Chapter 7

Final discussion

7.1 Conclusions

The aim of this project was to produce a functional EcoR124I$_{NT}$ R-M system so that biophysical and structural studies could be undertaken. The results obtained would allow comparison of EcoR124I$_{NT}$ with other systems such as the wild-type EcoR124I R-M system and the AhdI R-M system. The subunit organisation of the M.AhdI MTase resembles a Type I MTase yet the ENase R.AhdI is a typical Type II ENase (Marks *et al*., 2003).

S3 and S11 were expressed and purified according to published methods (Smith *et al.*, 1998). New purification methods were developed for the M and R subunits to decrease the purification time as to minimise the possibility of degradation. All proteins were purified in their native forms and found to form complexes of S3/M and S3/M/R.

EMSA assays have previously shown that $(S3/M)_2$ is able to bind to a 30 bp duplex containing the symmetrical recognition sequence GAAN$_7$TTC (Smith *et al*., 2001). Therefore this sequence was used throughout to study the DNA binding and functional activity of S3/M and S3/M/R. The functional assays that were developed showed S3/M to have methylation activity *in vitro*, requiring SAM only as a cofactor, and S3/M/R to have ENase activity *in vitro* that required ATP and Mg^{2+} as cofactors but not SAM. A reaction pathway of supercoiled DNA to linear DNA to further cleavage of linear DNA was found with DNA substrates containing two recognition sites. The cleavage of the DNA substrate occurred approximately equi-distant between the two sites, but a broad distribution of sites was observed as is normally found for naturally occurring Type I enzymes (Szczelkun *et al*., 1997). The ocr (overcome classical restriction) protein was shown to cause almost complete inhibition of the MTase activity of S3/M at a 2:1 molar ratio of ocr to S3/M.

Structural characterisation of the EcoR124I$_{NT}$ R-M system by dynamic light scattering (DLS), sedimentation equilibrium (SE) and sedimentation velocity (SV) showed S11 to form a tetramer in solution. The M subunit was found to be dimeric in solution. The same stoichiometry has been found in the recently solved structure of the M subunit of EcoKI (Rajashankar *et al., to be published*). The stoichiometry of S3 was confirmed as being dimeric in solution (Smith *et al.*, 2001). The MTase which was formed by combining the M and S3 subunits was found to exist as a heterotetramer with the stoichiometry $(S3/M)_2$.

The R subunit was found to be monomeric in solution. This suggests the R subunits bind to the wild-type MTase M.EcoR124I one monomer at a time, rather than as a dimer. For the wild-type EcoR124I system, the first R subunit binds to the MTase with a high affinity to form the restriction deficient R_1 complex. In contrast, binding of the second R subunit is much weaker such that the R_2 complex readily dissociates into the R_1 complex and free R with a K_d of ~2.4×10^{-7} M (Janscak *et al.*, 1998). However in the case of EcoR124I$_{NT}$, that is symmetrical due to dimerisation of the S3, it would be thought that the R subunits would bind with equal affinities. Therefore, it was surprising that when the R subunit was added to the MTase to form the ENase, an R_1 complex and not an R_2 complex was found.

Preliminary results by small angle neutron scattering (SANS) showed that the 56 Å radius of gyration obtained for the fully protonated $(S3/M)_2$ complex was identical to that obtained by small angle X-ray scattering for the wild-type MTase M.EcoR124I (Taylor *et al.*, 1994). A change in shape of $(S3/M)_2$ was found upon DNA binding, with an overall decrease in the dimensions of the enzyme from 195 Å to 145 Å and a decrease in the radius of gyration from 56 Å to 49 Å. This decrease is comparable to the wild-type MTase. When measurements are taken for the deuterated $(S3/M)_2$ complex in 40 % D_2O, *i.e.* the M subunits are contrast matched out, the R_g of the S3 subunits is essentially independent of DNA binding (31 Å to 33 Å). Whereas measurements carried out for the deuterated $(S3/M)_2$ complex in 100 % D_2O, where the S3 subunits are contrast matched out, demonstrate a large decrease in the R_g decreases from 48 Å to 32 Å in the presence of DNA. Therefore the rearrangement of the subunits is

due to compaction of the M subunits, with no significant change in the structure or organization of the S3 subunits. An *ab initio* model obtained using the program DAMMIN (Svergun *et al.*, 1999) is presented to illustrate the structural change of $(S3/M)_2$ upon DNA binding. The change in shape upon binding of $(S3/M)_2$ to DNA is also supported by the SV experiments, whereby the frictional ratio of the MTase upon DNA binding decreased from 1.73 to 1.35, corresponding to a much more compact structure for the complex.

Binding of the R subunit to $(S3/M)_2$ also revealed a structural change. A decrease in the radius of gyration from 56 Å to 42 Å was found for the fully protonated samples measured in 100 % D_2O (and 48 Å to 38 Å for the deuterated complex measured in 100 % D_2O), indicating a further compaction of the $(S3/M)_2$ "core". In addition the D_{max} decreased from 195 Å to 140 Å in the presence of the R subunit. However no further change was observed upon DNA binding to S3/M/R. To confirm that the structural change was due to S3/M, further experiments would need to be carried out with deuterated R subunits with various combinations of deuterated and protonated subunits, it would be possible to determine which subunits were responsible for the observed structural change.

The radius of gyration of deuterated S3 obtained from scattering data measured in 40 % D_2O was in very close agreement with the value obtained by hydrodynamic modeling of the crystal structure of the S subunit of *M. jannaschii*, indicating the S3 dimer adopts a similar structure to that of a standard S subunit.

The S subunit of AhdI comprises a single TRD, which recognises the trinucleotide half-site GAC. Therefore the S subunit of AhdI is equivalent to the S3 subunit which recognises the half site GAA. The stoichiometry of M.AhdI is M_2S_2 (Marks *et al.*, 2003), which is therefore identical to that of the MTase of the $EcoR124I_{NT}$ R-M system, $(S3/M)_2$. Both MTases have now been shown to have MTase activity *in vitro* and have a similar domain organisation as shown by SANS (Kneale *et al., to be published).* It is possible that AhdI represents an evolutionary intermediate between Type I and Type II R-M systems.

7.2 Future work

Prior to the start of this project, there was no structural information on any Type I R-M systems. However recently the crystal structures of two S subunits and the M subunit of EcoKI have been solved (Kim *et al*., 2005; Calisto *et al*., 2005; Rajashankar *et al*., *to be published*). However there is still no crystal structure of either an MTase or an ENase from a Type I R-M system. Attempts were made to crystallise S3/M in the presence and absence of DNA without success. However it may be worthwhile attempting co-crystallisation of S3/M and/or S3/M/R with ocr in the place of DNA. This would eliminate the need to determine the correct length of DNA to use. It would first be of use to determine by sedimentation equilibrium (SE) and sedimentation velocity (SV) the stoichiometry of ocr to S3/M or S3/M/R. These experiments would also allow determination of the number of binding sites on the MTase and ENase for ocr. It has been suggested that there may be a secondary binding site, in EcoKI for ocr (Atanasiu *et al*., 2002). Fluorescent labelling of either ocr or the S3/M complex would allow measurement of the absorbance at a specific wavelength due to that component only, thereby simplifying the analysis. The stoichiometry could also be determined by isothermal calorimetry (ITC). It would also be interesting to determine by SANS and SV if ocr causes the same change in shape as DNA upon binding to the MTase.

Further investigation is required to determine the number of recognition sites required by S3/M/R for cleavage. It would be interesting to see if there is any preference for DNA substrates (either supercoiled or linear) containing one or two sites and what the different rates of reactions on these substrates are. Varying the concentrations of the cofactors ATP, SAM and $MgCl_2$ could also be investigated. Confirmation that it is the second adenine in 5'-GAA or 3'-GAA within the symmetrical recognition sequence is methylated also needs to be confirmed, although the ability of methylation to inhibit cleavage by EcoR1 suggests that it is.

Further investigation of the stoichiometry of S3/M/R by SE and SV is required to determine whether it forms a R_1 or R_2 complex. The results presented in this thesis suggest it forms a R_1 complex. However it would be interesting to see if in the presence of DNA, the formation of the R_2 complex is favoured.

In addition, formation of the wild-type EcoR124I R_2 complex is slow (Janscak *et al.,* 1998). Therefore different molar ratios of S3/M to R could be mixed (in presence or absence of DNA) and the effect of the length of incubation time investigated by DLS, SE and SV. Precise elucidation of the conditions required to form the R_1 and R_2 complex will be essential to any further SANS experiments on these complexes.