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Analysis of the subunit assembly of the type IC restriction–modification enzyme EcoR124I

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ABSTRACT

Type I restriction–modification (R–M) enzymes are composed of three different subunits, of which HsdS determines DNA specificity, HsdM is responsible for DNA methylation and HsdR is required for restriction. The HsdM and HsdS subunits can also form an independent DNA methyltransferase with a subunit stoichiometry of M₂S₁. We found that the purified EcoR124I R–M enzyme was a mixture of two species as detected by the presence of two differently migrating specific DNA–protein complexes in a gel retardation assay. An analysis of protein subunits isolated from the complexes indicated that the larger species had a stoichiometry of R₁M₂S₁, and the smaller species had a stoichiometry of R₁M₂S₁. In vitro analysis of subunit assembly revealed that while binding of the first HsdR subunit to the M₂S₁ complex was very tight, the second HsdR subunit was bound weakly and it dissociated from the R₁M₂S₁ complex with an apparent Kₐ of −2.4 × 10⁻⁷ M. Functional assays have shown that only the R₂M₂S₁ complex is capable of DNA cleavage, however, the R₁M₂S₁ complex retains ATPase activity. The relevance of this situation is discussed in terms of the regulation of restriction activity in vivo upon conjugative transfer of a plasmid-born R–M system into an unmodified host cell.

INTRODUCTION

Type I restriction and modification (R–M) systems protect the bacterial cell against invasion of foreign DNA (such as viruses) by cleaving DNA which lacks a target specific N6-adenine methylating. The second physiological role of these systems is to restore full methylation of the target sites on the host DNA after DNA replication. Type I R–M enzymes are composed of three different subunits (HsdR, HsdM and HsdS) encoded by the three hsd genes (for recent reviews see 1,2). All three subunits are absolutely required for restriction activity, while the HsdM and HsdS subunits are sufficient for modification activity and can also form an independent DNA methyltransferase (MTase). Type I R–M enzymes specifically recognise a non-palindromic DNA sequence (e.g. GAAnnnnnnRTCG for EcoR124I, where n is any base and R is a purine) but cleave DNA at a non-specific site distant from the recognition sequence using Mg²⁺, S-adenosylmethionine (AdoMet) and ATP as cofactors (3,4). Binding of the endonuclease to a non-modified recognition site activates a powerful ATPase activity (5), which fuels DNA translocation past the DNA–enzyme complex, while the enzyme remains bound to the recognition site (6,7). DNA is cleaved at positions where the DNA translocation stops due to a collision of two translocating enzyme molecules on two-site, linear DNA substrates (8,9), or due to the build-up of topological strain on circular molecules (10). The endonuclease does not turnover in the cleavage reaction, however, the ATPase activity continues for a long period of time after the cleavage is completed (3,5). DNA methylation activity of the type I R–M systems results in a transfer of a methyl group from AdoMet to the N-6 position of a specific adenine in each strand of the recognition sequence (11,12).

Type I R–M systems are grouped into four families based on allelic complementation, protein homologies and biochemical properties of the enzymes. Types IA, IB and ID R–M systems are chromosomally encoded (13,14) while most type IC R–M systems are carried on large conjugative plasmids (15–17). The type IA family is typified by the EcoKI and EcoBI enzymes, type IB by EcoAI and type IC by EcoR124I. EcoKI forms a stable R₂M₂S₁ complex; the independent EcoKI MTase (M₂S₁) is, however, a relatively weak complex, dissociating into an inactive M₁S₁ species and free HsdM subunit (18–20). The purified EcoBI restriction endonuclease has been described as existing in a number of different stoichiometric forms including R₂M₂S₁, R₁M₂S₁ and R₁M₁S₁ (21). The type IB restriction endonuclease EcoAI was found to be a weak complex that dissociated into MTase and HsdR subunit when purified, and the active endonuclease could only be studied following assembly in vitro (22). We have recently purified the EcoR124I restriction endonuclease from a cell culture harbouring a recombinant, two-plasmid system, which over-produces all three subunits. The stoichiometry of this endonuclease preparation appeared to be R₂M₂S₁ (23). The EcoR124I MTase has also been over-produced and purified to homogeneity (12,24). This enzyme was found to exist only in the M₂S₁ stoichiometry and no M₁S₁ complex has been detected (12).

The hsd genes of all R–M systems can be transferred into an unmodified host by conjugation, transformation or transfection despite the fact that the presence of a restriction enzyme in the recipient cell would be a lethal event (17,23,25–31). It has been reported that the appearance of restriction activity was delayed

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after conjugative transfer of EcoKI genes into an unmodified host, while the modification activity was expressed immediately after the conjugation (29). It was proposed that restriction and modification activities of the EcoKI–R–M system are regulated by a gene distant from the _hsd_ locus since the conjugative transfer of EcoKI _hsd_ genes into a mutant form of _Escherichia coli_ C (designated as _hsdC_) had a lethal effect on the cell (29). The same regulation mechanism was found to be employed in the type IB R–M systems (32). However, the conjugal transfer of _EcoR124I_ (IC) _hsd_ genes into the _hsdC_ mutant was not lethal and full restriction activity was detected six generations after the start of conjugation (32). Recently, it has been shown that ClpX and ClpP, components of the ClpXP protease, are necessary for efficient transmission of genes encoding the EcoKI and EcoAI R–M systems, suggesting protease-mediated post-translational control of restriction and modification (33). The involvement of protease control of restriction and modification would support the proposal that post-translational control of restriction activity of EcoKI could be related to subunit assembly (20).

In this paper we show that the purified _EcoR124I_ restriction endonuclease is a mixture of two species, which have a subunit stoichiometry of R₂M₂S₁ and R₁M₂S₁, respectively. Only the former species was found to have endonuclease activity. However, the R₂M₂S₁ complex is relatively weak, dissociating into free HsdR subunit and the restriction-deficient R₁M₂S₁ assembly intermediate, which appears to be a very tight complex. We discuss the relevance of this situation with respect to the propagation of a conjugative plasmid-borne R–M system into an unmodified host cell. We suggest that control of _EcoR124I_ endonuclease activity in _vivo_ is at the level of subunit assembly in a manner similar to that observed for _EcoKI_ (20).

**MATERIALS AND METHODS**

**DNA substrates**

Complementary HPLC-purified oligonucleotides used in the gel retardation assays were purchased from Cnachem Ltd. The _EcoR124I_ recognition site is shown in bold (top strand: 5′-CTACGGTACCGAAAAACGGTTGCGGCCCCCGCACG¬TTGC-3′), DNA concentration was determined from the absorption at 260 nm. The extinction coefficients of the oligonucleotides and the annealed oligoduplex were calculated as a sum of the contributions from individual nucleotides, taking into account hyperchromicity observed after digestion of the DNA to completion with snake venom phosphodiesterase (34). The oligoduplex (usually 2.5 µg) was 5′-end-labelled using [γ-32P]ATP (25 µCi) and T4 polynucleotide kinase. Unincorporated label was removed using ‘Nuctrap’ columns (Stratagene).

A 2891 bp plasmid containing a single _EcoR124I_ recognition site, pDRM-1R, (23) used in DNA cleavage assays was produced from _E.coli_ HB101 (35) and its covalently closed form was isolated of the DNA–protein complexes, the best fit between their densitometer traces of individual subunits (S:M:R) was calculated using Image Grabber 2.05b1 and NIH image 1.52 software. The ratio of the absorbance at 280 nm using molar extinction coefficients calculated as a sum of contributions from tyrosines and tryptophans in the predicted amino acid sequences [HsdR subunit; 91 900/M/cm, MTase (M₂S₁); 160 400/M/cm, endonuclease, 252 300/M/cm for R₁M₂S₁, 344 200/M/cm for R₂M₂S₁].

**Reconstitution of the endonuclease**

Fully active reconstituted _EcoR124I_ endonuclease, used in the DNA cleavage and gel retardation assays, was simply produced by mixing of the purified MTase (M₂S₁) and HsdR subunit in assay reaction buffers (see below). The concentration of active endonuclease was taken as the input concentration of the MTase.

**Determination of subunit ratio in the endonuclease–DNA complexes**

The two DNA–protein complexes observed with purified and reconstituted _EcoR124I_ endonucleases (Fig. 1) were separated in preparative amounts (2 µM 39mer DNA in 10 µl binding reaction) on a non-denaturing polyacrylamide gel. After staining the gel in ethidium bromide solution, fluorescent bands were excised from the gel, mashed and protein extracted with SDS–PAGE loading buffer. Protein extracts were subjected to SDS–PAGE together with a series of R/MTase mixtures of known molar ratios. After staining with Coomassie Blue R250, the gel was quantified by densitometry using Image Grabber 2.05b1 and NIH image 1.52 software. The ratio of densitometer traces of individual subunits (S:M:R) was calculated for each sample. To determine the stoichiometry of the species isolated of the DNA–protein complexes, the best fit between their S:M:R ratios and one of the values obtained with the R/MTase mixtures was determined.

**HPLC gel filtration**

HPLC gel filtration used a Rainin Dynamax 4.6 × 250 mm Hydropropore-5-SEC column and a guard column. Samples of 20 or 50 µl, in 20 mM Tris, 20 mM 2-[N-morpholine] ethane sulphonic acid (MES), 0.2 M NaCl, 10 mM MgCl₂, 7 mM β-mercaptoethanol, 0.1 mM EDTA, pH 6.5, were injected onto the column. The samples were equilibrated at room temperature for varying time periods up to 24 h prior to injection. The flow rate was 0.5 ml/min and detection was most sensitive and stable at 254 nm. The column was calibrated with a series of globular proteins of known molecular weight giving a linear calibration curve of log (molecular weight) as a function of elution time. Most of our samples after buffer exchange into the column buffer using PD10 Sephadex G50 columns (Pharmacia), contained trace amounts of a small molecule which we believe to be glycerol. This gave rise to a ‘solvent peak’ after ~6.5 min, the elution time of which served as an internal standard to correct for slight run to run variation in protein elution times. The apparent molecular weight of the proteins eluting from the column was calculated in the
titration of MTase with HsdR subunit, the HsdR subunit–MTase complex was present only as a partially resolved shoulder when HsdR subunit was in large excess of the MTase.

**DNA cleavage and ATPase assays**

DNA cleavage and ATPase activities of EcoR124I restriction endonuclease were assayed using plasmid pDRM-1R (single EcoR124I site) as described previously (23). DNA, usually at a concentration of 200 nM, was incubated either with an equimolar concentration of the MTase and increasing concentration of the HsdR, or with a range of concentrations of reconstituted endonuclease. After addition of ATP, the reactions were incubated for 5 min (sufficient time to reach the reaction endpoint). DNA cleavage activity (concentration of full-length linear plasmid DNA) and ATPase activity (concentration of inorganic phosphate) were measured as described previously (23).

**Gel retardation assay**

DNA binding reactions were usually performed in a volume of 10 µl in the presence of a buffer consisting of 50 mM Tris–HCl (pH 8.0), 50 mM NaCl, 10 mM MgCl₂, 1 mM DTT and 10% (v/v) glycerol. The end-labelled 39mer oligoduplex (see above) of known concentration was incubated with purified endonuclease, MTase and MTase/HsdR or endonuclease (E/Nase)/HsdR mixtures over a range of various concentrations and/or protein:protein ratios, respectively, at room temperature for 10 min. Unbound DNA and DNA–protein complexes were separated on a 5% non-denaturing polyacrylamide gel run, at 4°C, at 100 V in 40 mM Tris-acetate pH 8.0, 1 mM EDTA buffer (TAE). After electrophoresis, gels were dried and subjected to autoradiography or quantified using a PhosphorImager and Image Quant software.

**RESULTS**

**Gel retardation analysis of EcoR124I endonuclease reveals two molecular species**

We have previously purified the EcoR124I endonuclease following over-production of the protein by a recombinant, two-plasmid system consisting of pJS4M (MTase) and pACR124 (HsdR). Using a combination of data from gel filtration and densitometric scanning SDS–polyacrylamide gels stained with Coomassie Blue, we concluded that this enzyme had a stoichiometry of R₁M₂S₁ (23).

To investigate the DNA-binding properties of the over-produced EcoR124I endonuclease, the technique of gel retardation, with a 39mer oligoduplex carrying a single EcoR124I recognition site, was utilised. Unexpectedly, two DNA–protein complexes were observed after separation of bound and unbound DNA on a non-denaturing polyacrylamide gel (Fig. 1, lane 2). The complex with faster mobility (complex I) was preferentially formed (88% of bound DNA). When the plasmid pBGSR124 (23), a higher copy number plasmid, which produces a significantly higher level of HsdR than the plasmid pACR124, was used with the plasmid pJS4M in the two-plasmid over-producing system, the enzyme preparation showed a different ratio between the two complexes; 53% of complex I and 47% of complex II (Fig. 1, lane 4). To exclude non-specific DNA binding, the experiment was repeated in the presence of excess competitor DNA (poly dI–dC), but no change was observed in the two retarded DNA bands (data not shown).

Another possibility was that there were two stoichiometric species present in the endonuclease preparations. This was confirmed by the following results. Addition of excess HsdR to both endonuclease preparations resulted in the disappearance of the complex I and increased the concentration of the complex II (Fig. 1, lanes 3 and 5).

A 1:1 mixture of HsdR and the MTase (M₂S₁) produced only complex I, while the 6:1 mixture of HsdR and the MTase produced mostly complex II (Fig. 1, lanes 8 and 9). Finally, addition of the MTase to the mixture of the endonuclease and DNA led to the disappearance of complex II and increased the amount of complex I (Fig. 1, lane 6). These results indicate that the two observed species vary in the number of HsdR subunits, and the lower molecular weight species is preferentially formed at HsdR:MTase ratios < 1.

**Analysis of subunit stoichiometry in protein–DNA complexes of EcoR124I**

To investigate the stoichiometry of the two species present in the endonuclease preparations, the proteins bound in complexes I and II were extracted from the retardation gel and subjected to an SDS–PAGE analysis together with a series of R/MTase mixtures, including ratios 0.5:1, 1:1, 2:1, 3:1 and 4:1. The relative ratio of the three endonuclease subunits, in each sample, was determined from a densitometer scan of the polyacrylamide gel after staining the gel with Coomassie Blue (23). As can be seen in Figure 2, the ratio of subunits in the protein extracted from the complex I agrees with the subunit ratio in the 1:1 mixture of HsdR and MTase and the protein from complex II corresponds with the 2:1 mixture. Thus the protein species bound in complex I has a stoichiometry of R₁M₂S₁ and that bound in complex II has a stoichiometry of R₂M₂S₁.

**Analysis of interaction between the MTase and HsdR in the presence of DNA**

To investigate the endonuclease assembly in vitro, we performed a reconstitution titration of the MTase (M₂S₁) with the HsdR subunit, and followed this by a gel retardation assay using a 39mer oligoduplex carrying the EcoR124I recognition site.
Apparent molecular weight by

Expected molecular

162.2

401.5

220–230

380–420

dissociation into R 1 M 2 S 1 and HsdR within the gel matrix. 

subunit complexes present in a series of dilutions of a 1:2 mixture

suggests that the R 2 M 2 S 1 species is a much weaker complex than

a smear on the gel, lying between the two complexes. This

molar ratio of 2, and even above the molar ratio of 3 there was still

unpublished observations). The R 2 M 2 S 1–DNA complex started to

have been observed for

25% would be occupied with two HsdR subunits and 25% MTase

ratio of 1, 50% MTase would be occupied with one HsdR subunit,

ratio of 1, 50% MTase would be occupied with one HsdR subunit,

complex I. At an HsdR:MTase (R:M 2 S 1 ) molar ratio of 1, only

of complex I. As can be seen, increased concentration of HsdR resulted

in the disappearance of the M 2 S 1–DNA complex and appearance of

complex I. At an HsdR:MTase (R:M 2 S 1 ) molar ratio of 1, only complex I was present. This suggests that one of the two R-binding sites in the MTase is stronger than the other one since in the case of two equivalent binding sites, at an HsdR:MTase ratio of 1, 50% MTase would be occupied with one HsdR subunit, 25% would be occupied with two HsdR subunits and 25% MTase would bind no HsdR subunit. Equivalent binding sites for HsdR have been observed for EcoKI (L.Powell and D.Dryden, unpublished observations). The R 2 M 2 S 1–DNA complex started to appear only when the HsdR:MTase ratio was >1; while under these conditions the R 1 M 2 S 1–DNA complex disappeared. However, the R 2 M 2 S 1–DNA complex was not fully present at an HsdR:MTase molar ratio of 2, and even above the molar ratio of 3 there was still a smear on the gel, lying between the two complexes. This suggests that the R 2 M 2 S 1 species is a much weaker complex than the R 1 M 2 S 1 species and that the smear corresponds to its dissociation into R 1 M 2 S 1 and HsdR within the gel matrix.

In order to measure an apparent dissociation constant character-

izing the equilibrium between R 1 M 2 S 1 and R 2 M 2 S 1 species, the subunit complexes present in a series of dilutions of a 1:2 mixture

of the MTase and HsdR, starting from a protein concentration of

1 mM, were analysed by gel retardation assay in the presence of

20 nM 39mer oligoduplex. Figure 4a shows that at a protein concentration of 1 mM, the R 2 M 2 S 1 species was almost exclusively present. However, a faint band appeared on the top of the gel at high protein concentrations apparently due to protein aggregation or non-specific binding of a second endonuclease molecule. When the protein was diluted, the R 2 M 2 S 1 species dissociated to R 1 M 2 S 1 (seen as an increased concentration of the R 1 M 2 S 1–DNA complex) which appeared to be stable in the concentration range used in the experiment (10⁻⁶–10⁻⁹ M). The retardation gel was quantified (Materials and Methods) and the percentage of the R 2 M 2 S 1–DNA complex of the total bound DNA was plotted as a function of protein concentration (Fig. 4b). Only data for protein concentrations >20 nM were used for plotting since at lower concentrations dissociation of the DNA–protein complex occurred (Fig. 4a). An apparent equilibrium dissociation constant of the R 2 M 2 S 1 complex was determined as the concentration of enzyme where 50% of R 2 M 2 S 1–DNA was dissociated to R 1 M 2 S 1–DNA. The value was estimated at ~2.4 × 10⁻⁷ M. Similar dilution experiments were also performed with a 1:1 mixture of MTase and HsdR (containing only R 1 M 2 S 1). No dissociation of R 1 M 2 S 1 complex into the MTase and HsdR was observed even at the lowest protein concentrations detectable by the gel retardation assay (10⁻⁹ M) (not shown). Therefore, the R 1 M 2 S 1 species is apparently a very stable complex.

Table 1. Molecular weights of complexes found for the EcoR124I system

<table>
<thead>
<tr>
<th>Sample</th>
<th>Apparent molecular weight by HPLC gel filtration (kDa)</th>
<th>Expected molecular weight (kDa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>M 2 S 1 MTase</td>
<td>220–230</td>
<td>162.2</td>
</tr>
<tr>
<td>HsdR subunit</td>
<td>137–148</td>
<td>119.5</td>
</tr>
<tr>
<td>In vivo assembled R 1 M 2 S 1</td>
<td>280–300</td>
<td>281.8</td>
</tr>
<tr>
<td>In vivo assembled mixture of R 1 M 2 S 1 + R 2 M 2 S 1</td>
<td>333–350</td>
<td>281.8 + 401.5</td>
</tr>
<tr>
<td>In vitro assembled R 2 M 2 S 1 (freshly prepared)</td>
<td>290–315</td>
<td>401.5</td>
</tr>
<tr>
<td>In vitro assembled R 2 M 2 S 1 (after 24 h incubation)</td>
<td>380–420</td>
<td>401.5</td>
</tr>
</tbody>
</table>

aProduced by the pJS4M-pACR124 plasmid system.

bProduced by the pJS4M-pBGS124 plasmid system.

Figure 2. Subunit composition of the gel-retarded protein–DNA complexes of EcoR124I. The histograms compare relative ratios of subunits extracted from the two DNA–protein complexes of purified EcoR124I endonuclease produced by the pJS4M-pBGS124 plasmid system (complexes I and II shown in Fig. 1) to the relative ratios of subunits in a series of mixtures of HsdR and MTase, as determined by densitometric scanning of SDS-PAGE gels stained with Coomassie Blue. White bars, HsdS; grey bars, HsdM; black bars, HsdR. Panels 1–5 on the x-axis correspond to the following HsdR:MTase ratios: 0.5:1, 1:1, 2:1, 3:1, 4:1. Panels 6 and 7 are complex I and complex II, respectively. The data suggest that the stoichiometry of the species in complex I is R 1 M 2 S 1, and the stoichiometry of the species in complex II is R 2 M 2 S 1. 

Figure 3. Titration of EcoR124I MTase with HsdR followed by gel retardation assay. A mixture of 200 nM MTase and 200 nM 39mer oligoduplex, containing one EcoR124I recognition site, was incubated with increasing concentrations of HsdR subunit at room temperature for 10 min. DNA–protein complexes and free DNA were separated on a non-denaturing polyacrylamide gel as described in Materials and Methods. The HsdR:MTase ratio in individual reactions and the position of DNA–protein complexes on the gel are indicated.
Analysis of interactions between the MTase and HsdR in the absence of DNA

To analyse endonuclease assembly in the absence of DNA, we used HPLC gel filtration. The apparent molecular weights of various subunit complexes of EcoR124I from an HPLC gel filtration column were determined from their elution times and are given in Table 1. It can be seen that the MTase and HsdR subunit eluted from the column earlier than expected, giving a slightly higher weight. Similar behaviour was observed for the EcoKI enzyme. The endonuclease purified from cells containing the pJ34M-pACRB124 plasmid-expression system had an apparent mass intermediate between R1M2S1 and R2M2S1 and formed a slightly asymmetric peak suggesting that it was a mixture of these two species. A freshly prepared mixture of MTase with a 5-fold excess of HsdR subunit, eluted with an apparent weight close to that expected for R1M2S1. However, the same mixture after a 24 h incubation at room temperature showed the formation of a higher molecular weight species with a weight equal to that expected for the R2M2S1 endonuclease. Figure 5a shows the elution profiles of the HsdR subunit, MTase and a mixture of these two components freshly prepared and after 24 h. The titration of a constant concentration of MTase with the HsdR subunit also showed the dramatic change in molecular weight of the complex upon prolonged incubation (Fig. 5b). Therefore, HPLC data indicated that the formation of the R2M2S1 complex is a slow process under the conditions used. One possible explanation of this observation is that the presence of 5% glycerol in the samples slows diffusion; although it seems unlikely that this would be a large effect. Alternatively, it appears likely that the presence of DNA in the sample grossly changes the rate of attainment of thermodynamic equilibrium. This idea is supported by the fact that DNA cleavage appeared in a very short time following mixing of MTase and HsdR in the presence of DNA (see the next section).

DNA cleavage and ATPase activities of MTase–HsdR complexes

To investigate enzyme activities of the R1M2S1 and R2M2S1 species, DNA cleavage and ATP hydrolysis were assayed following a reconstitution titration of the MTase with HsdR in the presence of a plasmid DNA substrate. The DNA used contained a single EcoR124I recognition site. This DNA readily undergoes primary cleavage to produce full-length linear DNA. Secondary processing of the linear product is observed only at very high enzyme:DNA ratios. Reaction products (linear DNA and inorganic phosphate, respectively) were quantified by densitometric scanning of agarose gels and malachite green assay as described elsewhere. A gradually increasing concentration of HsdR subunit was added into a 1:1 mixture of the MTase and DNA, which were present at a concentration of 200 nM to ensure full DNA binding. The same concentration conditions were also used in the reconstitution titration followed by a gel retardation assay (Fig. 3). At an HsdR:MTase ratio <1, where only the R1M2S1 complex was detected by a gel retardation assay, no DNA cleavage occurred (Fig. 6a). However, ATPase activity gradually increased with HsdR concentration (Fig. 6b). It is important to note that the HsdR subunit by itself shows only a weak DNA/A/Mg2+-dependent ATPase activity which is 100 times lower than that of theendonuclease. DNA cleavage appeared only when the R2M2S1 complex was formed (Fig. 6a). Both activities approached saturation level at an HsdR:MTase ratio of 4–5, where the gel retardation assay indicated that the R2M2S1 species was fully present. These data indicate that only the R2M2S1 species is capable of DNA cleavage.

DISCUSSION

The gel retardation and DNA cleavage experiments presented in this work revealed that the active form of the type IC EcoR124I restriction endonuclease has a subunit stoichiometry of R2M2S1. It is assembled from the trimeric MTase (M2S1) and the HsdR subunits through a stable intermediate with a stoichiometry of R1M2S1, which does not cleave DNA. While binding between the MTase and the first HsdR subunit is apparently very stable,
Figure 5. Elution profiles from the HPLC gel filtration column and titration of MTase with HsdR followed by HPLC gel filtration. (a) From left to right the traces show MTase (420 nM), HsdR subunit (1 µM), MTase (420 nM) freshly mixed with a 5-fold excess of HsdR subunit, and MTase (420 nM) incubated with a 5-fold excess of HsdR subunit for 24 h before application to the column. The numbers are the elution times, in minutes, of the peaks. (b) The molecular weight, determined by HPLC gel filtration, of the complex formed between MTase and HsdR subunit as the HsdR subunit concentration is increased. The MTase concentration was 420 nM throughout. The samples were applied to the column immediately after mixing (□) or after 24 h incubation (○). The lines drawn are only to guide the eye.

Our results indicate that the two HsdR subunits interact differently with the MTase in the assembly of the EcoR124I endonuclease. A simple model for assembly would be that there are two equivalent HsdR-binding sites on the MTase, but that the presence of one bound HsdR subunit affects the binding of the second HsdR subunit perhaps by partially blocking the second site, or by means of a conformational change at the second site. This model would require that the R1M2S1 complex should be a 1:1 mixture of two species with occupation of either of the two

interaction between the R1M2S1 intermediate and the second HsdR subunit is much weaker. The R2M2S1 complex dissociated into the R1M2S1 complex and the HsdR subunit with an apparent Kd of ∼2.4 × 10−7 M (Fig. 4).

In contrast to EcoR124I, the R2M2S1 complex of EcoKI endonuclease, a member of the type IA family, is relatively stable and both HsdR subunits appear to be bound to the MTase with similar affinities (20; L.Powell and N.Murray, personal communication).

Our results indicate that the two HsdR subunits interact differently with the MTase in the assembly of the EcoR124I endonuclease. A simple model for assembly would be that there are two equivalent HsdR-binding sites on the MTase, but that the presence of one bound HsdR subunit affects the binding of the second HsdR subunit perhaps by partially blocking the second site, or by means of a conformational change at the second site. This model would require that the R1M2S1 complex should be a 1:1 mixture of two species with occupation of either of the two

HsdR-binding sites. A second model would require that the two sites are not equivalent and that the assembly is inherently asymmetric with only one type of R1M2S1 complex being formed. However, the data presented in this work are not sufficient to definitely determine which model is applicable.

Since binding of the first HsdR subunit to the MTase is much stronger than binding the second HsdR subunit to the R1M2S1 intermediate, the in vivo formation of the R2M2S1 complex will be dependent on the ratio between the HsdR subunit and the MTase in the cell. At an HsdR:MTase ratio of <1, only the R1M2S1 complex will be present. The R2M2S1 complex will be formed only when the HsdR:MTase ratio is >1. Indeed, when the ratio between MTase and HsdR was varied in vivo by using different recombinant two-plasmid systems, the resulting endonuclease preparations differed in ratio between the species (Fig. 1). The purified endonuclease produced from hsd genes under the control of their natural promoters was also found to be a mixture of R1M2S1 and R2M2S1 species (J.Dreier, personal communication). This shows that the heterogeneity of the stoichiometry of EcoR124I endonuclease assembled in vivo was not a result of using an artificial expression system. The presence of two non-equivalent binding sites for HsdR has also been shown using the technique of surface plasmon resonance (38). This

Figure 6. Titration of EcoR124I MTase with HsdR, followed by measurement of DNA cleavage and ATPase activity. A mixture of 200 nM MTase and 200 nM pDRM-1R DNA was incubated with increasing concentrations of HsdR in the presence of 0.2 mM AdoMet and 10 mM ATP. Concentration of linear DNA produced after 5 min incubation (□) and the rate of ATP hydrolysis (○) was plotted against HsdR:MTase molar ratio. Reaction products were quantified as described elsewhere (23). The lines drawn are only to guide the eye.
clearly supports the data presented in this paper and has shown that the two sites differ in binding affinity by at least two orders of magnitude. Since two separate techniques have demonstrated the equilibrium between the R2M2S1 and R1M2S1 forms of the endonuclease, we believe the slow assembly of the R2 complex in the absence of DNA, as measured by HPLC gel filtration, either reflects the experimental conditions used, or the absence of DNA greatly decreases the rate for attainment of thermodynamic equilibrium.

Why should the restriction endonuclease of the EcoRI241 R–M system be such a weak complex and the stable assembly intermediate R1M2S1 not be able to cleave DNA? We suggest this may reflect a very sensitive mechanism for regulation of restriction activity following conjugative transfer of the hsd genes into a non-modified recipient cell. The EcoRI241 R–M system was originally found on the conjugal plasmid R124 (16) and this presents an unusual problem for the recipient cell. If the R124 plasmid entered a new host and immediately produced an active endonuclease, the unmodified host DNA would be restricted and the host cell would not survive. However, this does not happen and the host chromosome is modified following conjugation. Despite various attempts to show transcriptional or post-transcriptional control of this phenomenon for the EcoRI241 R–M system (32; K.Firman, unpublished observations), there is no evidence of such control. Nor is there any evidence of such control for the other type I R–M systems. Therefore, we propose that this control is exercised at the level of subunit assembly. The M2S1 MTase is a very tight complex and it will be assembled first in the cell. Since the MTase can form a tight complex with one HsdR subunit, which does not cleave DNA, all the HsdR subunits will initially be trapped in this inactive complex. Therefore, the MTase (and possibly the R1M2S1 complex) can modify and protect the host DNA. The weak endonuclease complex R2M2S1 can be formed only when a large amount of HsdR has built up in the cell, which is unlikely during the early stage of establishing the R–M system in a new host. When cellular levels of individual subunits were altered by using different promoters and expression vectors, it was observed that excess HsdR over MTase was lethal, unless the MTase was already established in the cell (23). This implies that the function of the EcoRI241 endonuclease in the cell is finely balanced and absolutely dependent upon subunit concentration. Control of restriction and modification via subunit assembly has also been recently proposed for the EcoK restriction endonuclease (20), suggesting this mechanism may be common to all type I systems. In addition, the EcoRI241 system shows a relatively high methylation activity with non-modified DNA (23) when compared to that of EcoKI (11). This will also ensure that a non-modified chromosome in the recipient cell can be readily modified following conjugal transfer of the hsd genes.

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REFERENCES