Purification and properties of the *Mbo*II, a class-IIS restriction endonuclease

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ABSTRACT

After five purification steps a homogeneous preparation of endonuclease *Mbo*II was obtained, and several properties of the enzyme were determined. *Mbo*II is a monomer, with Mr under native and denaturing conditions being $47-49 \times 10^3$ Da. Endonuclease *Mbo*II is a basic protein (pI 8.3) which remains active when Mg^{2+} is replaced by Mn^{2+}, Co^{2+}, Ca^{2+}, or Fe^{2+}. *Mbo*II exhibits a star activity in the presence of some of the following reagents or ions: DMSO, glycerol, ethanol (and Co^{2+} or Mn^{2+} at pH 6). *Mbo*II does not bend DNA and is heat sensitive, losing activity after 15 min at 50°C.

INTRODUCTION

Class II-S is a group of 32 restriction enzymes which have a number of common features (see ref.1 for a review). Most of them recognize asymmetric recognition sequence, which indicates that they might act as monomers and they cleave DNA at a specified distance from their recognition sequence (1,2). The enzymes which have been studied bind to their recognition sequence even when the DNA has previously been cleaved, indicating that binding and cleaving domains have separate functions (3–5). Not much is known about their physico-chemical properties; five of them have been described as monomers, the increase in molecular weight appears to be directly proportional to the distance between the recognition and cleavage sites (1,2). The most intensively studied enzyme from this group is *Fok*I (1–9), and its properties seem to be analogous to those found for *Mbo*II. Whereas the *Fok*I R-M system has been cloned in a number of laboratories, (6,9; T. Kaczorowski, unpublished results; L. Wu and S. Chandrasegaran, unpublished results), *Mbo*II had not been cloned until recently (10). Endonuclease *Mbo*II (ENase), together with a number of other class-IIS enzymes, was used for numerous applications which are based on unique properties of these enzymes (1,11–13).

The aim of the present study was to evaluate several physico-chemical and enzymatic properties of *Mbo*II in order to compare them with other class-IIS endonucleases.

MATERIALS

Bacterial strains and plasmids

Moraxella bovis strain ATCC 10900 was obtained from Dr E.Falsen (University of Göteborg, Sweden). The following medium was used for bacterial growth: 1% trypton, 0.5% yeast extract, 0.2% lactic acid, 0.2% NaCl, 0.44% Na_2HPO_4 pH 7.2. The early stationary phase bacteria were stored as a cell paste at −70°C. The following plasmids were used in this work: pNH20 (12) and pBend2 (14).

Enzymes and Proteins

All restriction enzymes were purchased from BRL, T4 polynucleotide kinase, T4 DNA ligase, mung bean nuclease were from New England Biolabs. Calf intestinal alkaline phosphatase was obtained from Boehringer Mannheim. *Escherichia coli DnaK* protein was from J.Osipiuk (University of Gdansk, Poland). Protein standards used in polyacrylamide electrophoresis and isoelectric focusing originated from Pharmacia, those used in gel filtration and sedimentation were from Boehringer Mannheim.

DNA

λ DNA purification, plasmid isolation and DNA fragments electroelution were done as described by Sambrook *et al.*, (15).

METHODS

Purification of *Mbo*II Endonuclease
*Mbo*II enzyme was purified in five steps, each carried out at 2–6°C.

Step I-Sonication of bacteria. M.bovis frozen cell paste (200 g) was suspended in 600 ml of 10 mM KPO_4 pH 7.5, 2 mM EDTA, 0.5 mM PMSF, 6.5 mM β-mercaptoethanol buffer then thawed at 4°C and sonicated.

Step II-Phosphocellulose chromatography. After centrifugation the supernatant (580 ml) was applied onto a 160 ml phosphocellulose (P-11, Whatman) column (5 cm×8 cm), previously equilibrated with buffer A, composed of 10 mM KPO_4 pH 7.5, 5% glycerol, 50 mM KCl, 6.5 mM β-mercaptoethanol, 1 mM EDTA. After washing with 1600 ml of

buffer A, the enzyme was eluted with a linear gradient of 50 to 600 mM KCl (1600 ml) in buffer A at a flow rate 50 ml/h. Fractions containing *Mbo*II enzyme were pooled (230 ml) and dialysed exhaustively against buffer B (2×2.5 l, overnight) composed of 5 mM KPO$_4$ pH 7.9, 5% glycerol, 6.5 mM β-mercaptoethanol, 1 mM EDTA, 0.01% Triton X-100 (Calbiochem) until the conductivity corresponding to 30 mM buffer was reached.

Step III-DEAE-cellulose chromatography. Dialysed active fractions were loaded on the DEAE-cellulose (DE-52, Whatman) column (26 cm × 15 cm, 90 ml volume), previously equilibrated with buffer B. The column was washed with 150 ml of buffer B at a flow rate 18 ml/h and then the enzyme was eluted with buffer B containing 50 mM KCl. Active fractions were collected (35 ml), and loaded directly on the next column.

Step IV-Blue Trisacryl chromatography. The Blue Trisacryl (IRF Biotechnics) column (2.6 cm × 10.5 cm—55 ml volume) was equilibrated with buffer C composed of 10 mM KPO$_4$ pH 7.5, 10% glycerol, 70 mM KCl, 6.5 mM β-mercaptoethanol, 1 mM EDTA, 0.01% Triton X-100, the protein was loaded and the *Mbo*II protein was eluted with a 500 ml linear gradient of 0−600 mM KCl in buffer C at flow rate 30 ml/h. The active fractions

were pooled, combined together (40 ml), and dialysed against 1 l of buffer C without Triton X-100 and KCl (buffer D).

Step V-CM-Sephadex chromatography. Dialysed preparation of *Mbo*II was applied onto a CM-Sephadex (Pharmacia) column (1.3 cm × 11.8 cm). After washing with two column volumes of buffer D, proteins were eluted with gradient 0 to 600 mM KCl (150 ml) in buffer D at a flow rate of 7.5 ml/h. Active fractions were analysed on 10% SDS-polyacrylamide gel. The homogeneous fractions were collected, concentrated with polyethyleneglycol 20000 (Serva). The final preparation of the enzyme was dialysed against a storage buffer containing 10 mM Tris-HCl pH 7.4, 50 mM KCl, 1 mM EDTA, 1 mM DTT, 50% glycerol (4.5 ml). *Mbo*II activity was determined in 10 mM Tris-HCl pH 7.4, 10 mM KCl, 10 mM MgCl$_2$ in a total volume of 20 μl for 1 h at 37°C. One unit of activity was defined as the minimal amount of enzyme required to completely digest 1 μg of lambda DNA in 1 h. Protein concentrations were determined by Bradford method (16), using a kit from Bio-Rad.

Gel electrophoresis

Enzyme purity was determined by polyacrylamide gel electrophoresis in the presence of SDS as described previously (17). The electrophoresis was carried out at 60 mA constant

Table I. Purification of *Mbo*II endonuclease from 200 g of *M.bovis*

Purification step	Total units ($\mu \times 10^3$)	Total protein (mg)	Specific activity (μ/mg)	Yield (%)	Purification factor
I. Crude lysate*	219	11300	19	100	1
II. Phosphocellulose	219	106	2070	100	108
III. DEAE-cellulose	136	32	4250	62	223
IV. Blue-Trisacryl	50.4	2.5	20200	23	1061
V. CM-Sephadex	22.5	0.135	166600	10	8768

* Because of the presence of nonspecific nucleases, exact determination of units of activity, in crude lysate, was not possible; therefore, the yield of *Mbo*II from phosphocellulose was arbitrarily set at 100%.
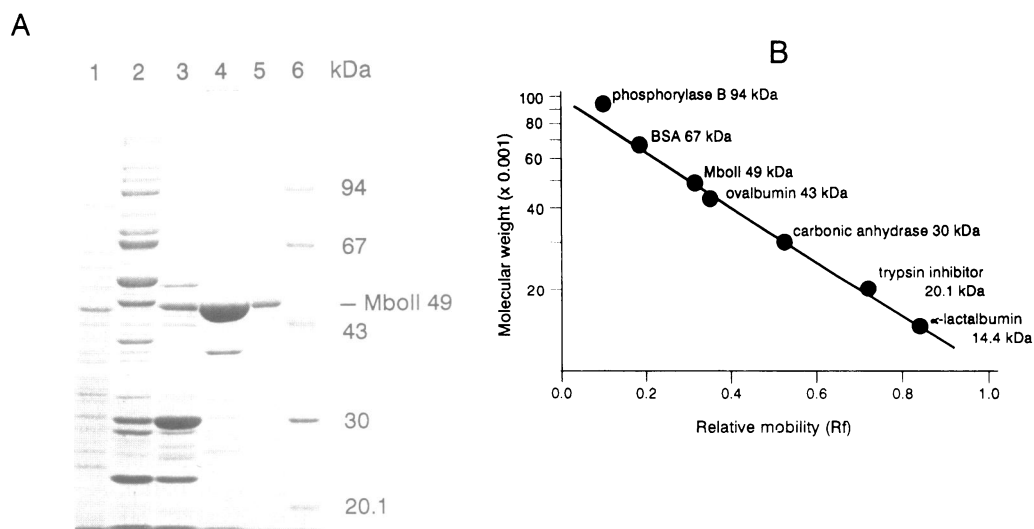
A

B

Figure 1. (A) Succesive steps in the purification of the *Mbo*II ENase. 10% SDS-polyacrylamide slab gel electrophoresis of: line 1: lysate after centrifugation; line 2: after P-cellulose chromatography; line 3:, after DEAE-cellulose chromatography; line 4: after Blue-Trisacryl chromatography; line 5: after CM-Sephadex chromatography; line 6:molecular weight standards. The gel was stained with Coomassie brilliant blue. (B) Determination of molecular weight of the *Mbo*II ENase under denaturing conditions. SDS-polyacrylamide gel electrophoresis (10%) of purified *Mbo*II protein in the presence of standard proteins.

power, then the gel was stained with Coomassie brilliant blue R-250. In order to estimate the molecular weight of *Mbo*II under denaturing conditions, 2.5 μg of purified enzyme was applied to 10% SDS-polyacrylamide gel together with protein standards. Isoelectric focusing on 5% polyacrylamide gel (0.2 mm× 10 mm×160 mm) was carried out in a pH gradient (3.5 to 10), using Pharmalyte (LKB) sixteen times diluted in 10% glycerol. As an anolyte we used 0.09 M H₂PO₄ and as a catholyte 0.1 M NaOH. After 40 min. prefocusing at 400−2500 V at 4°C, the samples (0.2 μg *Mbo*II) were focused at 1100−2500 V for 105 min. in the presence of protein standards. After focusing, the gel was fixed with 10% trichloroacid and 25% isopropanol solution and stained with Coomassie brilliant blue R-250.

The cross-linking reaction was performed essentially as described (18). The following proteins: *Mbo*II, *Dna*K and *Eco*RV were incubated at 30°C for 10 min in 25 μl reaction mixture in standard buffers. Glutaraldehyde (1.6 μl of 2.5% solution) was added to each reaction mixture and incubated for an additional 2 min at 30°C, then sodium borohydride (2.5 μl of 1 M solution) was added and incubation continued for another 20 min., at 4°C. The reaction was terminated by the addition of 8 μl 1 M Tris-HCl pH 7.5 and incubation for 5 min. at 4°C. Samples were electrophoresed in 5% SDS-polyacrylamide gel electrophoresis as described (19) at 110 mA for approximately 6 h, and silver stained (20).
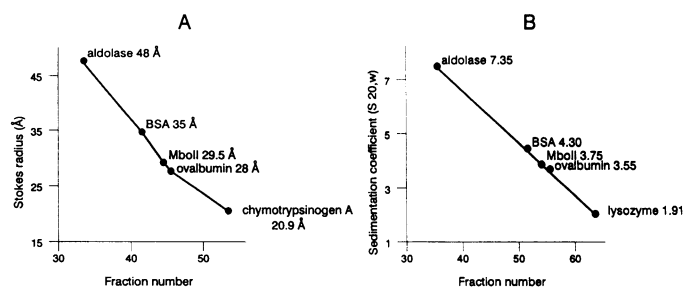

Figure 2. Determination of the subunit composition of the *Mbo*II ENase. (**A**) Estimation of Stokes radius of *Mbo*II enzyme by gel filtration on a Sephacryl S-200 column. The column was calibrated with proteins of known Stokes radius. (**B**) Estimation of sedimentation constant of *Mbo*II enzyme by glycerol gradient sedimentation (15−35%). Markers with known sedimentation coefficients ($s_{20,w}$) were used.
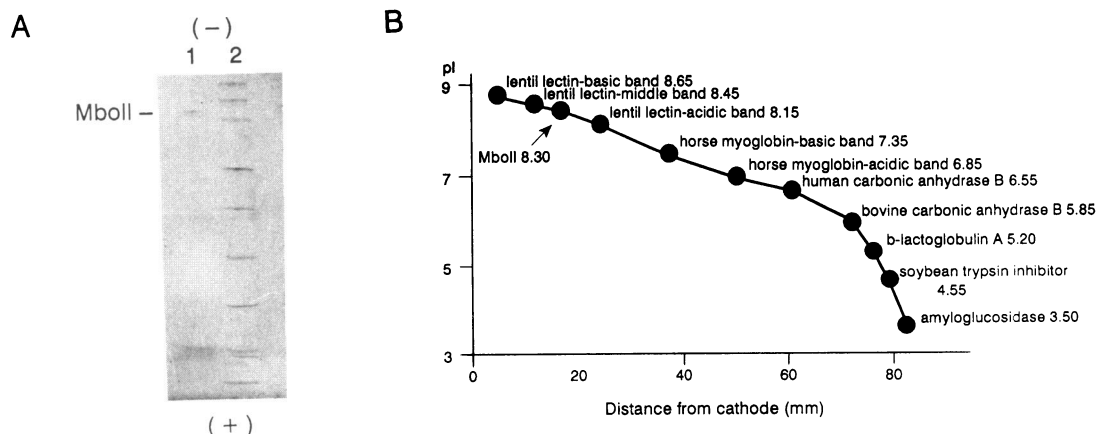
Determination of Stokes radius by gel filtration

A sample (0.5 ml) of purified *Mbo*II enzyme (10 μg protein) was loaded on a Sephacryl S-200 (Pharmacia) column (1.6 cm× 90 cm). The column was previously equilibrated with 20 mM KPO₄ pH 7.5, 200 mM KCl, 10 mM MgCl₂, 5% glycerol, 1 mM β-mercaptoethanol. The same buffer was used for elution of the protein at a flow rate of 5.4 ml/h. Fractions (1 ml) were collected and assayed for protein and enzymatic activity. The column was calibrated with proteins of known Stokes radius (22,23).

Glycerol gradient sedimentation

In order to estimate the sedimentation coefficient, purified *Mbo*II protein (3 μg) was sedimented along with various protein markers through an 11 ml linear 15−35% glycerol gradient (21) in 10 mM KPO₄ pH 7.0, 10 mM KCl, 10 mM MgCl₂, 1 mM β-mercaptoethanol in a Beckman SW41Ti rotor for 39 h at 36 rpm at 4.5°C. Samples (250 μl) were loaded on the top of the gradient (22,23). Fractions (78) were collected and the positions of the standard proteins were monitored by 10% SDS-polyacrylamide gel electrophoresis. Additionally, the fractions were assayed for *Mbo*II activity.

Star activity determination

1 μg of pNH20 plasmid (containing a single *Mbo*II site, 12) and 30 units of *Mbo*II enzyme were incubated with various amounts of organic solvents in 40 μl of standard buffer. Incubation was carried out for 4 h at 37°C. Samples were analysed by 2% agarose gel electrophoresis with ethidium bromide for star activity determination (15).

Construction of plasmid pBend2-44 for DNA bending determination

The pBend2 vector (14) was linearised by the *Xba*I and the cohesive ends were removed. The 44 bp *Kpn*I-*Bam*HI DNA fragment containing a single *Mbo*II site was excised from pNH20 plasmid (12), and, after removing the cohesive ends, inserted into dephosphorylated pBend2 vector. *E. coli* RR1 transformed with ligated DNA was plated on LB-agar containing ampicillin (100 μg/ml). Different DNA fragments containing a single GAAGA binding site were isolated by digesting of pBend2-44 with proper sets of restriction enzymes and the resulting DNA

Figure 3. (**A**) Determination of the isoelectric point of the *Mbo*II ENase. Isoelectric focusing was carried out on 5% polyacrylamide gel at 3.5−10 pH range. Line 1: *Mbo*II ENase; line 2: pI markers. (**B**) Estimation of the isoelectric point based on data from panel **A**.

fragments were isolated by gel electrophoresis and labelled at the 5' ends by T4 polynucleotide kinase. Binding of *Mbo*II to DNA wasperformed in 10 mM Tris-HCl pH 7.0, 10 mM KCl, 0.1 mM EDTA, and 50 μg BSA per ml. The incubation was at 21°C for 20 min. Nondenaturing gel electrophoresis of DNA-protein complexes was carried out on 5% polyacrylamide gel (30:0.4 w/w acrylamide:bis) in Tris-borate/EDTA buffer pH 8.3 at 4°C (15). The gel was dried and autoradiographed.

Other methods

Divalent cations requirement of the *Mbo*II endonuclease was investigated in two different buffers; in 10 mM bis-Tris pH 6.0 which prevents precipitation of the majority of metallic cations, and in 10mM bis-Tris pH 7.0 which is optimal for *Mbo*II digestion. In both cases digestion was carried out at 37°C in 15 μl reaction mixture for 3 h using 1 unit of *Mbo*II (9.2 ng) and 5 ng of 171 bp ^{32}P labelled DNA fragment. The 171 bp *Hae*III-*Hind*III DNA fragment (containing a single 5'-GAAGA site) was excised from the pNH20 (12). DNA labelling at 5' termini was done using [γ-^{32}P]ATP (5000 Ci/mmol, Amersham) and T4 polynucleotide kinase (15). Labelled DNA was purified on an NASC PREPAC column (BRL), according to the manufacturer's instruction. After incubation, the samples were analysed on 5% polyacrylamide gel in Tris-borate/EDTA buffer pH 8.3 and then dried and autoradiographed. Level of activity of the *Mbo*II enzyme in different digestion conditions was estimated by densitometry of the autoradiograms.

RESULTS AND DISCUSSION

Purification of *Mbo*II ENase

The purification procedure described in METHODS is summarized in Table I. As can be noticed, we did not follow the preliminary steps proposed by Gelinas *et al.*(24) and Bocklage *et al.*, (10)
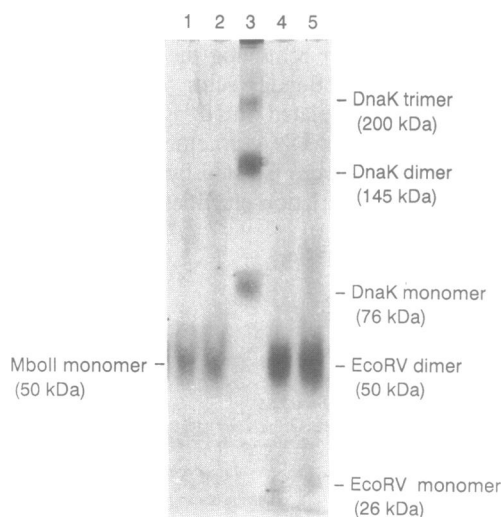
(streptomycine sulfate and ammonium sulfate treatment of crude lysate). The crude lysate obtained by us after sonication was directly applied onto a phosphocellulose column. Two peaks of endonucleolytic activity were observed. The first rather broad peak of *Mbo*I ENase was eluted between 100−200 mM KCl. The second, containing *Mbo*II ENase was covered by peaks of nonspecific nucleases and was eluted at about 250 mM KCl. During relatively long dialysis required for the KCl removal from the enzyme preparation, precipitation of hydrophobic proteins was observed. After short centrifugation the supernatant was applied to the next column containing DEAE cellulose. Optimalization of conditions for efficient binding of *Mbo*II to this bed is very critical. The best results were obtained at pH 7.9 and ionic strength below 30 mM. Denaturation of the enzyme observed at higher pH is consistent with the observation that proteins are usually unstable in solutions with pH close to their pI values. DEAE cellulose is known to cause electrostatic interactions between proteins and absorbent (Donnan effect,25) to prevent this, nonionic detergent (Triton X-100) was used. Elution of the *Mbo*II protein from a third bed, Blue-Trisacryl, required about 200 mM KCl concentration and 0.01% Triton X-100. During this step we were able to remove the majority of contaminating proteins, but the *Mbo*II ENase still contained traces of nonspecific nucleases. After the last step, CM-Sephadex chromatography (elution at 220 mM KCl), the *Mbo*II ENase was purified to homogeneity (with an overall yield of 10%) (Fig. 1A, line 5). No loss of activity was observed when the enzyme was concentrated and stored in 50% glycerol at −20°C.

Size and structure of the *Mbo*II enzyme

The *Mbo*II is a third enzyme belonging to class IIS, purified to homogeneity and characterized in our laboratory, the other two being: *Fok*I and *Mme*I. All three of them recognize asymmetric (5 and 6 nucleotides) DNA sequences but they differ in the distance from a recognition to a cutting site: *Mbo*II cleaves 8/7 nt from recognition sequence, *Fok*I 9/13, *Mme*I 18/20 (26−28).

Figure 4. Cross-linking of *Mbo*II enzyme with glutaraldehyde. The 5% SDS-polyacrylamide gel was silver stained. The gel shows a cross-linking reaction of the following proteins*: line 1: *Mbo*II ENase under optimal conditions (10 mM KCl); line 2: *Mbo*II ENase under high salt buffer conditions (550 mM); line 3: *Dna*K protein as the standard control; line 4: *Eco*RV ENase under optimal conditions (150 mM KCl); line 5: *Eco*RV ENase under high salt buffer conditions (550 mM). *) The reaction was performed in two different buffer conditions. The molecular weights of proteins were determined in the presence of standard proteins.
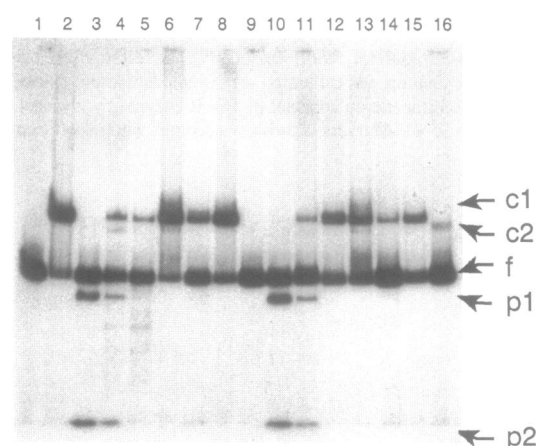

Figure 5. Autoradiogram showing divalent metal cation requirement of the *Mbo*II ENase. DNA used in this assay was prepared as described in METHODS. Line 1: untreated 171 bp DNA; line 2: DNA with *Mbo*II without divalent cations; lines 3−9 show digestion at buffer pH 6.0 and lines 10−16 show digestion at buffer pH 7.0 (all cations at 10 mM concentration): lines 3 and 10: Mg^{2+}; lines 4 and 11: Mn^{2+}; lines 5 and 12: Co^{2+}; lines 6 and 13: Fe^{2+}; lines 7 and 14: Ca^{2+}; lines 8 and 15: Zn^{2+}; lines 9 and 16: Cu^{2+}. *Abbreviations*: f, free 171 bp DNA; c1, retardation effect of mobility of complex of 171 bp DNA with *Mbo*II ENase; c2, retardation effect of mobility of complex 140 bp DNA (p1) with *Mbo*II ENase; p1, 140 bp DNA of digestion product; p2, 31 bp DNA of digestion product.

Molecular weight (M_r) of MboII estimated under denaturing conditions (10% SDS-polyacrylamide gel electrophoresis) is equal to 49000 ± 2000 Da (Fig. 1B) (the molecular weights of FokI and MmeI are $64000-66000$ Da [2,6,8] and 101000 Da [Tucholski,J., Skowron,P. and Podhajska,A.J., unpublished results] respectively). The M_r of a native form of MboII as estimated by gel filtration on Sephacryl S-200 (Stokes radius equals 29.5 ± 1.5 Å, Fig. 2A) and glycerol gradient sedimentation ([$s_{20,w}$] equals 3.75 ± 0.1, Fig. 2B) is about 47000 ± 3000 Da, according to calculation method of Siegel and Monty (21). M_r of MboII equal 48617 calculated for the cloned MboII ENase coding for 416 amino acids (10), is very close to M_r determined by us in native state and under denaturing conditions.

Since native molecular weight is almost equal to that determined by SDS gel electrophoresis, we conclude that, like FokI and MmeI, MboII in its native form is also a monomer. The isoelectric point of MboII determined by IEF equals 8.3 (Fig. 3), which means that it is a basic protein similar to FokI (2,7).

Direct proof that MboII exists predominantly as a monomer was obtained by glutaraldehyde cross-linking reaction, which allows for the detection of protein complexes in solution (18). The reaction was performed both in optimal conditions for MboII activity and in high ionic strength buffer (Fig. 4, lines 1 and 2, respectively). Under these conditions MboII protein exists predominantly as a monomer. Two well known, complex forming proteins, EcoRV ENase (29,30) and E. coli DnaK protein (31,32) were used as a control. EcoRV tested by Wolfes et al.,(33) (photochemical cross-linking to DNA) and shown to exist predominantly as a dimer, (Fig. 4, lines 4 and 5). DnaK, which is known to produce multimeric forms at high concentration of the protein (32), gave us three bands corresponding to DnaK monomers, dimers and trimers (Fig. 4, line 3).

Characterization of MboII activity optima

The purified enzyme was exposed to several factors which influence the MboII endonucleolitic activity, i.e.: pH, ionic strength, divalent cations, organic solvents, temperature. The pH found as optimum was 7.0, although activity was observed in the pH range 6.0 to 8.0 (data not shown). The effect of KCl was estimated at various $(0-320$ mM$)$ KCl concentrations. The enzyme has a maximum activity in a narrow range from 5 to 30 mM KCl, and exhibits a tendency to a rapid decrease of activity even when moderate increase of KCl concentration over 30 mM is applied (data not shown). As with other types of II ENases, the activity of MboII is influenced by divalent cations.
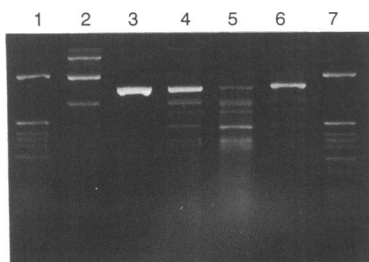
By using specific experimental conditions we were able to distinguish and show on gel two separate activities of MboII enzyme: binding, and cleaving of DNA fragment in the presence different divalent cations. Binding is demonstrated as the retardation of migration of the ^{32}P-labelled DNA fragment (c1 and c2 bands in Fig. 5). Cleaving activity is visualised by the appearance of additional bands (Fig. 5, p1 and p2 bands), below the position of the undigested DNA fragment marked 'f' in this figure. This experiment proves that the binding occurs in the presence of all divalent cations except for Cu^{2+}. However, the cleaving activity was visible clearly only in the presence of Mg^{2+}, Mn^{2+} and Co^{2+}, and in the case of Zn^{2+} and Cu^{2+} even traces of digestion products have not been seen. In the of two ions: Fe^{2+} and Ca^{2+} the c2 band located between c1 and f is visible (Fig. 5), we interpret this as the product of cleavage retarded in gel migration by a bound enzyme. Additional support of our interpretation was achieved when we removed the retarded band from the gel, deproteinized the DNA and reran it on gel. The DNA was then placed in the position of the p1 fragment (data not shown). The Cu^{2+} ion is a confusing exception because it forms DNA complexes visible as a well defined band even without the MboII enzyme added (data not shown). Similar to other authors (2,34−37) we noticed that other divalent cations substituting for Mg^{2+} ions usually decrease endonucleolytic activity. We also observed that the presence of Co^{2+} or Mn^{2+} at pH 6.0 influenced the specificity of MboII DNA recognition (Fig. 5, lines 4 and 5), as was observed for several other enzymes in case of Mn^{2+} (35,38,39). MboII turned out to be heat sensitive: preincubation of the enzyme for 15 min. at 45°C causes 55% loss of activity and at 50°C no trace of activity remained after 15 minutes incubation (data not shown). Based on our data the optimal conditions for catalysis for MboII are as follows: 10 mM Tris-HCl pH 7.0, 10 mM KCl, 10 mM MgCl$_2$ and 37°C. These conditions do not differ much, except for pH requirement, from those recommended by other manufactures. We also observed that MboII, like many other restriction enzymes (38,39), displays a star activity when exposed to certain chemicals
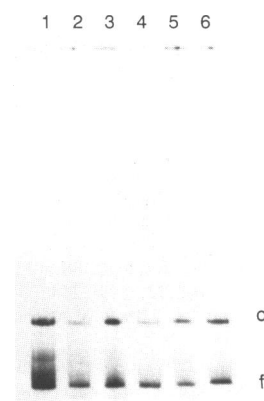

Figure 7. Autoradiogram of 5% polyacrylamide gel electrophoresis of circularly permuted MboII-DNA complexes. The DNA fragments (161 bp) were generated from pBend2 vector with the following restriction enzymes: line 1, MluI (22 bp)*; line 2, StyI (47 bp); line 3, XhoI (59 bp); line 4, EcoRV (69 bp); line 5 StuI (74 bp); line 6, BamHI (49 bp). Abbreviations: c, complexed DNA; f, free DNA.*) The values in parentheses present distances from the center of GAAGA binding site to the nearest end the given DNA fragment. *) The values in parentheses present distances from the center of GAAGA binding site to the nearest end the given DNA fragment.

Figure 6. Cleavage of pNH20 DNA by MboII under conditions which induce star activity. Lines 1 and 7: pBR322 digestion HinfI; line 2: pNH20; line 3: pNH20 digestion MboII under standard conditions; line 4: sample with 15% ethanol; line 5: sample with 20% DMSO; line 6: sample with 30% glycerol. All samples (40 μl) were analysed on 2% agarose gel with ethidium bromide.

(Fig. 6). This property is weakly expressed in low concentrations of reagents and the following concentrations: 20% DMSO, 30% glycerol and 15% ethanol. Complete inhibition of *Mbo*II activity was observed in 30% ethanol and 40% DMSO.

Bending of DNA by *Mbo*II enzyme

In order to find out if *Mbo*II ENase induces bending of DNA, we cloned a 44 bp DNA fragment containing a single GAAGA site, to the pBend2 plasmid (14). This plasmid can generate a large number of DNA fragments of identical length in which a protein-binding nucleotide sequence is located in circular permutations. We showed that there is no difference in the mobility of DNA-protein complexes in polyacrylamide gel despite the binding position of MboII to a given fragment of DNA (Fig. 7). This result is different from those obtained for the following endonucleases: *Eco*RI bending angle of DNA $-52°$ and 66° (40,41, respectively), *Fok*I $-58°$ (5), *Rsr*I $-50°$ (42).

ACKNOWLEDGMENTS

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