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Assembly of EcoKI DNA methyltransferase requires the C-terminal region of the HsdM modification subunit

Lynn M. Powell[b], Erwan Lejeune[a], Farhana S. Hussain[b], Andrew D. Cronshaw[b], Sharon M. Kelly[c], Nicholas C. Price[c], David T.F. Dryden[a,*]

[a]*Department of Chemistry, The King's Buildings, University of Edinburgh, Edinburgh, Scotland EH9 3JJ, UK*
[b]*Institute of Cell and Molecular Biology, The King's Buildings, University of Edinburgh, Edinburgh, Scotland EH9 3JR, UK*
[c]*Division of Biochemistry and Molecular Biology, Institute of Biomedical and Life Sciences, University of Glasgow, Glasgow, Scotland G12 8QQ, UK*

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Abstract

The methyltransferase component of type I DNA restriction and modification systems comprises three subunits, one DNA sequence specificity subunit and two DNA modification subunits. Limited proteolysis of the EcoKI methyltransferase shows that a 55-kDa N-terminal fragment of the 59-kDa modification subunit is resistant to degradation. We have purified this fragment and determined by mass spectrometry that proteolysis removes 43 or 44 amino acids from the C-terminus. The fragment fails to interact with the other subunits even though it still possesses secondary and tertiary structure and the ability to bind the *S*-adenosylmethionine cofactor. We conclude that the C-terminal region of the modification subunit of EcoKI is essential for the assembly of the EcoKI methyltransferase.

Keywords: Restriction/modification system; DNA methyltransferase; Protein assembly; EcoKI

1. Introduction

DNA methyltransferases (mtases) use *S*-adenosylmethionine (SAM) to methylate adenine or cytosine in specific DNA target sequences [1]. Most of these mtases are part of DNA restriction and modification (RM) systems [2,3]. Type II modification mtases methylate each strand of the DNA target sequence independently, dissociating after each methylation event. Therefore, they cannot distinguish between the unmethylated target, which is the desired target for the restriction endonuclease, and the hemimethylated target produced after DNA replication, which is the desired target for the mtase. This inability to distinguish between unmethylated and hemimethylated DNA correlates with the structure of type II mtases which contain one target recognition domain (TRD) for DNA binding and one catalytic domain for binding SAM.

Abbreviations: GdmCl, Guanidinium chloride; mtase, Methyltransferase; M, modification; RM, restriction and modification; SAM, *S*-adenosylmethionine; S, specificity; TFA, trifluoroacetic acid; TRD, target recognition domain.

*Corresponding author. Tel.: +44-131-650-4735; fax: +44-131-650-6453.
E-mail address: david.dryden@ed.ac.uk (D.T. Dryden).

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Type I DNA modification mtases, on the other hand, are more complex than type II mtases and can distinguish between unmethylated targets and hemimethylated targets [2–4]. They comprise one sequence specificity (HsdS or S) subunit which contains two TRDs, one for each part of the bipartite target sequence and two modification mtase (HsdM or M) subunits, each with a catalytic, SAM-binding domain. The three subunits interact with each other to form different conformations on unmethylated or hemimethylated targets. The conformation adopted on hemimethylated targets is active for methylation while that adopted on unmethylated targets is virtually inactive. Unmethylated targets trigger the DNA restriction reaction in the presence of additional type I restriction endonuclease subunits. Each part of the bipartite target contains one methylatable adenine nucleotide, e.g. 5′-AAC N6 GTGC-3′ for EcoKI mtase, with adenine methylation targets located at the underlined locations on the appropriate strand. The N-terminal TRD of the S subunit and one of the M subunits recognise the AAC sequence and its methylation state, while the C-terminal TRD and the second M subunit recognise the sequence 5′-GCAC-3′ and its methylation state on the complementary strand. The EcoKI mtase shows a 50-fold preference for hemimethylated targets [5] which can be reduced by mutation in the N-terminal region of the M subunits [6]. The central third of the M subunits contains amino acid motifs for methylation and a tertiary structure model based on homology of this region with the catalytic domains of type II mtases has been constructed [7]. The C-terminal region of the M subunit of EcoKI is susceptible to removal by the protease elastase to leave a 55-kDa fragment, E55M, which is resistant to further proteolysis by elastase when SAM is present [8]. Thus, the M subunit appears to contain three distinct regions, an N-terminal region responsible for recognising the methylation status of the DNA target, a central region which catalyses methylation and a C-terminal region of undefined function.

In this paper, we investigate the properties of the E55M fragment and its interaction with a partially assembled, inactive form of the EcoKI mtase containing only one M and one S subunit. The M_1S_1 protein can bind DNA and SAM but is unable to methylate DNA in the absence of a second M subunit [9–11]. Our results show that E55M is still capable of forming a stable globular fold and binding SAM but has lost the ability to interact with M_1S_1 to form the EcoKI mtase. This suggests a role in subunit interactions for the C-terminal region of the M subunit.

2. Materials and methods

The M subunit, M_1S_1 and M_2S_1 forms of EcoKI were purified and their concentrations determined by absorption at 280 nm as previously described [10]. Twenty-five millilitres of M subunit at a concentration of 17.4 μM was then incubated in 20 mM Tris–HCl, 0.2 M NaCl, pH 8 buffer containing 0.32 mM SAM and 0.2 μM elastase at room temperature for 55 min before proteolysis was halted by the addition of 25 μl of 20 mM phenylmethylsulphonyl fluoride dissolved in ethanol. This time was sufficient to digest all of the M subunit to the E55M form along with two smaller fragments of masses 50 kDa and 24 kDa. The two smaller fragments total approximately 10% of the total protein with the majority of this in the 24-kDa form. The digested sample was concentrated to 2 ml and passed through a Superose 12 FPLC gel filtration column (Pharmacia). Fractions containing E55M were pooled, concentrated and stored at −20 °C in buffer supplemented with glycerol to 50% v/v. Samples for electrospray mass spectrometry were prepared by microbore, reverse-phase HPLC on an Applied Biosystems 130A separation system with an Aquapore RP300-C8 column. The initial solvent was 0.1% TFA, the final solvent was 0.08% TFA in 70% acetonitrile and a gradient from 10 to 70% of the final solvent was used to elute the protein. Mass spectrometry was performed using 50 μl of the protein sample mixed with 200 μl of 60% methanol in 1% v/v formic acid on a Thermoquest LCQ mass spectrometer. N-terminal sequencing of protein was performed with an Applied Biosystems 477A instrument. Analytical HPLC gel filtration of protein samples was performed in 20 mM Tris–HCl, 20 mM MES, 0.2 M NaCl, 10 mM $MgCl_2$, 7 mM 2-mercaptoethanol, 0.1 mM EDTA at pH 6.5 as

described [10]. SAM binding was assayed by mixing 10 μM M subunit or E55M with 0.24 μM [^{3}H]methyl-SAM (Amersham) in 50 μl of 20 mM Tris–HCl, 50 mM NaCl, 0.1 mM EDTA, pH 8 and applying to a gel filtration column (Pharmacia PD10) as described [12]. Fractions were collected and counted using a TopCount microplate scintillation counter (Packard). Circular dichroism spectra were obtained on a JASCO J600 spectropolarimeter at 20 °C with protein concentrations of 2 μM and 7 μM and cell pathlengths of 0.02 cm and 0.5 cm in the far and near UV regions, respectively. Secondary structure predictions used the PHD program [13] and general peptide properties were derived using pepinfo (http://www.ebi.ac.uk/emboss/pepinfo/). Fluorescence spectroscopy was performed with an Edinburgh Instruments FS-900 T-geometry fluorimeter. Folding studies utilised guanidine hydrochloride (GdmCl) to denature the M subunit and E55M. Samples of 0.1 ml containing 0.2 μM protein in 20 mM Tris, 20 mM MES, 0.2 M NaCl, 10 mM $MgCl_2$, 7 mM 2-mercaptoethanol, 0.1 mM EDTA at pH 6.5 were incubated overnight in the appropriate concentration of denaturant. Protein fluorescence emission spectra excited at 295 nm were collected and corrected for background fluorescence from the buffer and denaturant. The temperature was 25 °C. The ratio of intensity at 320 nm to that at 370 nm was plotted against denaturant concentration. Samples in high concentrations of denaturant were diluted to initiate refolding and it was found that the refolding curve was identical to the unfolding curve (data not shown). This indicates that the denaturation is reversible and that equilibrium folding models can be applied. The data were analysed using GRAFIT (Erithacus Software). Fluorescence anisotropy measurements were performed with a 5-nM solution of a 21 base-pair DNA duplex containing the EcoKI target sequence, labelled at the 5′ end of the top strand with hexachlorofluorescein, in 20 mM Tris–HCl, 50 mM NaCl, 6 mM $MgCl_2$, 7 mM 2-mercaptoethanol at pH 8 as described [11,14]. The DNA solutions were supplemented with 100 μM SAM and either E55M or M subunit to 1 μM as appropriate and titrated with a concentrated solution of M_1S_1. These data were analysed using GRAFIT (Erithacus Software) with the full quadratic form of the single-site binding equation as in these experiments it cannot be assumed that the amount of unbound enzyme is equal to the total amount of enzyme.

3. Results

3.1. Identification of E55M fragment

The N-terminus of E55M was previously determined to be MNNND by automated Edman degradation identifying the normal starting sequence of the M subunit [8]. Mass spectrometry of E55M showed roughly equal amounts of two species with masses of 54613 ($n=3$) and 54696 ($n=3$) suggesting some heterogeneity within the single elution peak recovered from the reverse-phase HPLC preparation. None of the fragments expected to be produced by the digestion of the M subunit by elastase are predicted to have exactly the above masses. However, the fragments with the predicted mass closest to those observed are from Met1 to Ala486 with a predicted mass of 54690 and from Met1 to Leu485 with a predicted mass of 54619. Elastase cleaves effectively after both alanine and leucine residues and these fragments are within 0.01% of the observed masses. These masses are also within the fragment size range estimated previously by SDS-PAGE [8]. Therefore, we conclude that E55M is a mixture of fragments Met1 to Leu485 and Met1 to Ala486. Assuming that the fragment comprises amino acids 1–486, an extinction coefficient of 61 600 M^{-1} cm^{-1} at 280 nm was calculated from the known amino acid composition of the fragment.

3.2. Structural analysis of E55M and comparison to the structure of the M subunit

If one is to draw conclusions regarding the function of the C-terminal region of the M subunit and the consequences of its removal to form E55M, one has first to prove that the structure of E55M is very similar if not identical to the equivalent region of the M subunit. To address this, we have performed a series of spectroscopic and biochemical experiments.

Far-UV CD spectroscopy of the M subunit and E55M fragment reveals that the secondary structure content of E55M is high and not radically different from that of the whole M subunit implying that the folding of E55M is similar to that of the M subunit, (Fig. 1a). Application of the CONTIN procedure [15] to the spectra over the range 190–240 nm gives the following percentages of secondary structure for the M subunit and E55M, respectively; α-helix 28% and 32%, β-sheet 37% and 43%, remainder 35% and 25%. It is apparent that the secondary structure content of the M subunit and E55M are very similar. Secondary structure predictions concur with this result. Respectively, for M and E55M, the predicted amounts are; α-helix 23.4% and 21.1%, β-sheet 4.1% and 4.5% and remainder 72.5% and 74.4%. The amount of α-helix is close to that determined experimentally but the proportion of β-sheet is underestimated for both proteins. The near-UV CD spectra (Fig. 1b), although relatively weak, show that E55M still possesses significant tertiary structure.

Despite the similarity in the circular dichroism spectra, it is possible that E55M has substantial tertiary structural differences from the M subunit. Such changes would almost certainly have a major effect on the stability of the protein towards denaturants. To address this issue, the unfolding and refolding of the M subunit and E55M in GdmCl were compared. As the concentration of denaturant increased, the fluorescence spectrum shifted to longer wavelengths and increased in intensity as expected if buried fluorophores were becoming exposed to the aqueous environment (data not shown). E55M must, therefore, possess tertiary structure in addition to the secondary structure observed by far UV CD as the tryptophan fluorescence would show little change in the absence of such structure.

It was apparent from changes in total fluorescence emission intensity (data not shown) and from the ratio of intensity at 320 nm to that at 370 nm (Fig. 2), that the unfolding of both the M subunit and E55M was not well described as a two-state transition between a native and an unfolded state. The data were well described by a three-state model, which invokes a folding intermediate, as was found for the folding of the complete EcoKI mtase [9]. The relative contributions to the observed fluorescence of the three states, at a particular denaturant concentration, are different between the M subunit and E55M (Table 1).

It is apparent that E55M is slightly less stable than the M subunit as denaturation occurs at a lower concentration of GdmCl. Although the relative stabilities of the native state, intermediate state and the unfolded state of E55M and the M subunit are different, the total free energy change from native to unfolded states is the same. The intermediate in the unfolding of E55M is less stable than the intermediate formed during the unfolding of the M subunit presumably reflecting the loss of interactions between amino acids in the missing part of the C-terminal region and the rest of the protein.

Although the above results clearly indicate that E55M has a stable tertiary structure, it is still possible that this structure differs significantly from that of the M subunit. If this were the case, then the differences would very probably affect the SAM-binding domain as this domain comprises over 40% of the primary structure of the complete subunit (and even more of E55M). The addition of SAM to the M subunit stabilises the subunit so that most of it, except the C-terminal 43 amino acids, is resistant to elastase digestion. In the absence of SAM, elastase can digest the whole M subunit and no fragments are stable during extended periods of proteolysis [8]. This result strongly implies that the E55M fragment still possesses sufficient tertiary structure identical to that of the M subunit to be able to bind SAM.

Proof of SAM-binding by E55M was sought by gel filtration chromatography of a mixture of [^{3}H]methyl-SAM with either the M subunit or E55M. It has previously been shown that SAM binds sufficiently strongly to EcoKI mtase to elute with the protein ahead of unbound SAM [12]. Similarly, SAM was observed to co-elute with both the M subunit and E55M as a separate peak before unbound SAM, (Fig. 3). It is also possible to covalently cross-link [^{3}H]methyl-SAM to the M subunit [12] using UV irradiation. We observed

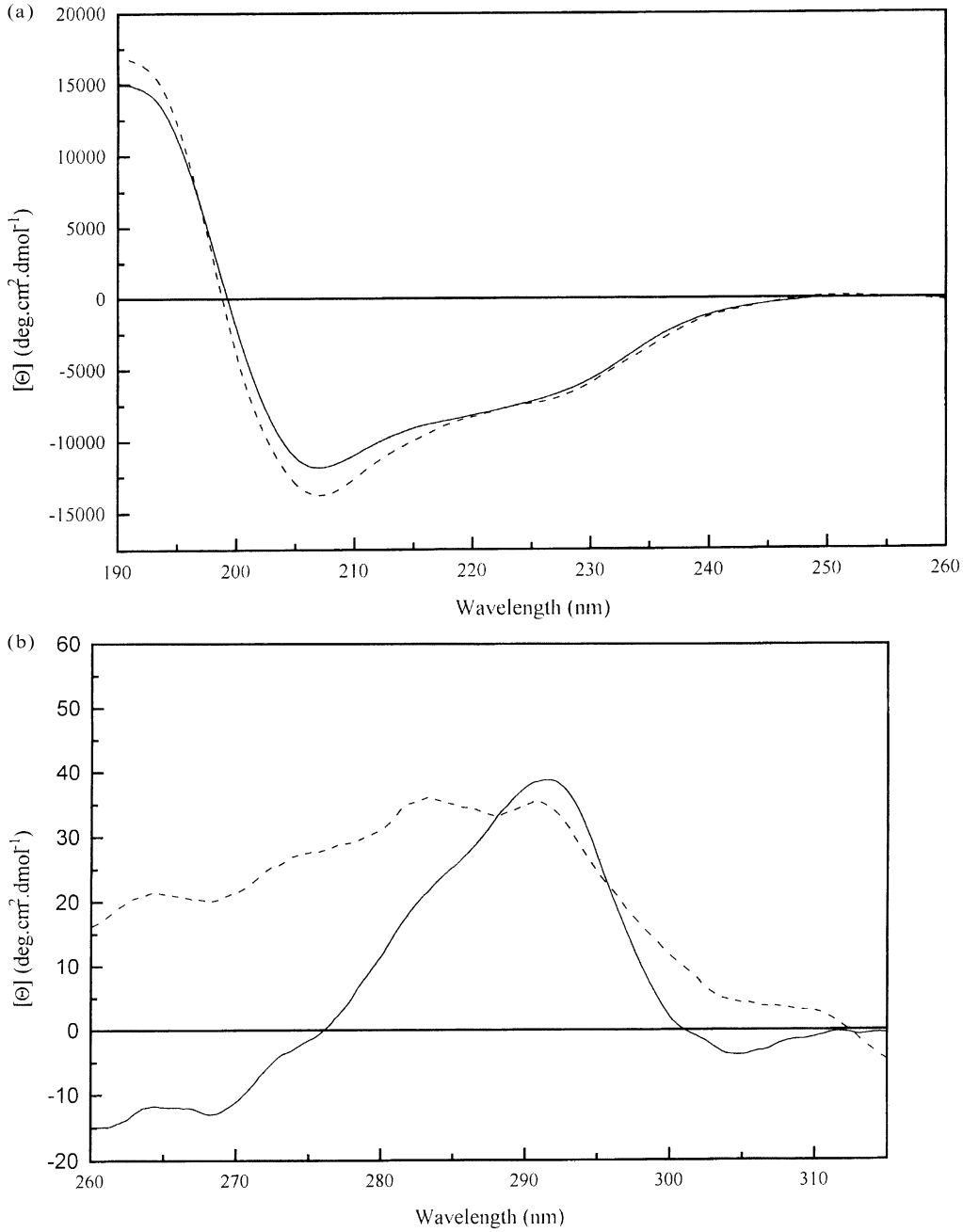

Fig. 1. Circular dichroism spectra comparing E55M with the M subunit. (a) Far UV CD spectra of 2 μM M subunit (-) and E55M (--). (b) Near UV CD spectra of 7 μM M subunit (-) and E55M (--).

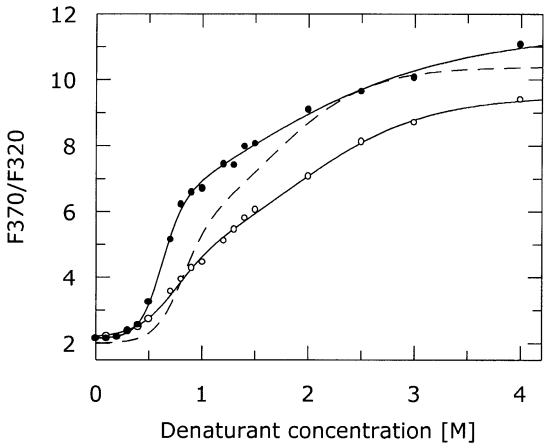

Fig. 2. GdmCl-induced denaturation of the M subunit and E55M as monitored by the ratio of fluorescence emission at 370 nm to that at 320 nm, F_{370}/F_{320}. M subunit data is shown as open circles, E55M data as closed circles. The fitted curves are derived from the three-state folding model as described. The curve describing denaturation of the M_2S_1 mtase, as determined previously [9], is shown as a dashed line.

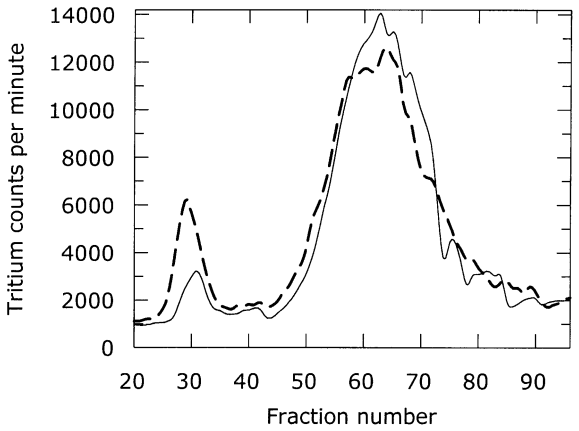

Fig. 3. Scintillation counting of fractions eluting from a gel filtration column from samples of [^{3}H]methyl-SAM mixed with either M subunit (-) or E55M (--). Protein-bound SAM elutes around fraction 30 for both proteins while unbound SAM elutes around fraction 60.

that this cross-linking also occurred to E55M (data not shown).

These results indicate that E55M retains the ability to bind the cofactor. This is consistent with sequence comparisons of type I M subunits with type II mtases which indicates that the M subunits of the EcoKI system contain a SAM-binding catalytic domain spanning approximately amino acids 146 to 364 [7]. If the structure of E55M is markedly different from that of the M subunit, such changes must be confined to regions outside the SAM-binding domain. Although the N-terminal region may have an altered structure, we have no assay for such a change at present. However, since this region has an assigned function in the recognition of DNA methylation [6], we believe it is more likely that any structural differences between the M subunit and E55M are confined to the C-terminal region which has been subjected to proteolysis.

3.3. The role of the C-terminal region of the M subunit

Having determined that E55M has a stable secondary and tertiary structure similar to that of the M subunit and can bind the cofactor, we examined the ability of E55M to interact with the

Table 1
Free energy of denaturation of the M subunit and E55M

Sample	Folding transition			
	Native to intermediate		Intermediate to denatured	
	Free energy kJ mol^{-1}	Slope kJ mol^{-1} M^{-1}	Free energy kJ mol^{-1}	Slope kJ mol^{-1} M^{-1}
M subunit	9.4±0.7	12.9±1.2	7.5±0.4	4.0±0.3
E55M	14.7±1.2	22.5±2.1	3.4±0.3	2.6±0.3
M_2S_1 [9]	17.7±1.4	22.0±1.8	5.5±1.3	4.1±0.5

Values were determined by fitting the three-state model to the F_{370}/F_{320} data obtained by GdmCl denaturation at pH 6.5.

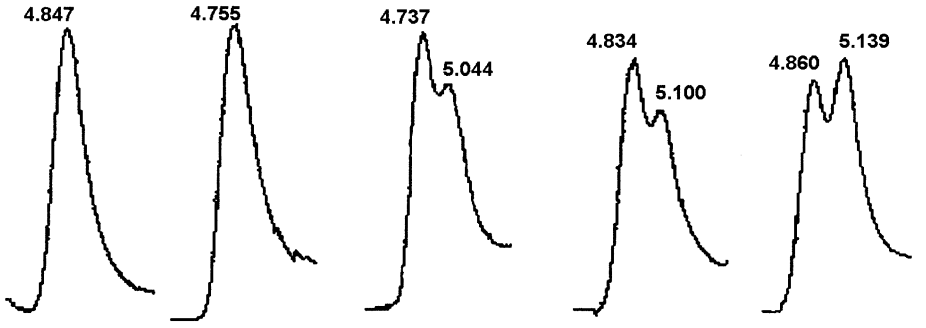

Fig. 4. HPLC gel filtration elution profiles of, from left to right, 750 nM M_1S_1, 750 nM M_1S_1 plus 900 nM M subunit, 750 nM M_1S_1 plus 3 μM M subunit, 750 nM M_1S_1 plus 900 nM E55M and 750 nM M_1S_1 plus 3 μM E55M. For each separate trace, elution time increases from left to right corresponding to a decrease in molecular mass. In the absence of interactions, M_2S_1 (169 kDa) elutes at approximately 4.74 min, M_1S_1 (110 kDa) at 4.85 min, M subunit (59 kDa) at 5.05 min and E55M (54.7 kDa) at 5.10 min. The mixtures of M_1S_1 with the M subunit form a M_2S_1 peak, whilst mixtures of M_1S_1 with E55M fail to form a peak eluting earlier than either protein alone indicating that M_1S_1 and E55M do not associate under these experimental conditions.

other components of the EcoKI mtase, namely the second M subunit and the S subunit.

It has previously been shown using analytical gel filtration chromatography that the purified M subunit, when mixed with purified M_1S_1, will lead to the reconstitution of the M_2S_1 mtase with a dissociation constant between 15 and 100 nM [10]. As shown in Fig. 4, it was observed that a mixture of purified E55M and M_1S_1 prepared under the same conditions as the M and M_1S_1 mixture failed to show assembly of an E55M–M–S complex as indicated by the absence of a complex of higher mass than that of M_1S_1 alone. This result strongly suggests that removal of the C-terminus of the M subunit has removed a region involved in the assembly of the EcoKI mtase. However, gel filtration measurements of interactions are semi-quantitative unless one utilises more complex large-zone methods [16] so a direct equilibrium method was sought.

As gel filtration chromatography is not an equilibrium technique, different molecular weight components are constantly being separated from each other [16], thus any weak association between E55M and M_1S_1 may not be stable enough to persist as the components pass through the column. The measurement of fluorescence anisotropy for mixtures of M or E55M with M_1S_1, using labelled DNA as a probe for assembly, provides an equilibrium method for determining the presence of interactions between these proteins. The fluorescence signal only arises from the DNA and any proteins bound to it. These proteins are the mtase, M_1S_1 and any complex between M_1S_1 and the M subunit or E55M. The M subunit and E55M by themselves lack DNA binding ability. The value of fluorescence anisotropy of labelled DNA duplex will increase due to the reduction in rotational motion of the fluorophore as the binding of protein to the DNA increases the molecular mass. The titrations shown in Fig. 5 demonstrate that M_1S_1 binds more weakly to the DNA duplex than the complete M_2S_1 mtase with dissociation constants of 25.3 ± 3.9 nM and 1.3 ± 0.4 nM, respectively. These values are similar to those found previously [11]. When an excess of M subunit is present, it can be seen that the binding of M_1S_1 to the DNA is enhanced. This can only be due to the formation of M_2S_1 mtase. The dissociation constant for DNA binding by this mixture of M subunit with M_1S_1 is 2.7 ± 0.7 nM. This binding constant is very close to that observed with the mtase and indicates that the M subunit has been able to bind to the M_1S_1 dimer to form the mtase. The dissociation constant for the binding of the M subunit to M_1S_1 in the absence of DNA is in the range of 15 nM to 100 nM [10]. The presence of DNA has been calculated to enhance this affinity still further [11]. As the M subunit is present in huge excess (1 μM), the equilibrium will be strongly in favour of assembly

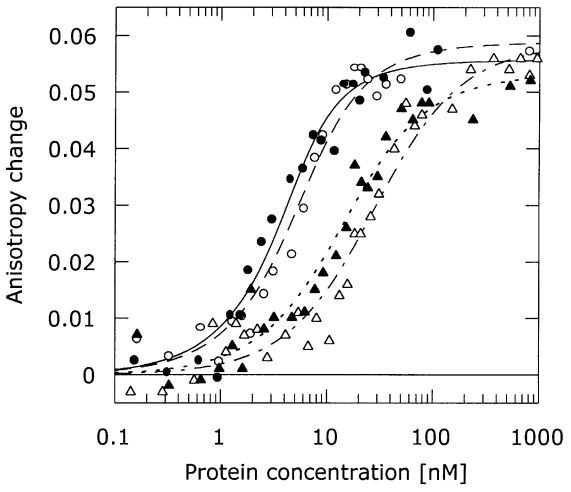

Fig. 5. Anisotropy increase of 5 nM fluorescently-labelled DNA as a function of protein concentration in the presence of 100 μM SAM. Proteins used were M_2S_1 (●), M_1S_1 (△), M_1S_1+M subunit (○) and M_1S_1+E55M (▲). The M subunit and E55M were present at concentrations of 1 μM. The binding of M_1S_1 to the DNA is significantly enhanced by the presence of M subunit but not by E55M.

of the mtase. This enhancement of M_1S_1 binding to DNA is not observed when E55M is added in huge excess to a solution of M_1S_1 and DNA. The dissociation constant for binding to DNA is calculated to be 23.7 ± 5.6 nM. This is identical within experimental error to that observed for M_1S_1 alone binding to DNA and indicates that E55M has no ability to enhance DNA binding by M_1S_1 even when present at a concentration of 1 μM. When this result is taken together with the observed lack of assembly in the gel filtration chromatography experiments, it is clear that E55M has no ability to bind to M_1S_1 even in the presence of DNA and SAM, both of which have previously been demonstrated to stabilise the association of M_1S_1 and M subunit [11]. It is possible that other regions of the M subunit are also involved in forming the interface with M_1S_1 but these are not sufficient for assembly in the absence of the C-terminal region.

4. Discussion

Our results show that the E55M fragment lacking the C-terminal 43 or 44 amino acids of the M subunit of the EcoKI mtase is a structurally stable protein capable of binding the mtase cofactor SAM and possessing both secondary and tertiary structure features not significantly different from the intact M subunit. The loss of the C-terminal fragment does, however, lead to a marked loss of the ability of E55M to interact with the rest of the mtase. This is in contrast to the M subunit itself which can bind well to the M_1S_1 species to form an active trimeric mtase. This implies that the C-terminal part of the M subunit is a major factor responsible for forming the interface with the other two subunits. Thus, virtually all domains of the M subunit of EcoKI mtase now have defined functions. Amino acids 1–153 are responsible for defining the preference of EcoKI for methylating hemimethylated DNA targets [6], amino acids 154–364 form a SAM binding catalytic domain with the same structure as the catalytic domain of monomeric type II mtases [7] and amino acids 486–529 are vital for the attachment of the M subunit to the remainder of the mtase. At present, because we cannot isolate the S subunit alone due to its insolubility, we cannot determine whether the C-terminal part of the M subunit interacts with both subunits of M_1S_1 or only with one of them. The isolation of temperature sensitive mutants of the EcoKI system provides support for the assignment of a subunit interface function to the C-terminus of the M subunit. Although two of these mutations were located within the S subunit [17], one has been located in the M subunit at amino acid 406 (Firman and Zinkevich, personal communication). This mutation in the M subunit may also be part of the C-terminal domain identified in this work as forming contacts with the other subunits of the EcoKI mtase.

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