Holliday Junction Resolution Is Modulated by Archaeal Chromatin Components in Vitro*

The Holliday junction-resolving enzyme Hjc is conserved in the archaea and probably plays a role analogous to that of Escherichia coli RuvC in the pathway of homologous recombination. Hjc specifically recognizes four-way DNA junctions, cleaving them without sequence preference to generate recombinant DNA duplex products. Hjc imposes an X-shaped global conformation on the bound DNA junction and distorts base stacking around the point of cleavage, three nucleotides 3' of the junction center. We show that Hjc is autoinhibitory under single turnover assay conditions and that this can be relieved by the addition of either competitor duplex DNA or the architectural double-stranded DNA-binding protein Sso7d (i.e. by approximating in vivo conditions more closely). Using a combination of isothermal titration calorimetry and fluorescent resonance energy transfer, we demonstrate that multiple Hjc dimers can bind to each synthetic four-way junction and provide evidence for significant distortion of the junction structure at high protein:DNA ratios. Analysis of crystal packing interactions in the crystal structure of Hjc suggests a molecular basis for this autoinhibition. The wider implications of these findings for the quantitative study of DNA-protein interactions is discussed.

Holliday junction-resolving enzymes catalyze a key step in the pathway of homologous recombination, cleaving four-way DNA junctions that link recombination intermediates to release heteroduplex DNA products (reviewed in Refs. 1 and 2). The first junction-resolving enzyme was identified in bacteriophage T4, in which the gene49 product was shown to cut branched DNA species and play a role in both viral DNA recombination and packaging (3, 4). Subsequently, resolving enzymes have been identified in all domains of life, including recently the archaea (5, 6), archaeal viruses (7), and pox viruses (8). Recent biochemical evidence suggests that the mammalian resolving enzyme exists in a complex with a junction-specific branch migration apparatus (9), which is analogous to the interaction of the Escherichia coli resolving enzyme RuvC with the RuvAB complex (10). The structures of four junction-resolv-

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ture of the bound junction into an X shape with distortion of base pairing around the site of cleavage (16). The structures of Hjc from *Pyrococcus furiosus* and *S. solfataricus* have been solved recently (17, 18) and have confirmed the relationship of the enzyme with the nuclease superfamily.

In this study, we investigate the activity of *Sulfolobus* Hjc under conditions approximating those found *in vivo*. In the course of this work, we uncovered an unexpected autoinhibitory feature of Hjc, which we have defined at a molecular level using a variety of biophysical techniques. We find that both double-stranded DNA and the archaeal dsDNA3-binding protein Sso7d can ameliorate this inhibitory phenomenon. The results demonstrate that caution should be applied when attempting to draw conclusions about the activity of junction-resolving enzymes based on simplified assay systems.

**EXPERIMENTAL PROCEDURES**

Expression and Purification of Recombinant Hjc—Recombinant Hjc was expressed in *E. coli* strain BL21 (DE3) Codon Plus RIL (Stratagene), and the protein was purified as described previously (19). In brief, E. coli lysate containing recombinant Hjc was first subjected to SP-Sepharose high performance 26/10 column chromatography (Amersham Biosciences, Inc.) using Buffer A (50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM dithiothreitol) and a linear elution gradient of 0–1 mM NaCl. Hjc activity peak fractions were concentrated and loaded onto a 26/70 gel filtration column (Superdex 200 Hi-Load, Amersham Biosciences, Inc.) that was developed with Buffer A containing 300 mM NaCl. Major protein absorbance peak fractions were pooled and shown by SDS-PAGE to contain essentially homogeneous Hjc protein. This enzyme was used for all subsequent analyses. The concentration of Hjc was estimated using the extinction coefficient ε280 (1 mg/ml) = 0.16 calculated from the amino acid composition of the protein.

Purification of Native Sso7d from *S. solfataricus*—The *S. solfataricus* P2 biomass was supplied by Dr. Neil Raven, Centre for Applied Microbiology and Research, Porton Down, UK. Cell lysis, centrifugation, and ultracentrifugation were performed using a variety of biophysical techniques. We find that both double-stranded DNA and the archaeal dsDNA3-binding protein Sso7d can ameliorate this inhibitory phenomenon. The results demonstrate that caution should be applied when attempting to draw conclusions about the activity of junction-resolving enzymes based on simplified assay systems.

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**RESULTS**

**Relationship of Hjc Concentration and Activity**—We have shown previously that Hjc can form multiple complexes with a four-way DNA junction, as judged by electrophoretic retardation analysis, with at least two distinct complexes apparent that may correspond to one and two dimers of Hjc respectively bound to each junction (19). In this study, we set out to characterize the activity of the enzyme in more detail and to investigate activity under conditions approaching those *in vivo*. We first examined the effect of increasing concentrations of Hjc on the junction-resolving activity (Fig. 1). Titration of Hjc against a fixed concentration of 100 nM junction resulted in a biphasic rate curve with maximal activity at 400 nM Hjc dimer. Cleavage activity was strongly inhibited above 400 nM Hjc, reducing to 1% of the maximal rate at 2 μM Hjc. Parallel analysis of the enzyme-junction complexes formed was carried out by electrophoretic retardation analysis. Titration of Hjc into the junction resulted in formation of a complex (complex I) corresponding to one dimer of Hjc bound per junction, reaching a maximum level at 400 nM Hjc, which is in good agreement with the conditions under which maximal enzyme activity was found. Further increases in the enzyme concentration led to the formation of more highly retarded complexes corresponding to larger protein-DNA ratios. Comparison of the gel electrophoretic retardation and activity data (Fig. 1, A and B) revealed that the initial accumulation of complex I was associated with the greatest junction-resolving activity of Hjc, whereas formation of higher complexes had a detrimental effect on the enzyme activity.

**Effect of Competitor Duplex DNA on Hjc Activity**—We next examined the effect of the competitor duplex DNA on the Hjc-junction complex formation and catalytic activity. The presence of a large excess of calf thymus dsDNA competitor over radio-

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1 The abbreviations used are: dsDNA, double-stranded DNA; FRET, fluorescence resonance energy transfer; ITC, isothermal titration calorimetry.

CGTCAACGATCGAGTCCTCGG; x strand, CGGAGACTGCGATGGTGGAGG; GTCCTGGAGACCCTGGAGGAGG.

**Isothermal Titration Calorimetry**—ITC experiments were carried out using a VP-ITC device (MicroCal, Northampton, MA). All solutions were degassed. Hjc and DNA samples were extensively dialyzed against 20 mM Tris-HCl buffer, pH 8.0, containing 200 mM NaCl, 15 mM MgCl2. The binding experiments were performed at 25 °C, under which no hydrolysis of the junction by thermophilic Hjc was detected. A 370-μl syringe with stirring at 400 rpm was used to titrate Hjc into a cell containing ~1.4 ml of DNA solutions. Each titration consisted of a preliminary 1-μl injection followed by up to 30 subsequent 10-μl injections. Calorimetric data were analyzed using MicroCal ORIGIN software. All measurements, including parameter fits, are presented as means of at least duplicate experiments.

**Assay of Hjc Activity**—The specific cleavage of 5′-32P-labeled four-way junction (100 nM) by Hjc was assayed in the presence or absence of 120 μg/ml competitor calf thymus DNA (Sigma) by first forming Hjc-junction complexes in the binding buffer (20 mM Tris-HCl, pH 7.5, 50 mM NaCl) and then initiating hydrolysis by the addition of 15 mM MgCl2 and incubating at 55 °C for 1 min. The reactions were stopped by adding a formamide/EDTA loading mix and heating to 95 °C for 5 min. Products were analyzed by denaturing gel electrophoresis and phosphorimaging as described previously and quantified by calculating the logarithm of the ratio of total:uncut substrate (13).
Fig. 1. Hjc activity and Hjc-junction complexes as a function of Hjc concentration. Radioactively $^{32}$P-labeled four-way DNA junction (100 nM) was incubated with increasing concentrations of Hjc. Free junction and Hjc-junction complexes were separated by electrophoresis in 5% polyacrylamide in TBE buffer. The hydrolysis of the corresponding Hjc-junction complexes was assayed by adding 15 mM MgCl$_2$ to the preformed complexes and incubating the reaction mix at 55 °C for 1 min. The cleavage products were analyzed by denaturing gel electrophoresis and phosphorimaging. A, titration of Hjc into the junction in the absence of a competitor dsDNA followed by non-denaturing gel electrophoretic retardation analysis. B, quantitative results of Hjc activity at corresponding concentrations of Hjc and junction. C, titration of Hjc in the junction in the presence of 120 μg/ml competitor calf thymus dsDNA. D, quantitative results of Hjc activity at corresponding concentrations of Hjc and junction.

Fig. 2. Effect of Sso7d on Hjc activity. Radioactively $^{32}$P-labeled four-way junction (100 nM) was incubated with a fixed concentration of 1 μM Hjc at 55 °C for 1 min in the presence of 15 mM MgCl$_2$, and the cleavage products were analyzed by denaturing gel electrophoresis and phosphorimaging. Under these conditions, autoinhibition by Hjc resulted in negligible quantities of cleaved-junction product. Increasing concentrations of the dsDNA-binding protein Sso7d were included in the incubation, resulting in a progressive stimulation of junction cleavage by Hjc.
comparison of the two binding thermograms immediately revealed a marked difference. The first higher affinity endothermic binding event detected with the Hjc-junction interaction was clearly absent in the Hjc-dsDNA interaction. This difference is due presumably to the specific binding of Hjc to the branch point of the junction in complex I. The binding of the higher complexes of Hjc to the junction molecule is reminiscent of that of Hjc-dsDNA interactions, suggesting that higher complexes of Hjc are accommodated on the double-stranded arms of the junction.

**FRET Studies Reveal Distinct Junction Conformations for Different Hjc Complexes**—We have shown previously, by both gel electrophoretic retardation analysis (16) and FRET (21), that the conformation of four-way DNA junctions is significantly altered upon the binding of one dimer of Hjc. A fixed four-way junction (Figure 3) in complex with Hjc adopted an X-shaped 2-fold symmetrical structure with the B/H arms and R/X arms subtending acute angles (Fig. 4A). In the present study, we addressed the question of whether higher complexes can further distort the junction conformation. We therefore repeated FRET experiments in the presence of a large molar excess of Hjc dimer over the DNA junction. The data obtained for Hjc concentrations higher than those of the junction displayed striking differences from those obtained under equimolar conditions (Fig. 4). In particular, the significant increase in FRET for BH and RX vectors indicated that these arms were driven closer in the higher Hjc complexes. The fluorescence anisotropy of fluorescein was unchanged in the presence of a large excess of Hjc, indicating that the mobility of the donor was unaffected by Hjc binding and suggesting that the increase in FRET efficiency was likely to be due to a shortening of the end-to-end distances.

**A Molecular Explanation for Autoinhibition by Hjc**—Similarities in the crystal packing of two crystal forms of S. solfataricus Hjc (17) and comparison with the P. furiosus Hjc structure (18) offer a possible explanation for the observed concentration-dependent inactivation. Both hexagonal and cubic crystal forms of Hjc contain similar crystal contacts involving loops between strands D-E and H-I (Fig. 5). These strands form a pair of pincers that interact with the equivalent residues from a partner subunit. This part of the molecule comprises the region of largest deviation between the Sulfolobus and Pyrococcus Hjc structures. Furthermore, the residue at which the deviation begins, at the N-terminal end of strand D, is the catalytically implicated Lys-57. It is conceivable that this interaction is responsible for the autoinactivation of Hjc at elevated concentrations. The observations that the same fold is observed in two unrelated crystal forms, accompanied by an increase in the secondary structure of the relevant loops and that one of the major components of the interaction is an otherwise exposed hydrophobic residue, Ile-62, suggest that it is a conserved rather than a fortuitous interaction.

**DISCUSSION**

We have shown that several dimers of the Holliday junction-resolving enzyme Hjc can bind to a single synthetic four-way junction in vitro. In addition to targeting the junction specifically to form an enzyme-substrate complex, further molecules of Hjc can associate, probably through a combination of DNA-protein and protein-protein interactions. These higher order complexes are inhibitory to DNA cleavage, possibly because of the extreme distortion of the four-way junction conformation (Fig. 4). Inhibition can be relieved either by adding duplex DNA competitor or by the dsDNA-binding protein Sso7d. At a molecular level, Sso7d probably competes with extra molecules of Hjc for binding sites on the duplex DNA arms, thus preventing the formation of inhibitory complexes, whereas competitor duplex DNA may “soak up” the extra Hjc molecules that would otherwise bind on the duplex arms of the junction. We do not see a synergistic effect when both duplex DNA and Sso7d protein are added to Hjc cleavage reactions, which makes sense if both are functioning to relieve Hjc inhibition through essentially the same mechanism.

The crystal structure of Sulfolobus Hjc gives a strong indication as to how Hjc dimers might interact with one another through a pair of interlocking loops to generate larger complexes. What we do not yet know is whether this secondary interaction has a functional role. It may, for example, represent a means by which the nuclease activity of Hjc is repressed in the absence of Holliday junctions to ensure that a nonspecific cleavage of phosphodiester bonds does not occur. If so, this mechanism is presumably not shared by Pyrococcus Hjc because the interaction surface does not exist for that enzyme. Mutagenesis studies are under way to assess the importance of the secondary interface for the structure and function of Sulfolobus Hjc.

In conclusion, the reconstitution of an approximation for
Holliday Junction Resolution in Archaeal Chromatin

Fig. 4. FRET analysis of Hjc-junction complexes. The six possible donor acceptor-labeled DNA species were used to examine the global structure of the junction bound to Hjc. The data are plotted as histograms of FRET efficiency ($E_{FRET}$) for the six end-to-end vectors. A, fluorescent junction (25 nM) preincubated with 25 nM Hjc dimer. B, schematic to show deduced global structure of the DNA junction upon binding of one dimer Hjc. In this complex, Hjc exhibits optimal activity. Arrows indicate the positions of Hjc cleavage. C, fluorescent junction (25 nM) preincubated with 150 nM Hjc dimer. D, schematic of the global conformation of the junction upon binding multiple Hjc dimers. In this complex, Hjc fails to cleave the DNA possibly because of the inaccurate positioning of the enzyme catalytic center over the targeted scissile bond. E, plot of $E_{FRET}$ for BH vector as a function of Hjc concentration in the absence of magnesium ions.

Fig. 5. Structural basis for autoinhibition by Hjc. Backbone traces for Sulfolobus Hjc (blue) and Pyrococcus Hjc (green) in the region proposed to be involved in autoinactivation. A symmetry related Sulfolobus Hjc molecule (gray) is shown, and the approximate position of the crystallographic 2-fold axis is marked (ellipse). Ile-62 of both molecules is shown in a stick representation. Single uppercase letters mark the relevant secondary structure elements in Sulfolobus Hjc. Inset, relative location in the global structure. This figure was prepared using Molscript (26) and Raster3d (27).

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archaeal chromatin in vitro has a significant influence on the activity of the Hjc enzyme. These observations highlight the complexity of DNA-protein interactions in vivo in which DNA is tightly packaged in chromatin and in which structures such as Holliday junctions must constitute a tiny proportion of the total nucleic acid present. Similar situations may be encountered for other enzymes that recognize a particular DNA structure or sequence during DNA replication, transcription, or repair, and there is evidence that chromatin structure can modulate DNA repair pathways both in vitro and in vivo (reviewed in Ref. 25).
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