mutation Lys86 → Gln was introduced, which enabled metal bridge contacts to form, resulting in crystals that diffracted to 1.9 Å. Although the mutant was designed to crystallize from $CdSO_4$, it did not do so. Rather, $CaCl_2$ gave large crystals which were isomorphous with rat and horse L ferritin crystals. In these latter crystals, Ca^{2+} is coordinated between Asp84 and Gln86, providing the rationale for the mutation.

4.3.8. Engineering heavy-atom sites

Another application of protein engineering to crystallography involves the mutation of wild-type residues to cysteines, thus creating potential heavy-atom binding sites (reviewed by Price & Nagai, 1995). This was first systematically investigated by Sun *et al.* (1987), who made five cysteine mutants of T4 phage lysozyme. They demonstrated that modification of the protein usually, but not always, introduced differences in isomorphism with the wild type. When the lack of isomorphism was not large, its effects could be reduced by comparing the mutant crystal with and without heavy atoms. The authors suggested that serine would be an attractive site for substitution, since it is structurally similar to cysteine and has a high probability of being on the protein surface.

However, in the absence of a known structure, the choice of a successful cysteine substitution site involves some luck. A general sense of the success rate of this approach can be gauged from three studies. Martinez *et al.* (1992) prepared 14 mutant forms of *Fusarium solani* cutinase in which each serine was replaced by cysteine. Four of these gave isomorphous crystals and led to useful derivatives with mercuric acetate. Nagai *et al.* (1990), as part of an attempt to crystallize a domain of the U1 small RNA-binding protein, engineered ten mutants to give cysteine replacements for polar side chains; of these, four yielded mercury derivatives that were isomorphous with the native protein. Finally, in a study of the ribosomal protein L9 (Hoffman *et al.*, 1994), eight cysteine mutants were prepared, but only one crystallized well and was isomorphous with wild-type crystals. In addition, two methionine mutants were engineered, and both crystallized isomorphously to the wild type (discussed below).

When the protein being examined belongs to a homologous superfamily, the sequences can be analysed to provide likely sites. For example, in a structure determination of ribosomal protein L6 (Golden *et al.*, 1993), a heavy-atom binding site was constructed with the mutant Val124 → Cys. This site was chosen because it is a cysteine residue in other L6 proteins. The mutant protein crystallized with the same space group and cell dimensions as the wild type. It was reacted with parachloromercuribenzoate to provide a heavy-atom derivative. However, at high resolution, the crystals were not isomorphous with the wild type, so a derivative was prepared by replacing the two methionines with selenomethionine, illustrating a second approach to engineering heavy-atom sites, discussed below. The structure was ultimately solved with a combination of multiple isomorphous replacement, anomalous scattering and solvent flattening. The same approach was used for OmpR (Martínez-Hackert *et al.*, 1996), in which cysteine residues were similarly engineered into positions determined by comparison with other proteins of the superfamily.

The increasing popularity of multiwavelength anomalous dispersion (Karle, 1980; Hendrickson, 1991; Hendrickson & Ogata, 1997) for phase determination, using selenomethionine (Se-Met) in place of methionine, has led to the engineering of proteins to create selenomethionine sites. The original substitution of Se-Met for Met was described by Cowie & Cohen (1957). The potential for crystallography was demonstrated for thioredoxin (Hendrickson *et al.*, 1990) and was used to solve the structure of ribonuclease H (Yang, Hendrickson, Crouch & Satow, 1990; Yang, Hendrickson, Kalman & Crouch, 1990). Methods for preparing Se-Met-substituted proteins are reviewed by Doublié (1997). Budisa *et al.* (1995) have also reported successful incorporation of telluromethionine into a protein, although this approach is not yet routine.

Since the frequency of methionines in proteins is about 1 in 60 (Dayhoff, 1978; Hendrickson *et al.*, 1990), it is not unusual for the protein being studied to contain no methionine residues. A number of investigators have introduced methionine into a protein sequence so that it can subsequently be replaced by Se-Met. These include Leahy *et al.* (1994), who crystallized domains FN7–10 of human fibronectin. Attempts to obtain mercury-soaked diffraction-quality crystals of FN7–10P, a double mutant that resulted from a (yet another!) PCR error, were unsuccessful, as were attempts to solve the structure by molecular replacement. They therefore prepared a mutant in which three residues (two leucines and one isoleucine) were substituted with methionine. Diffraction-quality data were subsequently obtained from the Se-Met derivatives.

Sometimes the protein cannot be crystallized satisfactorily in the Se-Met form, and further modification is required. The Se-Met derivative of UmuD′, an *Escherichia coli* SOS response protein, did not crystallize under conditions that gave native crystals (Peat *et al.*, 1996). Comparison with homologous proteins indicated that two of the Met sites were either conserved or replaced by hydrophobic residues. The third site, Met138, was variable and often replaced by a polar residue. The authors hypothesized that this methionine might, therefore, be surface-exposed, rendering the Se-Met version highly susceptible to oxidation and heterogeneity. When this penultimate Met was mutated to Met138 → Val or Met138 → Thr, these mutant proteins yielded crystals both with and without introduction of Se-Met.

As a final note, it is worth returning to the study involving the crystallization of the U1A/RNA complex (Oubridge *et al.*, 1995), in which the authors comment: ‘In retrospect it is clear that too much was assumed about interactions within crystals, and that the “design” of good crystals *per se* was not feasible . . .. It may be that almost anything can be crystallized to give well ordered crystals as long as enough constructs are tried; however, one only knows the right condition when the crystals are obtained.’

Acknowledgements

We gratefully acknowledge the kind assistance of B. Cudney, A. D’Arcy, J. Ladner and A. McPherson in the early stages of researching this article, and A. McPherson and K. Nagai for reviewing the manuscript. We also thank A. Stock and S. Hughes for their help in coordinating references and for sharing their manuscript prior to publication.

references