Determination of Metal Ion Binding to *Hinc*II

N.C. Horton, C. Etzkorn, E. Little, J. Crotty
Department of Biochemistry and Molecular Biophysics, University of Arizona, Tucson, AZ, U.S.A.

Introduction

Data were collected in two separate trips during this period for three projects: (1) the role of indirect readout by type II restriction endonucleases, (2) the modulation of specificity in a tetrameric type II restriction endonuclease, and (3) the role of dynamics in enzyme catalysis.

Methods and Materials

HincII/DNA/Mn^{2+} Data Set

Protein purification and crystallization of *Hinc*II bound to 5'-GCCG**GTCGA**CCGG were performed as previously described [1]. A crystal of *Hinc*II bound to DNA and Mn^{2+} was prepared by soaking a crystal in stabilization buffer (25% PEG at 4K, 0.1 M sodium citrate [pH 5.5], 0.15 M NaCl) containing 50 mM $MnCl_2$ for 24 hours at 17°C. The crystal was then exchanged into a cryoprotection buffer (25% PEG at 4K, 0.1 M sodium citrate [pH 5.5], 0.15 M NaCl, 30% glycerol) and flash-frozen in liquid nitrogen. The crystal was sent to APS for data collection at beamline 17-BM. Data collection was performed while maintaining the crystal at 100K. Image processing and data reduction were performed with HKL2000 [2].

Q138F HincII/DNA/Mg^{2+} Data Set

Protein purification was performed as previously described [1]. A new crystal form of a mutant of *Hinc*II (Q138F) was prepared by mixing with DNA (5'-GCCG**GTCGA**CCGG) and 50 mM $MgCl_2$ by the hanging-drop method by using 25% PEG at 4K, 0.1 M Tris-HCl (pH 8.5), 0.15 M NaCl as the precipitating agent. The crystal was then exchanged into a cryoprotection buffer (25% PEG at 4K, Tris-HCl [pH 8.5], 0.15 M NaCl, 30% glycerol) and flash-frozen in liquid nitrogen. The crystal was sent to APS for data collection at beamline 17-BM. Data collection was performed while maintaining the crystal at 100K. Image processing and data reduction were performed with HKL2000 [2].

H127A M.HhaI/DNA/SAH Data Set

Protein purification was performed as previously described [3]. Crystals of mutant (H127A) M.*Hha*I/DNA/SAH were prepared as described [3]. The crystal was then exchanged into a cryoprotection buffer (1.75 M ammonium sulfate, pH 5.5, 30% glycerol) and flash-frozen in liquid nitrogen. The crystal was sent to APS for data collection at beamline 17-BM. Data collection was performed while maintaining the crystal at 100K. Image processing and data reduction were performed with HKL2000 [2].

Phase Determination-Molecular Replacement (MR) Data Sets

Phasing was performed for the *Hinc*II/DNA/$MnCl_2$, Q138F *HincII*/DNA/$MgCl_2$, and H127A M.*Hha*I/DNA/SAH data sets by using MR as implemented in CNS [4] and search models derived from previously solved structures.

Model Building and Refinement

The MR data sets were refined by using CNS [4].

Results

HincII/DNA/Mn^{2+} Data Set

Analysis showed the crystals to be in the $I2_12_12_1$ space group with cell dimensions of 66.8 Å, 178.2 Å, 157.3 Å, 90°, 90°, 90°. The data are 89.3% complete to 2.5 Å, with an R_{merge} of 6.4%. The structure was solved by MR and refined to a crystallographic R value of 21.3% with an R_{free} of 27.0%. Analysis of the electron density maps shows that the DNA is cleaved, and two to three Mn^{2+} ions are bound in each active site.

Q138F HincII/DNA/Mg^{2+} Data Set

Analysis showed the crystals to be in the $P4_32_12$ space group with cell dimensions of 59.76 Å, 59.76 Å, 164.88 Å, 90°, 90°, 90°. The data set are 100.0% complete to 2.5 Å, with an R_{merge} of 7.6%. The structure was solved by MR and partially refined with a crystallographic R value of 29.0% and R_{free} of 35.1%. Examination of the electron density map shows that the DNA and Mg^{2+} are not visible, and the protein takes on a more closed conformation. The mutation of glutamine to phenylalanine at position 138 is clearly visible.

H127A M.HhaI/DNA/SAH Data Set

Analysis showed the crystals to be in the R32 space group with cell dimensions of 98.73 Å, 98.73 Å, 323.33 Å, 90°, 90°, 120°. The data are 95.8% complete to 2.5 Å, with an R_{merge} of 7.0%. The structure was solved by MR and refined to a crystallographic R value of 23.2% with an R_{free} of 26.6%. Difference (Fo-Fc) electron density maps showed the presence of the histidine side chain at position 127. Communication with collaborators and subsequent mass spectrometry analysis confirmed the wild-type sequence of this protein.

Discussion

Determination of Metal Ion Binding to HincII

The type II restriction endonuclease, *Hinc*II, cleaves duplex DNA containing the sequence GTYRAC (Y = C,T; R = A,G) in a divalent-cation-dependent manner. *In vivo*, the divalent cation utilized by the type II restriction endonucleases is Mg^{2+}; however, Mn^{2+} and Co^{2+} have been found, in some cases, to confer activity although with lower values of k_{cat} [5]. In some cases, the presence of a divalent cation is required not only for the cleavage activity but also for DNA sequence specificity. In addition, Ca^{2+} can "substitute" for Mg^{2+} in providing this specificity, which involves also greatly increasing the affinity of the enzyme for DNA, without, however, conferring cleavage activity. The 3-D crystal structures of 13 type II restriction endonucleases have been determined, and, in five cases, the positions of divalent cations in the enzyme substrate and/or enzyme product complexes have been determined. The positions of these cations provide some insight into the roles of these ions in the catalysis of the DNA phosphodiester backbone cleavage reaction.

Currently, controversy exists over the roles of the cations in the catalytic mechanism. This may arise in part from the use of different enzymes in these studies; the catalytic mechanism and, in particular, the roles of the cations may not necessarily be conserved in each enzyme. However, even within the same enzyme *Eco*RV, four distinct mechanisms have been proposed [6-9]. These mechanisms have been proposed on the basis of not only crystal structures but also biochemical observations, such as the requirement for the 3′ phosphate [9], and stopped flow kinetic studies [8]. The biochemical and kinetic studies provide valuable information but limited atomic detail. On the other hand, the crystal structures suffer from the difficulty in trapping an active ground-state complex prior to cleavage. Product complexes show the positions of atoms after the reaction, but before cleavage, the reaction must be stalled in order to complete the structural analysis. Since the structural analysis generally takes several days (from the time the crystals are prepared to data collection), only relatively inactive complexes can be studied. This fact leaves open the possibility that the complexes observed are "off pathway" or in a nonproductive and irrelevant dead end. However, by the elucidation of many such complexes, one can, in principle, "tease apart" the irrelevant from the relevant to thereby obtain a cohesive picture of what the active pre-transition-state complex may look like.

Previous studies by Horton and others on the type II restriction endonuclease *Eco*RV are the most comprehensive structural studies of divalent cation binding to an enzyme in this class [6, 10, 11]. Recently, the crystal structure of the type II restriction endonuclease *Hinc*II bound to its recognition DNA sequence was solved [12]. Although these two enzymes display sequence identity (9%) well below that which indicates homology (25%), the similar DNA target sequence and pattern of cutting (they both cut DNA to leave blunt ends) suggested that they may have diverged from a common ancestor. In fact, the structure of *Hinc*II supports this hypothesis, showing the same topology and manner of binding to DNA. One of the structures described in this report is that of *Hinc*II bound to DNA with Mn^{2+} soaked into the crystals. Since the crystals grow at pH 5.5, it was unclear whether or not the Mn^{2+} would bind the enzyme, and if it did, if the DNA would be able to be cut. In addition, it was not known whether or not the crystal lattice supported cleavage activity, since that of the *Eco*RV enzyme bound to DNA does not [7]. However, the refined structure showed the presence of two to three Mn^{2+} ions per active site (four active sites are found in the asymmetric unit, two from each dimer), and it showed that the DNA is cleaved.

Proposed roles of the divalent cations in the cleavage of DNA by endonucleases can vary, as can the proposed number of divalent cations required for the catalytic mechanism. Mechanisms involving one, two, and even three divalent cations have been proposed for the enzyme *Eco*RV. Previous work by Horton and others supported the existence of three divalent cations in the active site during catalysis. An admitted weakness of the model was the inability to see all three divalent cations in the active site in one structure, which could simply be caused by the difficulties described above in trapping a pre-transition-state complex. The easily viewable enzyme product complex may have less opportunity to become "off pathway," since it does not contain any mutations or substrate modifications (since it need not be "trapped"). However, the divalent cation positions in the *Eco*RV wild-type product complex show very little similarity to any of the proposed divalent cation positions in the enzyme substrate complexes and may therefore actually be dissimilar to the product complex that occurs just after the cleavage reaction. The structure of *Hinc*II/DNA/Mn^{2+} found here, however, is a product complex with three divalent cation positions found in the exact locations of those proposed in the original model of three metal ions. Therefore, this structure puts the three-metal-ion mechanism on much firmer ground and should serve as a model for other divalent-cation-dependent endonucleases.

Role of Protein Dynamics in Enzyme Catalysis: Structure of H127A M.HhaI Bound to DNA and SAH

The goal of this project is to test the possibility that protein dynamics play an important role in enzyme catalysis. The approach is to create mutations in the enzyme far from the active site that have been suggested by modeling and computational studies to

disrupt the protein's intrinsic dynamic properties. These mutants would then be tested for any effect on catalysis, and the dynamic properties would be measured by some means, in this case, by any changes in the crystallographic Debye-Waller temperature factors. M.*Hha*I is a prokaryotic DNA methytransferase that utilizes the cofactor S-adenosylmethionine as a source of the methyl group that is transferred to a cytosine within its recognition sequence. The crystals here were of a mutant of M.*Hha*I provided by a collaborator (N. Reich, University of California, Santa Barbara) and were bound to DNA and the methyl depleted S-adenosylcysteine. The crystals diffracted very well, and the structure was easily solved by using MR. However, the electron density around the site of mutation showed the presence of the original wild-type sequence. This result has subsequently been confirmed by mass spectrometric analysis of the protein provided by the collaborator.

Structure of Unliganded Mutant Q138F HincII

Little is known about the early steps in the binding of DNA by sequence-specific DNA-binding enzymes. One question is whether they bind initially to undistorted DNA, and only then, in a second step, distort the DNA in order to form a sequence-specific complex, or alternatively, whether the distortion of DNA occurs concurrently with binding [13]. In addition, it is not known in many cases how the enzyme is distorted in order to bind DNA, and there is evidence that many DNA-binding enzymes undergo disorder-to-order transitions upon DNA binding [14]. One of the structures described in this report, that of unliganded Q138F *Hinc*II, provides some insight into how this enzyme changes conformation upon DNA binding, since previously only the structure of *Hinc*II bound to DNA had been determined. The unliganded enzyme shows that the two DNA-binding domains have shifted toward each other, occupying the space that is occupied by DNA in the cocrystal structure. The dimerization interface, which consists of a long carboxy terminal alpha helix from each monomer, is unchanged; however, the linkage between it and the DNA-binding domain contains a flexible loop that appears to be the part of the protein that allows the rigid body displacement of the domains of the protein. This structure has not yet been refined, and it remains to be seen if parts of it (particularly the DNA-binding loops) are disordered.

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