical work has already been amassed on a particular protein construct. The mutation of surface residues with high conformational entropy (Lys, Glu, Asn) to alanines has been found to increase the likelihood of crystallization (Longenecker *et al.*, 2001; Czepas *et al.*, 2004; Derewenda, 2004*a*; Derewenda & Vekilov, 2006). Although these mutations can have numerous biophysical effects (*e.g.* altering solubility and surface charge), it is often subsequently found

that these residues participate directly or indirectly in crystal contacts. For proteins with an unknown structure, the surface-entropy reduction prediction (*SERp*) server can be utilized to suggest high-entropy residues which are in close proximity in the sequence and are likely to be on the surface (Goldschmidt *et al.*, 2007). Finally, one may want a quicker and more direct method of altering the surface properties of a particular protein construct or several variants produced by previously mentioned methods. The reductive dimethylation of free amino groups (lysines, NH_2-termini) with formaldehyde and strong reducing agents (sodium borohydride or borane–dimethylamine) has been successfully used to crystallize recalcitrant proteins (Rypniewski *et al.*, 1993; Kobayashi *et al.*, 1999; Kurinov & Uckun, 2003; Schubot & Waugh, 2004; Walter *et al.*, 2006; Rauert *et al.*, 2007; Shaw *et al.*, 2007; Kim *et al.*, 2008). Through the modification of the high surface-entropy residue lysine, reductive dimethylation of a protein can significantly alter the surface properties at several sites, which can result in novel crystal contacts and/or change the crystallization kinetics.

The chromosomally encoded *Enterococcus faecalis* protein *Efs*Qnr has been demonstrated to provide intrinsic resistance to fluoroquinolones (Arsene & Leclercq, 2007), presumably through its interaction with DNA gyrase. *Efs*Qnr belongs to a family of proteins termed pentapeptide-repeat proteins (PRPs), the repeating signature sequence $(STAV)_1(D,N)_2(L,F)_3(STR)_4(G)_5$ of which folds into a right-handed quadrilateral parallel β-helix (Hegde *et al.*, 2005; Buchko *et al.*, 2006; Vetting *et al.*, 2006). Here, we demonstrate a novel modification of free protein amines by treatment with glutaraldehyde in a strongly reducing environment. This modification facilitated the crystallization and structure determination of *Efs*Qnr and may be broadly applicable to other proteins that resist crystallization.

2. Methods

2.1. Cloning and purification

Details of the cloning and purification of *Efs*Qnr will be described elsewhere.

Briefly, the gene for *Efs*Qnr (NCBI GeneID 1199794) was PCR-amplified from genomic DNA and ligated into the overexpression vector pET28a using *Nde*I and *Xho*I restriction sites, yielding a protein with an N-terminal thrombin-cleavable

Table 1
Data-collection and refinement statistics.

Values in parentheses are for the outer shell.

	*Efs*Qnr (modified)	Sm acetate
Space group	$P2_1$	$P2_1$
Unit-cell parameters (Å, °)	$a = 36.9$, $b = 63.1$, $c = 94.6$, $\beta = 96.1$	$a = 36.6$, $b = 65.5$, $c = 94.1$, $\beta = 97.5$
Resolution (Å)	20–1.6 (1.69–1.6)	38–2.0 (2.11–2.0)
Completeness (%)	94.7 (86.0)	100 (100)
Redundancy	2.9 (2.7)	7.5 (3.9)
Mean $(I)/\sigma(I)$	19.3 (3.1)	20.3 (4.1)
R_{merge}†	0.043 (0.253)	0.091 (0.322)
Wilson B factor (Å²)	22.4	15.4
Model and refinement data		
Resolution (Å)	20–1.6 (1.64–1.6)	
Unique reflections	51172 (3093)	
R_{cryst}‡ (%)	18.7 (36.0)	
R_{free}‡ (5% of data) (%)	22.1 (44.9)	
Contents of model		
Residues (1–211 + tag)	*A*1–*A*8, *A*13–*A*211, *B*4–*B*211	
Waters	417	
Other	4 Cl	
Total atoms	3910	
Average B factor (Å²)		
Protein	13.6	
Waters	29.7	
R.m.s.d.		
Bond lengths (Å)	0.014	
Angles (°)	1.59	
MOLPROBITY statistics		
Ramachandran favored/outliers (%)	98.2/0.0	
Rotamer outliers (%)	1.1	
Clashscore§	7.2 [85th percentile, $N = 718$, 1.35–1.85 Å]	
Overall score§	1.4 [92nd percentile, $N = 7200$, 1.35–1.85 Å]	

† $R_{merge} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where $I_i(hkl)$ is the intensity measurement for reflection i and $\langle I(hkl)\rangle$ is the mean intensity for multiply recorded reflections. ‡ R_{work}, $R_{free} = \sum_{hkl} ||F_{obs}| - |F_{calc}|| / \sum_{hkl} |F_{obs}|$, where the working and free R factors are calculated using working and free reflections, respectively. § As calculated using the *MOLPROBITY* server; N is the number of sample structures in the resolution range.

hexahistidine tag (tag sequence MGSSHHHHHHSSGLVP-RGSH-Met1). *Efs*Qnr was expressed in Mueller–Hinton broth at 298 K and induced overnight with 0.5 m*M* IPTG after the cells reached an A_{600} of 0.6. Recombinant *Efs*Qnr was purified to homogeneity by Ni–NTA chromatography followed by size-exclusion chromatography on a Superdex S75 column and the N-terminal His tag was cleaved using thrombin, leaving the native protein with an additional three amino acids at the N-terminus (GSH-Met1). *Efs*Qnr was concentrated by ultracentrifugation to 20–50 mg ml^{-1} in 20 m*M* Tris pH 8.0, 200 m*M* NaCl and stored as a 50%(*v*/*v*) glycerol stock at 253 K.

2.2. Treatment with glutaraldehyde

Reductive cyclic pentylation of free amines is based on the protocols for reductive dimethylation utilizing formaldehyde and a strong reducing agent (Rayment, 1997). *Efs*Qnr was extensively dialyzed against 50 m*M* HEPES pH 7.5. All experiments were carried out with *Efs*Qnr at a concentration of 10 mg ml^{-1} (3.2 m*M* free amine) and were incubated on a rocking platform shaker at 277 K in the dark. Stock solutions of 1 *M* borane–dimethylamine (DMAB; Sigma 180238) and

0.5 *M* glutaraldehyde [diluted from 25%(*w*/*v*) stock; Sigma G-5882] were freshly prepared. 20 µl DMAB was added per millilitre of protein solution followed by addition of 40 µl glutaraldehyde per millilitre of protein solution. This was incubated for 2 h prior to the addition of another aliquot of DMAB (20 µl per millilitre) and glutaraldehyde (40 µl per millilitre). After an additional 2 h incubation period, a final aliquot of DMAB (10 µl per millilitre) was added prior to incubation overnight (~16 h). The final concentrations of DMAB and glutaraldehyde were 50 and 40 m*M*, respectively. The reductive cyclic pentylation of lysozyme was performed in a similar manner using lysozyme (Sigma L6876) at 10 mg ml^{-1} (5 m*M* free amine).

Treated samples were diluted 1:10 in buffer *A* (100 m*M* HEPES, 1 *M* ammonium sulfate, 1 m*M* DTT, 1 m*M* EDTA pH 7.5), applied onto a Phenyl-Sepharose CL-4B (1.5 × 15 cm; GE Healthcare) column equilibrated with buffer *A* and eluted with a linear gradient from buffer *A* to buffer *B* (100 m*M* HEPES, 1 m*M* DTT, 1 m*M* EDTA pH 7.5) over ten column volumes. Peak fractions were pooled and dialyzed for 12 h against buffer *C* (15 m*M* HEPES, 1 m*M* DTT, 1 m*M* EDTA pH 7.5). Samples were concentrated to 10–20 mg ml^{-1} by Amicon filtration and snap-frozen in liquid nitrogen for storage at 193 K.

2.3. Crystallization

*Efs*Qnr was crystallized by vapor diffusion under oil in 96-well round-bottom plates (Costar-3795). Equal volumes of *Efs*Qnr (10 mg ml^{-1} in buffer *C* plus 400 m*M* ammonium sulfate) and precipitant [100 m*M* MES pH 6.0, 5–15%(*w*/*v*) PEG 6000, 1 *M* LiCl] were combined under 150 µl silicon oil (Fisher Scientific). Plates were stored at 277 K open to room humidity. Initial crystals, which appeared after significant incubation (1–2 months), were crushed and used as micro-seeds to streak-seed crystallization conditions identical to the initial screen (Stura & Wilson, 1991). Streak-seeded drops produced large thick plates that grew to maximum size (0.25 × 0.25 × 0.05 mm) in approximately 2–7 d. Crystals were incubated (30 s) in a solution of 100 m*M* MES pH 6.0, 15%(*w*/*v*)

PEG 6000, 1.5 *M* LiCl prior to being mounted on 0.2 mm LithoLoops (Molecular Dimensions Ltd) and undergoing vitrification by plunging into liquid nitrogen. Diffraction data were collected at 100 K on a Rigaku RU-H3R Cu *Kα* rotating-anode generator (50 kV, 100 mA) equipped with an R-AXIS IV^{++} detector (Rigaku/MSC) and Osmic Blue confocal mirrors. Data were processed and scaled using *MOSFLM* (Leslie, 2006) and *SCALA* (Evans, 2006).

2.4. Structure determination

The structure of *Efs*Qnr was determined by single-wavelength anomalous dispersion (SAD). A single crystal of *Efs*Qnr was soaked for 120 s at 277 K in a cryosolution doped with 100 m*M* samarium acetate. Heavy-atom substructure determination and phasing (overall SAD figure of merit = 0.27) were performed using *PHENIX* (Adams *et al.*, 2004). The automated fitting program *ARP/wARP* (Perrakis *et al.*, 1997) was utilized to produce an initial model starting with the solvent-flattened SAD phases. This model was sufficient to place the structure of MfpA, a related pentapeptide-repeat protein, into the data, which guided further model building. The final model was built by iterative cycles of model building in *Coot* (Emsley & Cowtan, 2004) and positional and restrained individual *B*-factor refinement in *REFMAC* (Murshudov *et al.*, 1997). The molecular-replacement program *AMoRe* (Navaza, 1994) and a partially built *Efs*Qnr model were used to phase a high-resolution data set. Waters were added at sites exhibiting peaks in $F_o - F_c$ (>3.5σ) and $2F_o - F_c$ (>1.0σ) maps if at least one hydrogen-bonding partner was present. There are two *Efs*Qnr molecules per asymmetric unit, yielding a solvent content of 48.5%. Noncrystallographic symmetry restraints were not utilized during refinement owing to the high resolution of the data. Translation/libration/screw (TLS) refinement was used in the last steps of refinement (Winn *et al.*, 2001). A total of four TLS groups were used per chain and the groups were determined by submission of the PDB file to the *TLSMD* server (Painter & Merritt, 2006). Model quality was checked using the *MOLPROBITY* web server (Davis *et al.*, 2004). Data-collection and model statistics
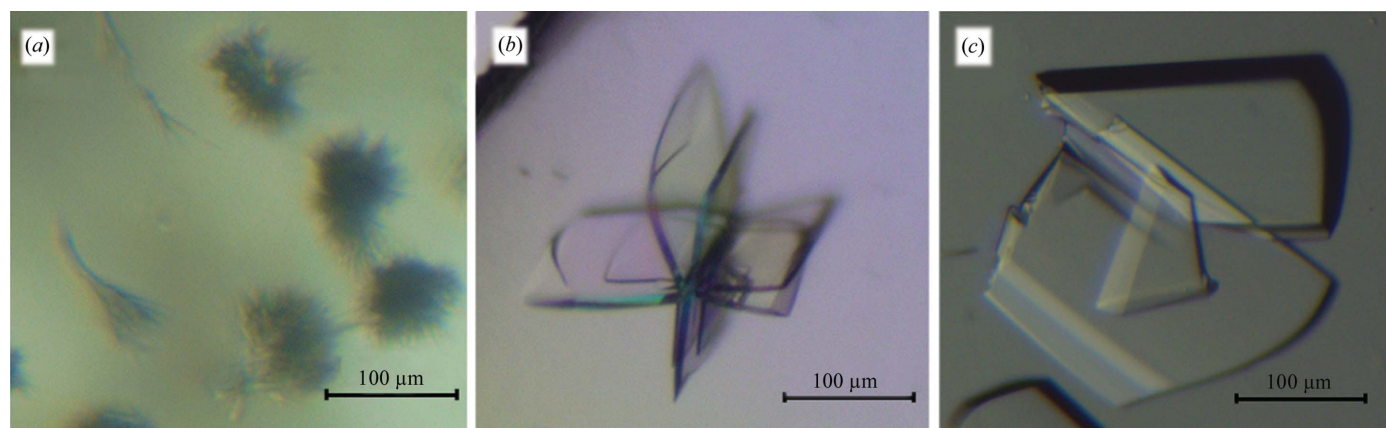

Figure 1
Crystals of *Efs*Qnr. (*a*) Crystals of unmodified *Efs*Qnr. Crystals of reductively pentylated *Efs*Qnr before (*b*) and after (*c*) crystal seeding.

are shown in Table 1. OMIT maps were utilized to check for crystallographic evidence of cyclic pentylation. All lysines were exchanged for serines and all atoms underwent randomized deviations, yielding an r.m.s.d. of 0.4 Å for the entire model to the parent structure. The omitted and shaken model underwent positional and constrained B-factor refinement prior to synthesis of OMIT maps.

2.5. Mass spectrometry

All mass spectra were acquired on a 9.4 T Fourier transform ion cyclotron resonance mass spectrometer (Ionspec, Lake Forest, California, USA). The protein was directly injected onto a C18 column at a rate of 10 µl min^{-1} in 50% acetonitrile containing 0.1% TFA.

3. Results

3.1. Crystallization of unmodified EfsQnr

The pentapeptide-repeat protein *Efs*Qnr (211 amino acids) was purified to homogeneity utilizing Ni–NTA chromatography. *Efs*Qnr was screened for crystallization utilizing approximately 600 solution conditions from various commercially available matrices (*i.e.* Hampton Research Crystal Screens I and II, PEG/Ion Screen *etc.*). Most of the conditions produced clear drops or precipitate, with only 2% of the conditions producing crystalline material. Over a period of 1–2 months, spherulite-shaped crystals appeared in drops containing medium-molecular-weight PEGs (2000–8000) in the pH range 5–7 (Fig. 1a). These crystals could not be improved through seeding and were not large enough for determination of their space group or diffraction quality.

3.2. Reductive pentylation

In an attempt to alter the crystallizability of *Efs*Qnr, its lysine side chains were modified by chemical modification with glutaraldehyde under reducing conditions. *Efs*Qnr was incrementally treated with glutaraldehyde in the presence of the strong reducing agent borane–dimethylamine (DMAB) over a period of 16 h. The reaction was quenched with 1 *M* ammonium sulfate and the protein was separated from free glutaraldehyde and DMAB by hydrophobic interaction chromatography (HIC). Hen egg-white lysozyme was treated using the same procedure in order to evaluate the universality of the technique. Neither *Efs*Qnr nor lysozyme produced any precipitation at the protein concentrations used (10 mg ml^{-1}) and protein recoveries were >80%, with no extraneous peaks in the HIC purification. SDS–PAGE of the proteins after modification suggested little or no intermolecular cross-linking (Fig. 2a). Analysis by Fourier transform ion cyclotron resonance mass spectrometry (FTICR-MS) was used to determine the molecular weights of treated and untreated samples (Figs. 2b–2e). Native *Efs*Qnr was determined to have a molecular mass of 24 533.0 Da (expected 24 518.1 Da). The cause of the +15 mass difference is not known, but it does not affect the final analysis of untreated and treated *Efs*Qnr. Glutaraldehyde/DMAB-treated *Efs*Qnr exhibited a single
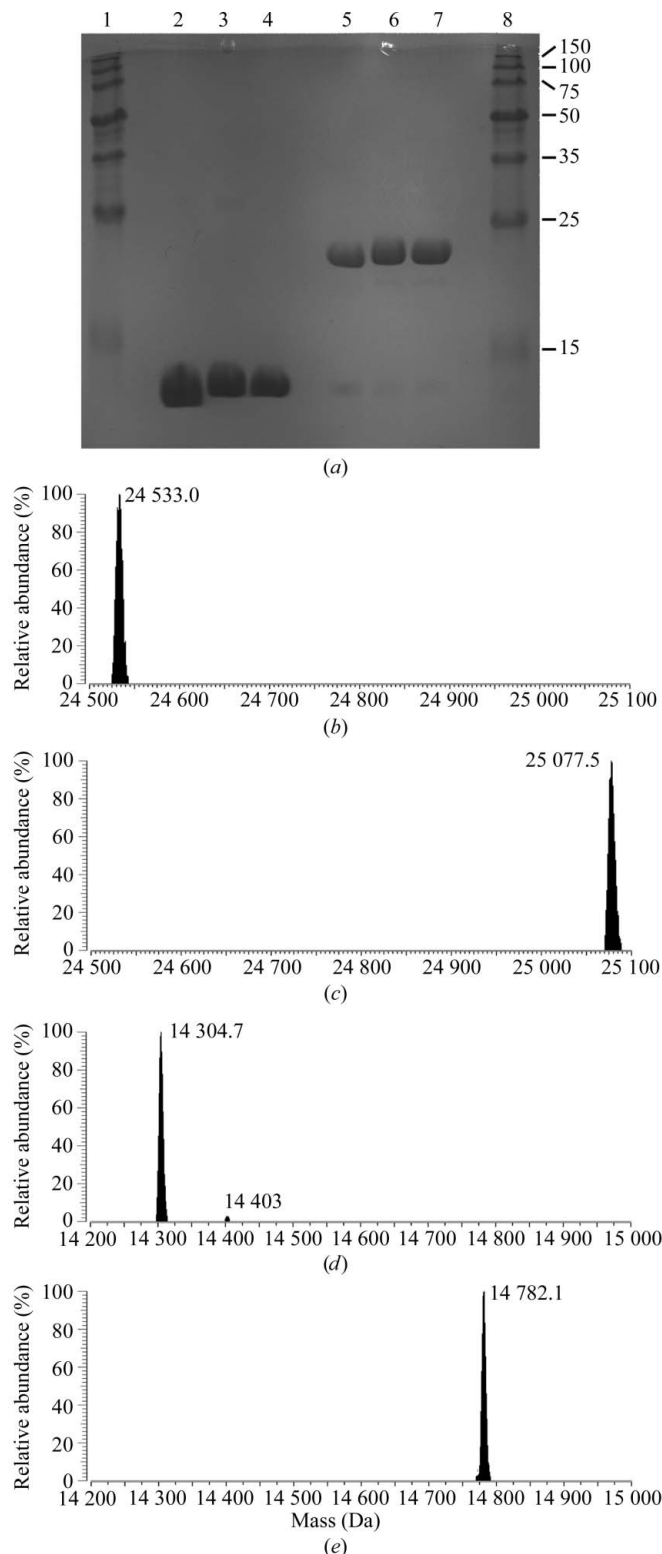

Figure 2
SDS–PAGE and deconvoluted FTICR-MS of protein samples. (a) Coomassie-stained SDS–PAGE of proteins before and after modification. Lane 2, native lysozyme; lane 3, modified lysozyme; lane 4, modified lysozyme after HIC purification; lane 5, native *Efs*Qnr; lane 6, modified *Efs*Qnr; lane 7, modified *Efs*Qnr after HIC purification. Lanes 1 and 8, 10–150 kDa molecular-weight markers. (b, c) FTICR-MS of *Efs*Qnr before (b) and after (c) modification by glutaraldehyde. (d, e) FTICR-MS of lysozyme before (d) and after modification (e) by glutaraldehyde. The measured masses are indicated.

peak in FTICR-MS with a mass of 25 077.5 Da, an increase of 544.5 mass units. *Efs*Qnr has eight free amines (seven lysines and the N-terminus), giving an increase of 68.1 mass units per free amine. Similarly, the mass of lysozyme increased from 14 304.7 Da (expected 14 303.8 Da) to 14 782.1 Da after glutaraldehyde/DMAB treatment, an increase of 68.2 mass units per free amine (+477.4 Da; six lysines and the N-terminus). Based on the well documented reductive dimethylation of free amines, the FTICR-MS data suggest that a single glutaraldehyde molecule is reacting with each amine, as shown in Fig. 3. The first reaction is similar to the reductive dimethylation reaction: attack of a primary amine on an aldehyde followed by dehydration, resulting in a Schiff base
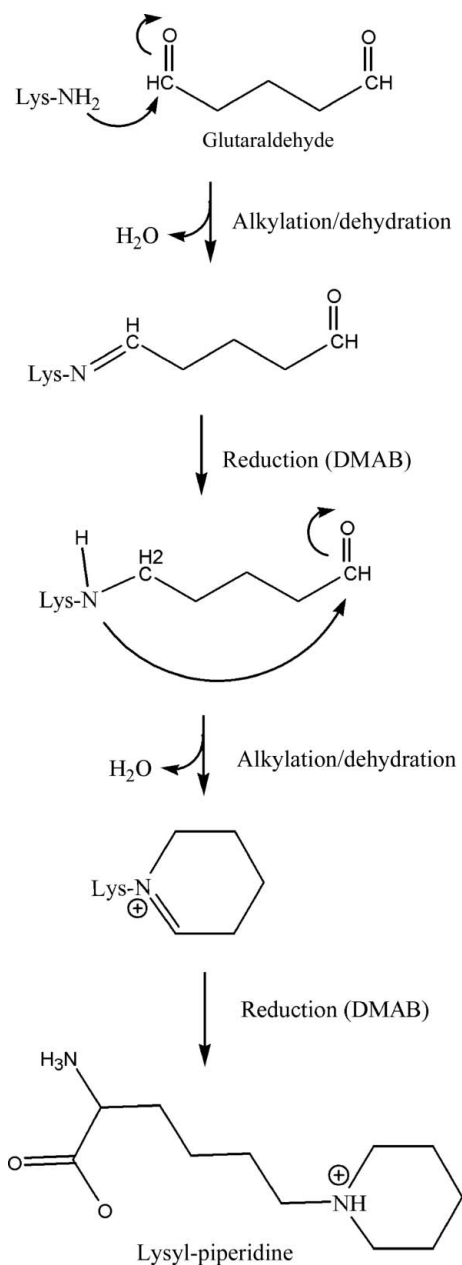

Figure 3
Reductive cyclic pentylation reaction scheme. Proposed reaction of glutaraldehyde with lysine/N-termini in the presence of a strong reducing agent.

which can then be reduced by a strong reducing agent. However, in the glutaraldehyde/DMAB reaction the next step is an intramolecular attack of the secondary amine on the remaining aldehyde moiety of glutaraldehyde followed by dehydration and reduction to produce a piperidine ring. The conversion of a ternary amine to a piperidine group would result in a mass difference of 68 Da ($+C_5H_{10}$, $NH_3 \rightarrow NH$) as observed in the FTICR data. We are subsequently terming this reaction the cyclic pentylation of free amines.

3.3. Structural evidence for cyclic pentylation

Glutaraldehyde/DMAB-treated *Efs*Qnr was rescreened for crystallization. Similar to unmodified *Efs*Qnr, modified *Efs*Qnr mostly formed clear drops or precipitate. Over a period of 1–2 months, crystals with a paddle-wheel morphology (interjoined plates, $0.1 \times 0.1 \times 0.02$ mm) formed in 10–15%(w/v) PEG 3350, 1 *M* LiCl, 0.1 *M* MES pH 6 (Fig. 1*b*). The paddle wheels were broken apart and the individual plates diffracted to 2.0 Å resolution. Crystal and diffraction quality were improved by streak-seeding crushed paddle wheels through an identically produced PEG 3350/LiCl/MES screen. Individual plates with enhanced thickness ($0.25 \times 0.25 \times 0.05$ mm) grew over a period of a week (Fig. 1*c*) and diffracted to 1.6 Å resolution. The structure of modified *Efs*Qnr was determined by single anomalous dispersion (SAD) phasing using data collected on a Cu *K*α home source from a samarium acetate-soaked crystal.

Structural analysis will be limited to the modified lysine residues; the full details of the *Efs*Qnr structure will be presented elsewhere. Briefly, *Efs*Qnr folds as a right-handed quadrilateral β-helix similar to that observed for other members of the pentapeptide-repeat family (PRPs) of proteins (Hegde *et al.*, 2005; Buchko *et al.*, 2006; Vetting *et al.*, 2006). There is a molecular dimer in the asymmetric unit, with a C-terminal dimer interface similar to that observed for the PRP protein MfpA (Hegde *et al.*, 2005). Three N-terminal residues remain from the thrombin-cleavable hexahistidine tag (Gly-Ser-His-Met1) used for purification. As such, the modified N-terminal residue is a cloning-artifact glycine and not the native methionine. The N-terminal residues are not observed in the electron-density maps, with the first modeled residues being Met1*A* and Thr4*B* (subunit designations are in given in italics). The lack of electron density for the N-termini suggests that they are solvent-accessible and available for modification. Apart from the N-terminal residues, all 211 residues were built into electron-density maps except for residues Pro9–Pro12 of subunit *A*, which are part of a flexible N-terminal fragment (see below). All seven lysines (Lys2, Lys110, Lys118, Lys150, Lys151, Lys170 and Lys193) are solvent-exposed with excellent electron density for the backbone atoms but weak electron density for most of their side chains, indicative of high mobility. This is consistent with all seven lysines being available for modification by glutaraldehyde as indicated by the FTICR-MS data. A select number of lysines are more ordered and exhibit electron density consistent with the proposed modification (Fig. 4). The

electron density for Lys2*A* is consistent with a piperidine ring in the chair conformation and sp^3 hybridization of the amine group, confirming successful reduction of the final Schiff base in the reaction. The nonpolar edge of the piperidine group lies in a hydrophobic pocket bounded by the peptide planes of two rungs of the PRP β-helix (Asp111–Leu113, Arg91–Thr93), the outward projecting side chains from those rungs and the side chain of Leu7 (Fig. 5*a*). Based on the pK_a values of substituted piperidines (*N*-methylpiperidine, pK_a 10.1; *N*-ethylpiperidine, pK_a 10.4; Hall, 1957) the amine would be protonated under the experimental conditions (pH 6.0) and be positively charged. In the case of Lys2*A* the tertiary amine of the piperidine group forms a charged pair ($d = 2.8$ Å) with the side chain of Asp111.

3.4. Change in crystallizability

Analysis of the position of the modified residues does not readily suggest a mechanism for the altered crystallization. While none of the modified residues directly participate in crystal contacts, Lys2*A* appears to be a residue which may influence the dynamics of a flexible N-terminus. The N-terminus (residues 1–16) runs down one of the faces of the β-helix prior to forming a cap on the N-terminal end of the β-helix (residues 17–26) and finally initiating the first coil (residues 27–47). The N-terminus takes alternative conformations in the two subunits of the dimer (Fig. 5*b*), with each making numerous and non-equivalent polar interactions with the face of the β-helix. The piperidine group of Lys2*A* forms intimate interactions with the face of the β-helix, while Lys2*B* is not visible in electron-density maps. Interestingly, each of

the N-termini is involved in extensive but non-equivalent crystal contacts. The *A*-subunit N-terminus interacts with one crystallographically related subunit, while the *B*-subunit N-terminus interacts with two crystallographically related subunits (Figs. 5*c* and 5*d*). It is possible that the dynamics of the N-terminus is important in the formation of a stable crystal lattice and that cyclic pentylation of Lys2 would affect crystal lattice formation. Alternatively, the cyclic pentylation of lysines in *Efs*Qnr may be acting in a negative fashion to disrupt non-ordered aggregation under those conditions which yielded crystals.

4. Discussion

In order for a covalent modification to be a useful technique for positively altering protein crystallization, the modification must be homogeneous. At first glance, treatment with glutaraldehyde would seem to be an unlikely choice to produce a homogeneous population, as glutaraldehyde has been used for decades as a protein cross-linking agent. However, in the limited tests performed here on two biophysically different proteins (*Efs*Qnr and lysozyme) the method appears to be robust in preparing homogenously modified protein with no cross-linking when used under reducing conditions. This surprising result most likely originates from several factors. Firstly, glutaraldehyde is in molar excess (10–40-fold) over free amine and once the first reaction takes place access to that amine by free glutaraldehyde is reduced. Secondly, the effective concentration of the bound aldehyde is vastly increased over free aldehyde, ensuring that the second reac-
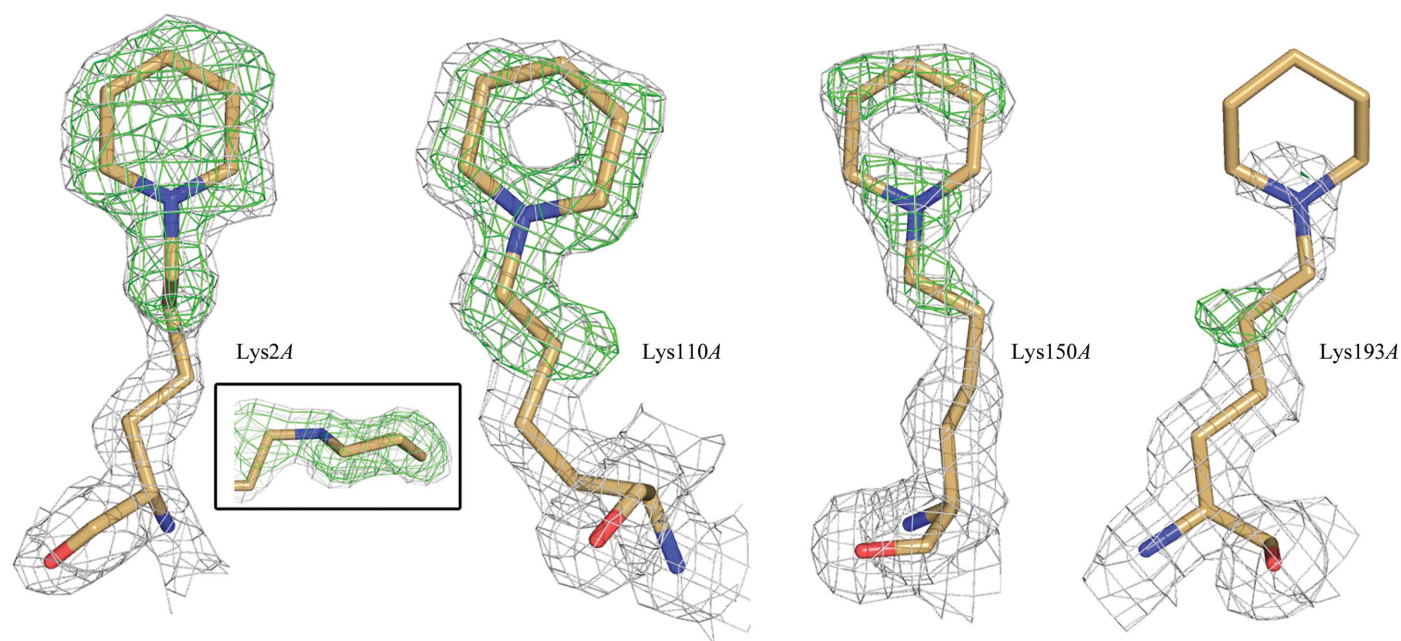

Figure 4
Electron density for the typical (Lys193*A*) and best ordered (Lys2*A*, Lys110*A*, Lys150*A*) lysine residues of *Efs*Qnr. The final $2F_o - F_c$ electron density contoured at 1σ is shown in gray. The OMIT $F_o - F_c$ electron density contoured at 2.5σ is shown in green. The boxed figure shows the electron density of Lys2*A* looking parallel to the piperidine ring. See §2 for the OMIT procedure.

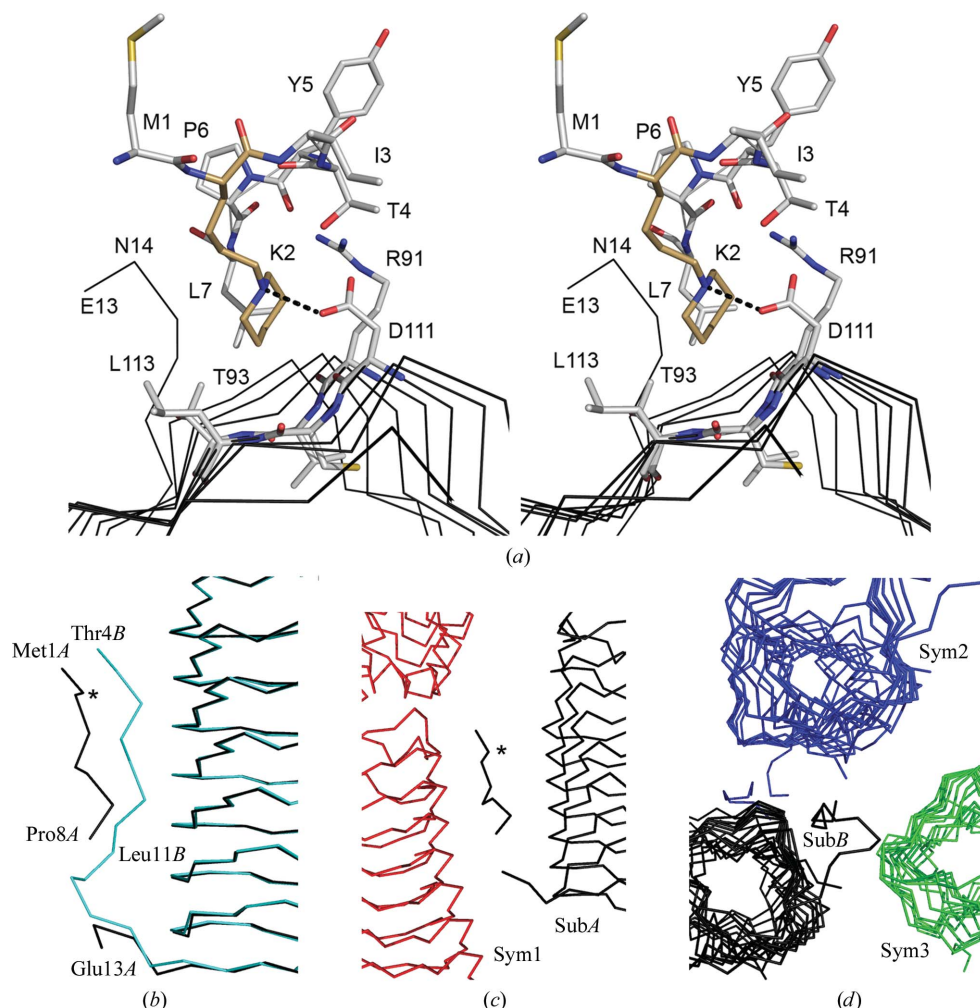**Figure 5**
Environment around the N-terminal residues. (*a*) Stereoview around Lys2*A* (brown C atoms) and its interaction with the surface of the β-helix. Atoms are colored by atom type. The hydrogen bond from Asp111 to Lys2 is shown as dotted line. (*b*) Alternate conformation for the N-terminus in the *A* (black trace) and *B* (cyan trace) subunits. The position of Lys2*A* is marked by an asterisk. (*c*, *d*) Interactions of the two N-termini with symmetry-related subunits.

tion is intramolecular rather than intermolecular. Finally, in the modification of free amines by formaldehyde the mono-methylated amine is more reactive to a second aldehyde (Means & Feeney, 1968; Rayment, 1997); if this holds true for modification by alternate aldehydes then the intramolecular reaction by a dialdehyde should be rapid.

The modification of free amines by cyclic pentylation is most likely to be sufficiently dissimilar to reductive dimethylation that the technique will generate a unique set of crystallization hits for recalcitrant proteins. The dimethylation of lysines produces a side chain with polarized methyl groups owing to the electron-withdrawing effect of the nitrogen. The dimethylated lysine is therefore able to make ionic inter-actions between the methyl groups and carbonyl/carboxyl O atoms of neighboring residues, an interaction that has recently been observed in crystal structures of dimethylated proteins (Shaw *et al.*, 2007; Kim *et al.*, 2008). In contrast, the cyclic pentylation of lysines produces a piperidine ring which, while retaining the charge of the lysine, buries that charge behind a

saturated hydrocarbon ring. Therefore, a protein that has been treated with glutaraldehyde/DMAB should have a more hydrophobic surface than a protein treated with formalde-hyde/DMAB and may form unique crystal contacts. Interest-ingly, glutaraldehyde/DMAB-treated lysozyme did not form crystals (data not shown), suggesting that treatment with glutaraldehyde can also have a negative effect on crystal-lization. However, it should be noted that the dimethylation of lysozyme also led to a decrease in crystallizability, with usable crystals requiring macroseeding techniques (Rypniewski *et al.*, 1993). A recent study of a large number of proteins resistant to crystallization indicated that approximately 7% could be rescued (lead to finished structures; Kim *et al.*, 2008). Inclusion of additional modification procedures such as cyclic pentyla-tion should increase this success rate.

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