DNA Binding Specificity of *Mun*I Restriction Endonuclease Is Controlled by pH and Calcium Ions: Involvement of Active Site Carboxylate Residues†

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ABSTRACT: Gel shift analysis reveals [Lagunavicius, A., & Siksnys, V. (1997) *Biochemistry 36* (preceding paper in this issue)] that at pH 8.3 in the absence of Mg^{2+}, *Mun*I restriction endonuclease exhibits little DNA binding specificity, as compared with the D83A and E98A mutants of *Mun*I. This suggests that charged carboxylate residue(s) influence the DNA binding specificity of *Mun*I. In our efforts to establish the determinants of *Mun*I binding specificity, we investigated the possible role of the ionic milieu, and we found that lowering pH or elevating Ca^{2+} levels per se induces specific DNA recognition by WT *Mun*I. In contrast to the binding experiments at pH 8.3, gel shift analysis at pH 6.5 indicated tight sequence-specific binding of WT *Mun*I in the absence of Mg^{2+}, suggesting that protonation of active site carboxylate residue(s) which manifest anomalously high pK_a value(s) control binding specificity. Interestingly, Ca^{2+} ion concentrations, which did not support DNA cleavage by *Mun*I also induced DNA binding specificity in WT *Mun*I at pH 8.3. To explore possible structural changes upon DNA binding, we then used a limited proteolysis technique. Trypsin cleavage of *Mun*I−DNA complexes indicated that in the presence of cognate DNA the *Mun*I restriction endonuclease became resistant to proteolytic cleavage, suggesting that binding of specific DNA induced a structural change. CD measurements confirmed this observation, suggesting minor secondary structural differences between complexes of *Mun*I with cognate and noncognate DNA. These results therefore suggest that binding of *Mun*I to its recognition sequence triggers a conformational transition that correctly juxtaposes active site carboxylate residues, which then chelate Mg^{2+} ions. In the absence of Mg^{2+} ions, at pH 8.3, conditions in which carboxylate groups would be expected to be completely ionized, electrostatic repulsion between charged carboxylates and phosphate oxygens is enhanced such as to interfere with specific DNA binding. Elimination of such repulsive constraints by replacement of carboxylate residues, by lowering pH, or by metal ion binding, then promotes *Mun*I binding specificity.

Type II[1] restriction endonucleases, in the presence of Mg^{2+} ions, cleave DNA with extreme specificity (Roberts & Halford, 1993). In the absence of Mg^{2+} ions, restriction enzymes are still able to bind DNA and form stable protein−DNA complexes (Roberts & Halford, 1993) but DNA is not cleaved. Interestingly, equilibrium-binding studies reveal differences between restriction enzymes with respect to DNA binding. In the absence of Mg^{2+}, *Eco*RI, *Rsr*I, and *Bam*HI bind preferentially to their respective recognition sequences (Terry et al., 1983; Aiken et al., 1992; Xu & Schildkraut, 1991), while *Eco*RV, *Taq*I, and *Cfr*9I are unable to discriminate between specific and nonspecific sequences under such conditions (Taylor et al., 1991; Zebala et al., 1992; Siksnys & Pleckaityte, 1993). Nevertheless, *Eco*RV, *Taq*I, and *Cfr*9I cleave DNA, in the presence of Mg^{2+} ions, with extreme specificity, suggesting that metal cofactor binding modulates the sequence specific interactions of these enzymes.

Crystallographic studies of *Eco*RI (Kim et al., 1990; Rosenberg, 1991) and *Eco*RV (Winkler, 1993) reveal that both enzymes possess similar active site architecture. Two acidic residues from the conserved restriction endonuclease sequence motif PDX_n(E/D)XK are included, D90 and E111 in *Eco*RI and D74 and D90 in *Eco*RV. The phoshate group of the scissile bond also contributes to the active site. Each of these groups is thought to be involved in the coordination of the metal ion at the catalytic centers of both enzymes. Indeed it has been argued that both enzymes use a similar chemical mechanism to cleave phosphodiester bonds (Jeltsh et al., 1992). Differences of mechanisms of specific DNA recognition however are described. In the case of *Eco*RI (Wright et al., 1989) and *Rsr*I (Aiken et al., 1991) recognition is thought to be mainly achieved by preferential binding to the cognate sequence, even in the absence of Mg^{2+}, with subsequent Mg^{2+}-induced "isomerization" to the active complex (Rosenberg, 1991). *Eco*RV, *Taq*I, and *Cfr*9I, on the other hand, do not demonstrate any specificity of binding in the absence of Mg^{2+} ions. Detailed biochemical studies of *Eco*RV reveal that it binds DNA nonspecifically in the absence of Mg^{2+} (Taylor et al., 1991), but manifestation of sequence specific cleavage is achieved through an increase of Mg^{2+} binding affinity in the complex with the cognate sequence (Taylor & Halford, 1989; Vermote & Halford,

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[1] Abbreviations: bp, base pair(s); CD, circular dichroism; EDTA, ethylenediaminetetraacetic acid; LB, Luria broth; MES, 2-(*N*-morpholino)ethanesulfonic acid; PAAG, polyacrylamide gel; SDS, sodium dodecyl sulfate; TAE, Tris-acetate buffer; TBE, Tris-borate buffer; WT, wild-type endonuclease.

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1992). These findings were consistent with crystallographic studies of complexes of *Eco*RV with cognate and noncognate DNA, which indicated that the DNA in the cognate DNA−enzyme complex was bent (Winkler et al., 1993). Such bending may promote the insertion of the scissile phoshate into the active site and also may create an optimal binding site for Mg^{2+}. The absence of such bending in noncognate DNA−enzyme complexes, would fail to create a favorable geometry for metal ion binding and prevent DNA cleavage (Winkler et al., 1993; Halford et al., 1993). Thus, despite the differences described, both the enzyme "isomerization" and the DNA bending hypothesis propose that optimal positioning of carboxylate residues for chelation of the metal cofactor is achieved only with cognate DNA binding, which is mediated by conformational rearrangement of either the enzyme and/or the DNA. The enzyme "isomerization" hypothesis, however, still lacks direct supportive experimental evidence. The importance of enzyme "isomerization" and/or DNA bending, therefore, in the development of binding specificity, as well as the role of such changes in the connection between recognition and catalysis, still needs elucidation.

The *Mun*I restriction endonuclease recognizes the palindromic hexanucleotide sequence C/AATTG and cleaves it as indicated (Stakenas et al., 1993). The enzyme exhibits intriguing protein sequence similarities with the *Eco*RI restriction enzyme (Siksnys et al., 1994) which recognizes the partially overlapping sequence G/AATTC. Recent mutational analysis of *Mun*I supported the suggestion that the acidic residues D83 and E98, from the proposed *Mun*I active site motif $P82DX_{14}EXK$, are essential for catalysis and presumably are involved in metal ion coordination (Lagunavicius & Siksnys, 1997). In addition, during the biochemical characterization of active site mutants of *Mun*I we found that single replacement of the presumed metal ion chelating residues D83 and E98 to alanine drastically affected binding specificity of the enzyme (Lagunavicius & Siksnys, 1997). Gel shift analysis revealed that in the absence of Mg^{2+}, *Mun*I bound cognate and noncognate DNA with equal affinity. The active site mutants D83 and E98A however exhibited specific DNA binding in the absence of Mg^{2+} ions. The development of specific recognition by replacement of one of the putative metal chelating residues by alanine therefore, suggested that in addition to their role in catalysis carboxylate residue(s) influence the specific binding of *Mun*I to DNA.

In this study we demonstrate that specific DNA recognition by WT *Mun*I can be induced by reducing pH to 6.5 or adding Ca^{2+} at pH 8.3. These data indicate that active site carboxylate residue(s) with anomalously high pK_a values may regulate the specificity of *Mun*I's binding to DNA. Data generated by the limited proteolysis of *Mun*I−DNA complexes together with CD data, suggest that the binding of *Mun*I to its cognate DNA triggers a conformational transition in the protein that correctly juxtaposes active site carboxylates to chelate Mg^{2+}. In the absence of Mg^{2+}, at pH 8.3, electrostatic repulsion between phosphate oxygens and enzyme carboxylates result to abolish specific DNA binding.

EXPERIMENTAL PROCEDURES

Enzyme Purification. Purification of the *Mun*I protein was performed according to the technique previously described by Stakenas et al. (1993). The cells were grown into late logarithmic phase in LB medium containing 50 mg/L Ap and 30 mg/L Cm with aeration, and were harvested by centrifugation. Crude cell extracts were obtained by sonication. These extracts were then applied to a heparin-Sepharose column and eluted using a NaCl gradient. Subsequently, chromatography on blue-Sepharose and phosphocellulose was applied. The fractions containing *Mun*I enzymes were pooled and dialyzed against the storage buffer (10 mM K_3PO_4, pH 7.4; 1 mM EDTA, 7 mM 2-mercaptoethanol, 50% glycerol) and stored at −20 °C . The protein was 99% homogeneous according to SDS gel analysis. Protein concentrations were determined spectrophotometrically at 280 nm using the extinction coefficient of 45 720 M^{-1} cm^{-1} for a monomer. The concentrations of *Mun*I are given in terms of dimeric protein.

DNA and Oligodeoxynucleotides. HPLC-purified oligodeoxynucleotides 5′- GCCAATTGGC-3′ and 5′-GCCAGCTGGC-3′ were purchased from MWG-Biotech and used without further purification. Both oligodeoxynucleotides were annealed by heating to 80 °C and were cooled to room temperature overnight. Concentrations were determined spectrophotometrically at λ of 259 nm.

Preparation of DNA Fragments for Gel Shift Assay. A ^{33}P-labeled 166 bp DNA fragment containing the recognition sequence of *Mun*I and a 174 bp DNA fragment lacking the recognition sequence of *Mun*I were obtained as previously described (Lagunavicius & Siksnys, 1997).

Gel Shift Assays. DNA-binding studies of WT *Mun*I and mutant proteins at pH 8.3 were performed as previously described (Lagunavicius & Siksnys, 1997).

Gel shift experiments in the presence of Ca^{2+} ions were performed as previously described, except that the binding buffer used contained Ca^{2+} (10 mM Tris-HCl, pH 7.5 at 37 °C, 50 mM NaCl, 10% glycerol, 10 mM $CaCl_2$), and the gel was run in TA buffer containing Ca^{2+} (40 mM Tris-acetate, pH 8.3, 10 mM $CaCl_2$).

DNA-binding studies of WT *Mun*I and mutant proteins at pH 6.5 were performed as previously described (Lagunavicius & Siksnys, 1997), except that MES-His buffer (30 mM MES, 30 mM His, pH 6.5, 10% glycerol) was used as a binding buffer and the gel was run in a MES-His buffer (30 mM MES, 30 mM His, pH 6.5).

*Limited Proteolysis of Mun*I *by Trypsin*. Limited proteolysis experiments of *Mun*I and its complexes with oligonucleotides were performed in a buffer containing 10 mM KH_2PO_4, 100 mM NaCl, pH 7.4, 0.5 mM EDTA at 22 °C. The concentration of *Mun*I endonuclease used was 8.6 μM. Trypsin (TPCK-treated, Worthington) treatment of *Mun*I complexes with oligodeoxynucleotides 5′-GCCAATTGGC-3′ and 5′-GCCAGCTGGC-3′ was performed in the presence of a 3-fold excess of oligodeoxynucleotides. Trypsin was used at a trypsin:*Mun*I ratio of 1:60. Reactions were stopped at fixed time intervals by addition of Pefabloc (Boehringer). The extent of reaction was monitored by electrophoresis in 12% SDS−polyacrylamide gels, according to the technique described by Laemmli (1970).

Oligodeoxynucleotide Quenching of MunI Fluorescence. Fluorescence quenching measurements were carried out using a Hitachi MPF-4 spectrofluorometer. Emission spectra of *Mun*I were recorded from 310 to 390 nm using an excitation wavelength 295 nm. The spectral band-pass used for excitation was 5 nm, and that used for emission was 8 nm.

The fluorescence emission maximum of *Mun*I was observed at 335 nm. Since no shift of fluorescence maximum was observed when the highest concentrations of specific and nonspecific oligodeoxynucleotides were used, fluorescence quenching experiments were performed directly, adding small aliquots of the oligodeoxynucleotide solutions (5′-GCCAAT-TGGC-3′ or 5′-GCCAGCTGGC-3′) to the protein solution in the cuvette, while monitoring fluorescence intensity at 335 nm. Protein was dissolved in a buffer containing 10 mM KH_2PO_4, 100 mM NaCl, pH 7.4, 0.5 mM EDTA. All experiments were performed at the temperature 22 °C. Fluorescence values were corrected for all volume increases.

Circular Dichroism. All WT *Mun*I spectra and those of complexes of *Mun*I with specific and nonspecific oligodeoxynucleotides were collected in 10 mM Tris, 50 mM NaCl, 1 mM EDTA, pH 7.5 at 20 °C in 0.1-cm cuvettes using Jobin-Yvon Auto-Dichrograph R.J Mark IV calibrated with (+)-camphor-10-sulfonic acid and epiandrosterone. The bandwidth used was 0.08 nm, the rate of scanning used was 0.05 nm/s, and the time constant used was 7 s. To derive protein-only spectra in the presence of oligodeoxynucleotide, oligodeoxynucleotide spectra were subtracted from protein−oligodeoxynucleotide spectra. The concentration of protein used in a typical experiment was 13 μM.

RESULTS

To test whether the state of ionization of active site carboxylate residues, or alternatively whether the presence of metal ions, affected *Mun*I endonuclease DNA binding affinity, binding studies of WT *Mun*I at different pH values, and in the presence of different concentrations of metal ions, were performed. Gel shift assays were used to monitor DNA binding by *Mun*I as described under Experimental Procedures.

pH Dependence of MunI Binding to Specific and Nonspecific DNA. To test whether pH influences *Mun*I DNA binding, *Mun*I was preequilibrated with specific or nonspecific DNA in a binding buffer at pH 6.5 (30 mM MES, 30 mM His, 1 mM EDTA, 10% glycerol). Samples were loaded on an 8% PAAG and electrophoresis was run at pH 6.5, using the same binding buffer without glycerol, to maintain pH at a constant value during the experiment. When *Mun*I was complexed to the 166 bp specific DNA at pH 6.5 and analyzed by electrophoresis, a DNA band with reduced mobility was observed, even at protein concentrations of 0.25 nM (Figure 1a, band 3). The amount of *Mun*I required to shift the band at pH 6.5 was similar to the amount of each of the D83A and E98A mutants, required to shift the band with specific DNA at pH 8.3 (Lagunavicius & Siksnys, 1997). The concentration of the initial complex (corresponding to band 3, Figure 1a) observed after complexing with specific DNA at pH 6.5, decreased progressively with the use of increasing concentrations of *Mun*I, and a second band (band 2, Figure 1a) with a highly reduced mobility, corresponding probably to the complex of *Mun*I with nonspecific DNA, appeared at the top of the gel. A similar binding pattern was characteristic of the D83A mutant at pH 8.3 (Lagunavicius & Siksnys, 1997). The nonspecific 174 bp DNA fragment, lacking the recognition sequence of *Mun*I, failed at pH 6.5 to generate an effective complex (Figure 1b). When the concentrations of protein used were increased though, a DNA band with a highly reduced electrophoretic

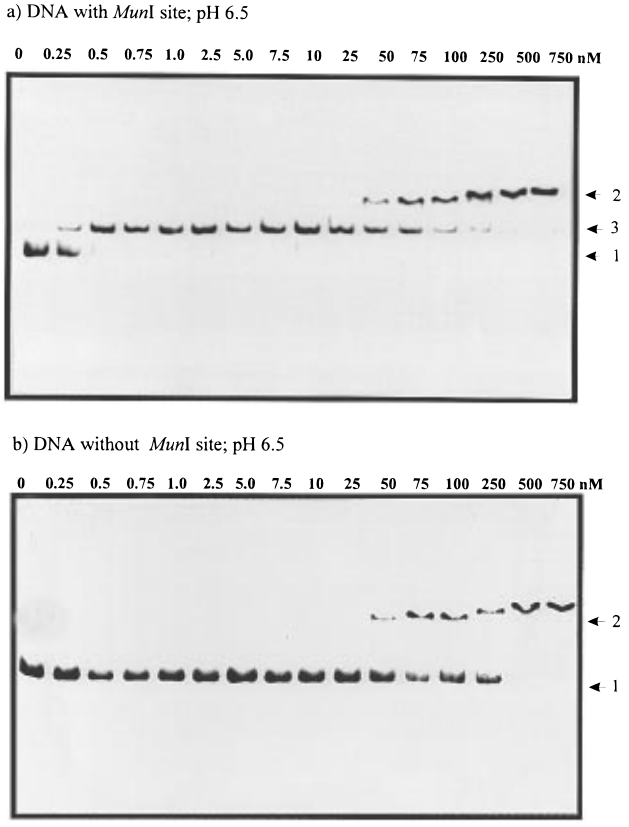

FIGURE 1: Binding of *Mun*I to specific and nonspecific DNA at pH 6.5. The 166 bp fragment containing the recognition sequence of *Mun*I (panel a) or the 174 bp fragment lacking the recognition sequence of *Mun*I (panel b) was used in binding experiments as specific and nonspecific DNA, respectively. The binding mixture contained 0.5 nM ^{33}P-labeled DNA and *Mun*I at concentrations indicated above each lane of the gel. After PAAG electrophoresis, gels were dried and subjected to autoradiography. Arrows on the right side of the gel indicate positions of DNA forms with different mobility.

mobility was observed (Figure 1b), one similar to that of the band 2 observed when complexes using specific DNA and high protein concentration were analysed (Figure 1a). Since at pH 6.5 at low *Mun*I concentrations, a tight effective complex [corresponding to band 3 (Figure 1a)] was observed only when the specific 166 bp DNA fragment was used, we conclude that the complex represents a complex of *Mun*I bound to its recognition sequence. Similar binding experiments at pH 5.5 led to a binding result indistinguishable from that observed when *Mun*I binding to the specific DNA fragment was analysed at pH 6.5 (data not shown).

Effect of Metal Ions on DNA Binding by MunI. Since the D83A and E98A residues have been implicated by mutational analysis to be the Mg^{2+} binding residues of *Mun*I, it is tempting to suggest that at higher pH values (e.g., pH 8.3), conditions under which specific *Mun*I−DNA complexes are not observed, Mg^{2+} ion chelation by WT *Mun*I could induce specific DNA binding. Unfortunately this suggestion cannot be addressed experimentally using gel shift assays, because the specific DNA fragment is cleaved rapidly in the presence of Mg^{2+} ions. We did find, however, that *Mun*I failed to cleave DNA in the presence of Ca^{2+} ions, an observation consistent with reports on *Eco*RV (Vipond & Halford, 1995). We therefore analyzed *Mun*I binding to DNA, by gel shift analysis, in the presence of Ca^{2+}. Addition of 10 mM $CaCl_2$ to the binding buffer at pH 8.3, led to the appearance of

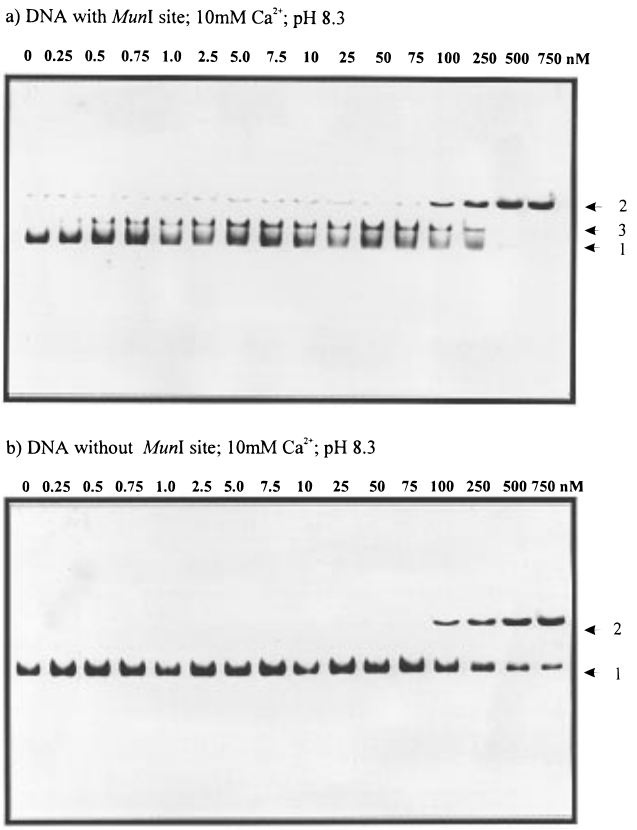

FIGURE 2: Binding of *Mun*I to specific and nonspecific DNA in the presence of Ca^{2+} ions at pH 8.3. The 166 bp fragment containing the recognition sequence of *Mun*I (panel a) or the 174 bp fragment lacking the recognition sequence of *Mun*I (panel b) was used in binding experiments as specific and nonspecific DNA, respectively. The binding reaction mixture contained 0.5 nM ^{33}P-labeled DNA and *Mun*I at concentrations indicated above each lane of the gel. The concentration of Ca^{2+} ions was 10 mM. After PAAG electrophoresis in the presence of 10 mM of $CaCl_2$, gels were dried and subjected to autoradiograpy. Arrows on the right side of the gel indicate positions of DNA forms with different mobility.

shifted bands (band 3, Figure 2a) in samples containing specific DNA fragments at low concentrations of *Mun*I. These bands were similar to the bands observed when the D83A and E98A mutants were used at pH 8.3, or WT *Mun*I was used at pH 6.5. Since these bands were missing when nonspecific DNA fragments were used (Figure 2b), we conclude that the bands observed with specific DNA, at low protein concentrations represent sequence-specific complexes of *Mun*I. In the presence of Ca^{2+}, however, a slight smear was observed between the specific complex (band 3, Figure 2a) and free DNA, in contrast to WT *Mun*I binding at pH 6.5, or D83A and E98A mutant binding at pH 8.3.

Limited Proteolysis of MunI and Its Complexes with Specific and Nonspecific DNA. Substrate binding frequently affects enzyme sensitivity to proteolytic cleavage (Citri, 1983). In addition, substrate-induced stabilization against proteolysis has previously been reported to be relevant to the folding−unfolding transitions in a number of DNA binding proteins (Mirset et al., 1993; Cohen et al., 1995). We therefore attempted to use the technique of limited proteolysis to characterize *Mun*I binding to specific and nonspecific DNA. Time course studies of trypsin cleavage of WT *Mun*I initially revealed a major band, with a molecular weight of approximately 20 kDa, which subsequently was gradually cleaved to smaller peptides (Figure 3a). After 22

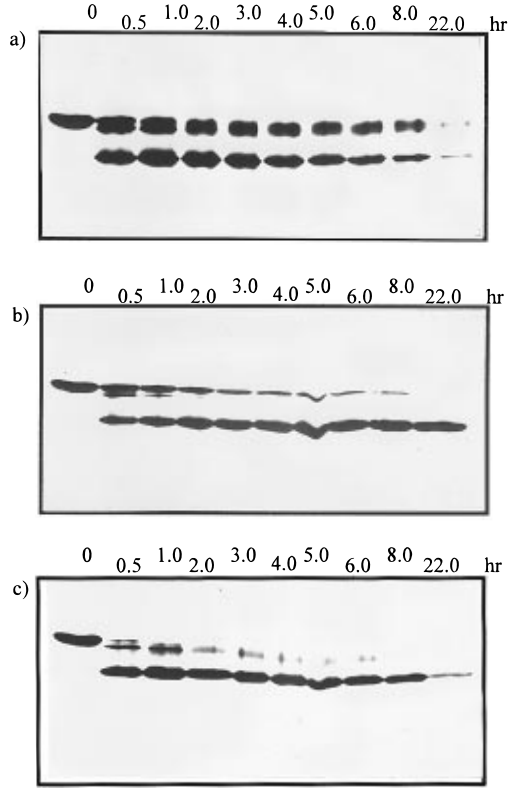

FIGURE 3: Limited proteolysis of *Mun*I by trypsin. WT *Mun*I (a), *Mun*I in the presence of 3-fold molar excess of specific oligodeoxynucleotide 5′-GCCAATTGGC-3′ (b), and *Mun*I in the presence of 3-fold molar excess of nonspecific oligodeoxynucleotide 5′-GCCAGCTGGC-3′ (c) were treated with trypsin as described under Experimental Procedures. Samples were removed at time intervals indicated above the lanes and analyzed in SDS−PAAG.

h of trypsin treatment, *Mun*I was almost completely digested to a mixture of short peptides that were not resolvable on PAAG, under our experimental conditions (Figure 3a). The *Mun*I trypsinolysis pattern did not change significantly in the presence of cognate DNA, as compared to noncognate DNA (Figure 3b,c). However, the rate of digestion of the 20 kDa fragment, obtained after the first cleavage steps, was greatly retarded in the presence of the specific oligonucleotide. Trypsin treatment of the *Mun*I complex with the synthetic oligonucleotide duplex 5′-CGCAATTGCG for 22 h yielded a 20 kDa polypeptide that was resistant to subsequent proteolysis (Figure 3b). The 10 bp oligonucleotide duplex 5′-CGCAGCTGCG, lacking the *Mun*I recognition sequence, provided only modest protection of the 20 kDa fragment from trypsin cleavage, as can be judged from its gradual disappearance (Figure 3c). Indeed, after 22 h approximately 90% of the 20 kDa fragment remained uncleaved in the complex with the specific oligonucleotide, while less than 10% of the same fragment remained after 22 h trypsinolysis of the complex with nonspecific DNA, an amount comparable to that seen in experiments using the WT enzyme (Figure 3a−c). A similar increase of resistance to proteolysis, in the presence of specific DNA, has been reported for the *Eco*RI restriction enzyme (Jen-Jacobsen et al., 1986).

To test whether the lack of protection afforded by the nonspecific oligonucleotide was due to a lower DNA-binding affinity, the binding of specific and nonspecific oligonucleotides to *Mun*I was evaluated using the fluorescence quenching technique (Carpenter & Kneale, 1994). The fluorescence

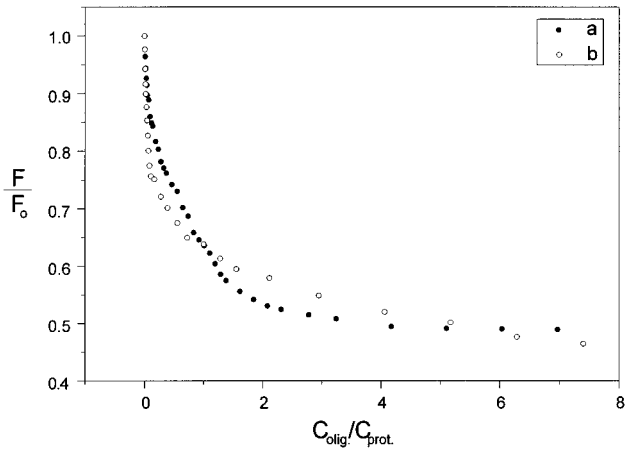

FIGURE 4: Fluorescence quenching of *Mun*I by specific and nonspecific oligodeoxynucleotides. Fluorescence quenching experiments were performed adding small aliquots of specific oligodeoxynucleotide (a) 5′-GCCAATTGGC-3′or nonspecific oligodeoxynucleotide (b) 5′-GCCAGCTGGC-3′ to the protein solution and monitoring fluorescence intensity change at 335 nm. Protein was dissolved in a buffer containing 10 mM KH_2PO_4, 100 mM NaCl, pH 7.4, 0.5 mM EDTA. All experiments were performed at the temperature 22 ± 1 °C.

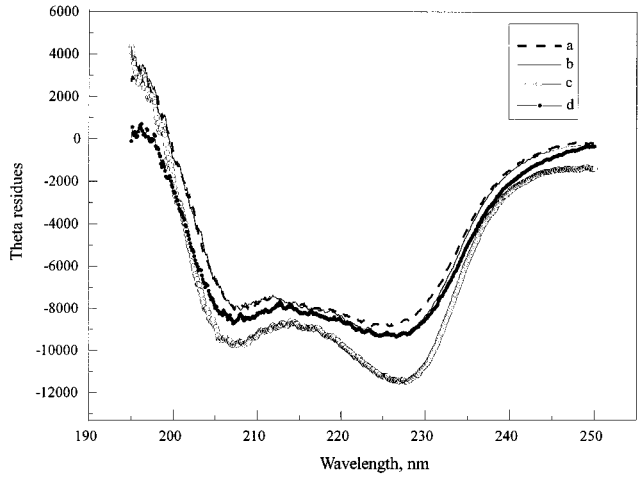

FIGURE 5: Circular dichroism spectra of *Mun*I and its complexes with specific and nonspecific DNA. All spectra of WT *Mun*I (a) and its complexes with nonspecific (b) oligonucleotide 5′-GCCAGCTGGC-3′, specific (c) oligonucleotide 5′- GCCAATTGGC-3′, and after addition of 10 mM of $MgCl_2$ (d) to the cuvette containing complex of *Mun*I with specific DNA were collected in 10 mM Tris, 50 mM NaCl, 1 mM EDTA, pH 7.5 at 20 °C in 0.1-cm cuvettes. The concentration of a protein in a typical experiment was 13 μM, 2-fold molar excess of oligodeoxynucleotide was used. To derive protein-only spectra in the presence of oligodeoxynucleotide, oligodeoxynucleotide spectra obtained under similar conditions was substracted from the protein−oligodeoxynucleotide spectra.

of *Mun*I was effectively quenched by addition of either the specific or the nonspecific oligonucleotide (Figure 4). Analysis of binding data revealed a 1:1 stoichiometry of *Mun*I binding both to specific and nonspecific oligonucleotides. Further, fluorescence quenching studies indicated that under the experimental conditions of limited proteolysis used, both specific and nonspecific oligonucleotides formed a complex with *Mun*I. This suggests that the protection of *Mun*I against trypsin cleavage in complexes with specific DNA, and the loss of that protection in complexes with nonspecific DNA, probably reflect different conformational states of the protein in the presence of cognate and noncognate DNA. Since the 20 kDa fragment of *Mun*I contains 22 sites of trypsin cleavage distributed randomly throughout the sequence, however, the observed proteolytic protection in the presence of specific DNA cannot be explained by simple protection of trypsin cleavage sites by DNA but probably reflects conformational change that occurs in the *Mun*I molecule upon binding to its recognition sequence.

Circular Dichroism Spectroscopy of MunI and Its Complexes with Specific and Nonspecific DNA. We employed circular dichroism spectroscopy in order to characterize the possible conformational change in *Mun*I, suggested in limited proteolysis experiments, circular dichroism being a sensitive method of monitoring possible folding−unfolding transitions in proteins with DNA binding (D'Amare et al., 1994; Petersen et al., 1995). *Mun*I was mixed with specific or nonspecific DNA in binding buffer (10 mM Tris, 50 mM NaCl, 1 mM EDTA, pH 7.5), and CD spectra were recorded. The CD spectra of WT *Mun*I and its complex with nonspecific DNA revealed few differences (Figure 5a,b). A slight increase of molar ellipticity at 208 and 228 nm was observed, however, in the presence of the specific oligonucleotide (Figure 5c), suggesting some increase in α-helical structure. The addition of 10 mM $MgCl_2$ (added directly to the cuvette), however, transformed specific *Mun*I−DNA complex spectra (Figure 5c) to those of nonspecific *Mun*I−DNA complex CD spectra (Figure 5d), to reflect the cleavage of specific oligonucleotide, in turn confirmed by HPLC analysis of the reaction mixture (S. Grazulis, personal communication). Addition of 10 mM $MgCl_2$ to the WT *Mun*I and to the nonspecific *Mun*I−DNA complex had no effect on the CD spectra recorded (data not shown).

DISCUSSION

On the basis of previously published biochemical data it was possible to separate restriction enzymes into two different groups: a group which bound cognate DNA sequences much more strongly than other sequences in the absence of Mg^{2+} ions (*Eco*RI, *Rsr*I, *Bam*HI) (Terry et al., 1983; Aiken et al., 1991; Xu & Schildkraut, 1991) and a group which did not bind specifically to cognate DNA in the absence of Mg^{2+} (*Eco*RV, *Taq*I, *Cfr*9I) (Taylor, 1991; Zebala et al., 1992; Siksnys & Pleckaityte, 1993). The extreme cleavage specificity of this latter group may therefore result from the binding of Mg^{2+} ions.

Gel shift analysis of DNA binding by WT *Mun*I suggests that *Mun*I might be assigned to the second group of restriction enzymes (Lagunavicius & Siksnys, 1997). Under experimental conditions, in the absence of Mg^{2+} ions, no distinct specificity for binding to its recognition sequence was observed. DNA binding studies of the cleavage deficient *Mun*I mutants, in which the catalytically essential *Mun*I residues D83 and E98 were replaced with alanine, indicated, however, that such replacement conferred DNA binding specificity in the absence of Mg^{2+} ions. Since the acidic residues D83 and E98 are thought to be located in the vicinity of the cleavable phosphate, and since these residues are thought to chelate Mg^{2+} at the active site, it was tempting therefore, to speculate that Mg^{2+} binding to carboxylate residues might reduce the negative charge at the active site and increase the affinity of the enzyme for it's recognition sequence.

It is not possible though to directly test whether Mg^{2+} ions might be instrumental in the development of specificity of *Mun*I using the gel shift assay, since in the presence of Mg^{2+}

ions, specific DNA is cleaved. Like *Eco*RV (Vipond et al., 1995), however, *Mun*I appeared to be cleavage deficient in the presence of Ca^{2+} ions. Consequently Ca^{2+} was employed, instead of Mg^{2+}, in WT *Mun*I DNA-binding studies. Gel shift experiments indeed revealed that the addition of Ca^{2+} conferred specificity on the DNA binding of *Mun*I (Figure 2). These experiments therefore suggest that binding of Ca^{2+} ions probably relieves unfavorable electrostatic repulsive forces between the active site carboxylates and the phosphate oxygen to enable development of a specific complex. Mg^{2+} ions may play a similar role during cleavage of the phosphodiester bond by *Mun*I at pH 8.3, where specificity is developed principally during the catalytic step.

Comparisons of the crystal structures of *Eco*RI (Kim et al., 1990; Rosenberg, 1991), *Eco*RV (Winkler et al., 1993), and *Pvu*II (Athanasiadis et al., 1994; Cheng et al., 1995) reveal that despite variations in recognized sequence and cleavage position, a sequence motif P(N)DX_n(D/E)XK, which lies in close proximity to the scissile phosphodiester bond, is conserved between them (Aggarwal, 1995). The acidic residues within this motif are thought to chelate Mg^{2+} ions, which are a necessary cofactor for catalysis. Our experimental evidence, suggests that carboxylate residues from the putative active site of *Mun*I (Lagunavicius & Siksnys, 1997) are essential in enzyme mediated catalysis and in addition, play an important role in the development of binding specificity. This conclusion is further supported by the results of pH dependence studies of *Mun*I DNA binding. Lowering the pH from 8.3, where no specific binding was observed, to pH 6.5, led to a drastic increase in *Mun*I binding affinity and specificity (Figure 1a). Formation of the specific WT *Mun*I/DNA complex at pH 6.5 suggests that protonation of carboxylate group(s) present at or in the vicinity of the active site of *Mun*I enables formation of a tight sequence-specific WT *Mun*I/DNA complex. The apparent pK_a of the group controlling specific complex formation is expected to be between 6.5 and 8.3. Thus, since both lowering the pH and replacement of D83 and E98 residues with alanine led to the development of specific DNA binding by *Mun*I, it is tempting to think that the prototropic equilibrium characteristics of the carboxylate groups define the specific binding of DNA by *Mun*I. If one assumes that protonation of active site Glu and/or Asp carboxylates controls *Mun*I DNA binding in the pH range 6.0−8.3 then anomalously high pK_a values (between 6.5 and 8.3) should be assigned to the carboxylate residues. Alternatively, other ionizable groups, such as His, might be involved in the prototropic equilibrium which controls specific DNA binding; however, this seems less probable. Indirect evidence supporting the fact that carboxylate residues may be involved in the prototropic equilibrium is derived from the pH-dependence studies of DNA binding by the D83A mutant. Gel shift analysis at pH 6.5 revealed that the D83A mutant bound the specific DNA fragment with the same affinity as did the WT protein (data not shown). We think that such a high carboxylate pK_a value at the active site seems plausible because of the local clustering of negatively charged residues at the Mg^{2+} binding site. It is well-known that the presence of neighboring carboxylate residues increases the pK_a value of carboxylic acid side chains in proteins (Fersht, 1985). Indeed, anomalously high pK_a values of carboxylate residues have previously been reported for the metal chelating acidic residues of RNAse H (Oda et al., 1994; Huang & Cowan, 1994) and the CheY protein (Lukat et al., 1990). In the case of enzyme interacting with the negatively charged DNA molecule, the pK_a shift at the active site of the enzyme might be further enhanced by the electrostatic effect of the proximity of the DNA phosphates, as was demonstrated recently for barnase (Gordon-Beresford et al., 1996). In the light of our experimental results, this is a likely scenario for *Mun*I. It is not yet certain, however, which carboxylates (D83 or E98) are protonated. Interestingly, X-ray analysis of the *Eco*RV complex with its substrate DNA (Kostrewa & Winkler, 1995) indicated that the D90 active site residue, which is homologous to the E98 residue of *Mun*I, should be protonated at pH 6.8, suggesting an anomalously high pK_a value for this residue.

It is tempting to speculate that pK_a values of active site carboxylates of other restriction endonucleases containing the PDX_n(E/D)XK motif might be also increased, the extent of the pK_a shift depending on the presence of other charged residues in the vicinity. If so, then the DNA binding properties of such restriction enzymes might also be modulated by pH, as are those of *Mun*I. The gel mobility shift assay has become probably the most popular method for studies of DNA binding by restriction enzymes (Taylor et al., 1995) and is most frequently carried out at pH 8.0−8.5 (1× TAE or TBE). At this pH value active site carboxylates might be ionized and might interfere with the specificity of binding, as observed in our studies on *Mun*I. Therefore, gel shift experiments performed at pH 8.0−8.5, might hide DNA binding specificity of restriction enzymes. We reported earlier that the *Cfr*9I restriction endonuclease does not show binding specificity at pH 8.5 in the absence of Mg^{2+} ions (Siksnys & Pleckaityte, 1993). Withers and Dunbar (1995) reported that the *Xma*I restriction enzyme, an isoschizomer of *Cfr*9I which manifests 80% homology with *Cfr*9I, binds specifically to its recognition sequence at lower pH values, however. Thus the difference in protonation state of the active site carboxylate might well be responsible for the binding differences observed.

The experimental evidence presented therefore suggests that protonation of carboxylate residues at *Mun*I's active site affects the specificity of *Mun*I's binding to DNA. But how does change of ionization state at the Mg^{2+} ion binding site influence DNA binding specificity? Limited proteolysis studies indicate (Figure 3) that *Mun*I, in the presence of the specific oligonucleotide, undergoes a structural transition that renders the protein molecule resistant to further proteolytic cleavage. Accordingly, CD data revealed increases in the α-helical content of the *Mun*I protein, suggesting that some of the protein regions become more ordered. These minor structural changes appeared to be reversible. Cleavage of the specific DNA fragment in the presence of Mg^{2+} led to the reduction of the α-helical content of the *Mun*I protein, to the extent characteristic for *Mun*I in the presence of nonspecific DNA. We therefore think that upon binding to its recognition sequence *Mun*I undergoes a conformational change which juxtaposes active site carboxylates to bind Mg^{2+} ions. Such positioning of carboxylate residues might be energetically unfavorable in the absence of Mg^{2+} ions, however, because of the repulsive interactions with phosphate oxygen atoms. In the case of specific DNA binding, the energy cost of forming a strained conformation might be approximately compensated for favorable interactions generated in specific binding mode. At higher pH values (for example, pH 8.3) where carboxylates are probably completely ionized, almost all binding energy gained from

specific interactions might be well consumed to fix metal chelating residues at the cleavable phosphate, leading to an apparent loss in binding affinity to the specific sequence. At lower pH values active site carboxylate residue(s) are protonated and promote specific binding. Thus carboxylate residue(s) at the active site of *Mun*I might well control both specific DNA binding and metal ion affinity. At lower pH values (6.5), protonated active site carboxylates may enhance specific DNA binding but might reduce the affinity for the metal ion at the active site. At higher pH values (pH 8.3), charged carboxylate residues might favor metal ion binding but under these conditions specific DNA binding may be reduced and probably not be fully compensated for by metal ion binding.

The chemical mechanism of phosphodiester bond cleavage by restriction endonucleases is still poorly understood. No amino acid residues have yet been identified, which might contribute to either water activation or leaving group protonation. It's possible that *aquo*-Mg^{2+} complexes fulfill both functions: a water from the coordination sphere might attack the phosphorous atom of the scissile bond, and another water molecule from the hydration shell might protonate the leaving group. However, we still lack crystallographic and biochemical evidence to support such a mechanism. The presence of a carboxylate residue with a pK_a value >6.5 in the *Mun*I active site, though, suggests that such carboxylate residues might well play an active role in catalysis, e.g., providing the proton for the leaving group after Mg^{2+} chelation of the active site carboxylate, as has been suggested for RNAse H (Oda et al., 1994).

NOTE ADDED IN PROOF

After this paper had been accepted for publication, a report appeared in the literature describing the DNA binding properties of *Eco*RV restriction enzyme under different environmental conditions (Engler et al., 1997). Similar to the results reported here for *Mun*I, decrease of pH promoted DNA binding specificity of *Eco*RV.

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