Biochemical characterization of the Hjc Holliday junction resolvase of Pyrococcus furiosus

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ABSTRACT
The Hjc protein of Pyrococcus furiosus is an endonuclease that resolves Holliday junctions, the intermediates in homologous recombination. The amino acid sequence of Hjc is conserved in Archaea, however, it is not similar to any of the well-characterized Holliday junction resolvases. In order to investigate the similarity and diversity of the enzymatic properties of Hjc as a Holliday junction resolvase, highly purified Hjc produced in recombinant Escherichia coli was used for detailed biochemical characterizations. Hjc has specific binding activity to the Holliday-structured DNA, with an apparent dissociation constant (Kd) of 60 nM. The dimeric form of Hjc binds to the substrate DNA. The optimal reaction conditions were determined using a synthetic Holliday junction as substrate. Hjc required a divalent cation for cleavage activity and Mg2+ at 5–10 mM was optimal. Mn2+ could substitute for Mg2+, but it was much less efficient than Mg2+ as the cofactor. The cleavage reaction was stimulated by alkaline pH and KCl at ~200 mM. In addition to the high specific activity, Hjc was found to be extremely heat stable. In contrast to the case of Sulfolobus, the Holliday junction resolving activity detected in P.furiosus cell extract thus far is only derived from Hjc.

INTRODUCTION
The Holliday junction is a critical intermediate structure formed during homologous recombination in which two homologous duplex DNA molecules are held together by a single-stranded crossover (1). In Escherichia coli the RuvA and RuvB protein complex or the RecG protein specifically binds to the Holliday junction and promotes branch migration. Then the Holliday junction is resolved by a specific endonuclease, RuvC (reviewed in 2,3). The biochemical properties of the RuvC endonuclease have been studied extensively. The crystal structure of RuvC was determined at atomic resolution and a putative model of RuvC complexed with the Holliday junction was also prepared to suggest the DNA cleavage mechanism (4,5).

The Holliday junction resolving enzymes are ubiquitous structure-specific endonucleases and their activity has been identified in a wide variety of organisms, from bacteriophage to mammals (6,7). However, besides E.coli RuvC, until recently the corresponding enzymes had been isolated and characterized only from bacteriophage T4 (endonuclease VII), bacteriophage T7 (endonuclease I), lambda prophage (RusA) and yeast mitochondria (endonucleases CCE1 and SpCCE1). These junction resolvases share certain biochemical properties, but little sequence homology.

The Archaea is now recognized as constituting a third major branch of life, together with Bacteria and Eukarya (8). Recently we identified a protein with Holliday junction resolving activity in a hyperthermophilic archaeon, Pyrococcus furiosus, and named it Hjc (Holliday junction cleavage) (9). The amino acid sequence of the Hjc protein is highly conserved in the archaeal genome, but not in Bacteria or Eukarya thus far. This year two different Holliday junction cleavage activities were reported in a cell extract of Sulfolobus solfataricus, one of which was the orthologous enzyme to Hjc (10,11).

In this study we have investigated the general biochemical and physical properties of the P.furiosus Hjc resolvase in more detail and will discuss the similarities and differences in comparison with other Holliday junction resolvases, especially S.solfataricus Hjc and Hje (Holliday junction endonuclease).

MATERIALS AND METHODS
Enzymes
Hjc was purified to homogeneity as described previously (9). T4 polynucleotide kinase, T4 DNA ligase and restriction endonucleases were obtained from Takara Shuzo (Kyoto, Japan).

DNA substrates
Synthetic DNA substrates were prepared by annealing of oligonucleotides, one of which had been labeled with 32P at its 5'-end, as described (9). The sequences of 20 oligonucleotides and combinations thereof that were used for preparation of DNA substrates with various structures are shown in Table 1. The four-way junctions 4Jh and 4Jhs contained homologous cores. The three-way junction 3Jh has a mobile homologous core and its conformation can change to a four-way junction.

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Other junctions, 4J, 4Jb (four-way) and 3J (three-way), do not have homologous cores and the junction centers are fixed.

**Endonuclease assay**

Hjc (10 or 25 nM) was incubated with various 32P-labeled DNA substrates at 10 or 100 nM in 40 µl of reaction buffer (10 mM Tris–HCl, pH 8.8, 10 mM MgCl2, 200 mM KCl, 1 mM DTT) at 56°C for 30 min. The reaction was stopped by adding phenol and the products were analyzed by PAGE in TAE buffer (40 mM Tris–acetate, pH 8.3, 1 mM EDTA) or by denaturing PAGE in TBE buffer (90 mM Tris–borate, pH 8.3, 2 mM EDTA) and visualized by autoradiography.

**Determination of Hjc cleavage sites**

3J, 3Jb and L10 (each at 10 nM), with a uniquely 32P-labeled arm, were incubated with or without Hjc (100 nM) at 56°C for 1 h in reaction buffer (40 µl), as described above. The reaction was stopped by phenol extraction and the products were analyzed by denaturing PAGE followed by autoradiography. GA ladders of each labeled oligonucleotide generated by the Maxam–Gilbert method were loaded alongside to provide markers.

**Ligation reaction**

32P-labeled 4Jhs (10 nM) was incubated with or without Hjc (1 µM) at 56°C for 1 h in reaction buffer (100 µl) as described above. The reaction was stopped by phenol extraction and ethanol precipitation. The product was dissolved in ligation buffer (50 mM Tris–HCl, pH 7.9, 10 mM MgCl2, 20 mM DTT, 1 mM ATP, 60 µg/ml BSA) and was incubated with or without T4 DNA ligase at 16°C for 2 h. The reaction products were analyzed by denaturing PAGE and autoradiography.

**Gel retardation assay**

Various concentrations of Hjc were incubated with 10 nM 32P-labeled 4Jb in 20 µl of binding buffer (20 mM Tris–acetate, pH 8.0, 1 mM DTT, 0.1 mg/ml BSA, 5% glycerol) for 10 min.
on ice and then 5 µl of loading buffer (20 mM Tris–acetate, pH 8.0, 10% glycerol, 0.1% bromophenol blue) was added. The complexes were analyzed by 6% PAGE in TGE buffer (50 mM Tris, 380 mM glycine, 2 mM EDTA).

**Construction of MBP–Hjc**

The hjc gene was amplified by PCR using the primers 5′-CGCA-CGAGGAAATTCCATGTATAGAAAAGGG-3′ and 5′-CAC-GAGTCCTGCAGTTATCATGATTTCCCC-3′. The PCR product was digested with EcoRI and PstI and was inserted into EcoRI/PstI ended plasmid pMAL-c2 (New England Biolabs, Beverly, MA). The resultant plasmid was designated pHJMAL. *Escherichia coli* JM109 carrying pHJMAL or pMAL-c2 (as a control) was grown in 100 ml of LB medium containing 100 µg/ml ampicillin to an OD₆00 of 0.7 and then the *malE–hjc* or *malE* gene was induced by adding isopropyl-β-D-thiogalactoside (IPTG) to a final concentration of 1 mM. After further incubation for 3 h the cells were harvested. The MBP (maltose binding protein)-fused Hjc and MBP were purified from *E.coli* cell extracts by amylose column chromatography according to the manufacturer’s instructions (New England Biolabs). The fractions containing MBP–Hjc or MBP were dialyzed against buffer B (50 mM Tris–HCl, pH 8.0, 0.1 mM EDTA, 0.5 mM DTT, 10% glycerol and stored on ice.

**Proteolytic digestion of MBP–Hjc fusion protein**

The linker region between the MBP and Hjc proteins contains the recognition sequence (Ile-Glu-Gly-Arg) for Factor Xa protease.

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**Figure 2.** Determination of the cleavage sites produced by Hjc. 3J (A), 3Jh (B) and L10 (C), each uniquely labeled with 32P at the 5′-end on the indicated strand, were incubated with (+) or without (−) Hjc (100 nM) and the reaction products were analyzed as described in Materials and Methods. GA lanes show the GA sequence ladder of each labeled oligonucleotide. Schemes of the central sequences of 3J (D), 3Jh (E) and L10 (F), indicating the cleavage sites. The cleavage sites are shown by arrowheads whose sizes represent the cutting efficiencies. The boxed areas indicates the mobile regions. 3Jh is able to change its configuration to a four-way junction.
Partial digestions of the fusion protein with factor Xa (New England Biolabs) were performed as described (9). Binding of these fusion proteins to the substrate DNA was analyzed by the gel retardation assay described above.

**Chromatography for detection of junction resolving activity**

*Pyrococcus furiosus* strain Vc1 (DSM3638) was cultured as described previously (12). A crude cell extract was prepared by sonication of the cells, followed by centrifugation. The cell extract from 2 or 0.7 g of cells was applied to a cation exchange (HiTrap SP, 5 ml) or anion exchange (HiTrap Q, 5 ml) column, respectively, pre-equilibrated with buffer A as described earlier (11) and developed with a linear gradient of NaCl (0–1.5 M for HiTrap SP and 0–1 M for HiTrap Q). Each fraction was subjected to cleavage assay with a synthetic Holliday junction as the substrate.

**RESULTS**

**Substrate specificity of Hjc resolvase**

The substrate specificity of Hjc was investigated using the highly purified Hjc protein and various forms of synthetic DNA under the standard assay conditions determined previously (9). As shown in Figure 1, Hjc efficiently cleaved four-way junctions and much less efficiently cleaved three-way junctions (both mobile and immobile). The cleaved band was detected from the looped-out DNA substrate when 10 times more Hjc (100 nM) was added (data not shown). Therefore, the exact cleavage sites of the three-way junctions and looped-out DNA were determined (Fig. 2). Both three-way junctions were cleaved at the same major cleavage sites as the four-way junctions (between the third and fourth nucleotides from the junction center), as shown previously (9). However, the mobile junctions were cleaved more efficiently at two other sites, which may reflect the change in the configuration of the substrate to the four-way junction, as shown in Figure 2E. The reason why the amount of the cleaved band from the mobile three-way junction was less than that from the immobile three-way junction, as seen in Figure 1, is possibly due to the configuration change to the four-way junction, in which the 32P-labeled oligonucleotide (oligo-2) used in Figure 1 was cleaved with less efficiency (Fig. 2A and B). The looped-out structure was also cleaved near the site of looping out (Fig. 2C and F).

To confirm that the nicked strand in the resolved duplex DNA is rejoined by subsequent ligation, as described previously (9), we analyzed the reaction products as described in Materials and Methods. Most of the major cleavage products from the junction cleaving reaction (37 bases) were converted to a longer strand (70 bases) by treatment with T4 DNA ligase, as shown in Figure 3. This result clearly shows that the cleaved strand is rejoined by ligation, which means that cleavage occurs at the symmetrically related sites of the two strands, to leave 5′-phosphate and 3′-hydroxyl termini.

**Reaction conditions**

The reaction conditions for Hjc were investigated in detail using the synthetic four-way junction 4Jh as described in Materials and Methods. The Hjc activity for this substrate was stimulated by alkaline pH, with an optimum at 8.0–10.0 (Fig. 4A).

Hjc requires MgCl2, with an optimum at 5–10 mM (Fig. 4B). MgCl2 can be replaced by MnCl2, even though cleavage efficiency is drastically decreased, however, no cleaved product was detected in reactions with CaCl2 and ZnCl2 (Fig. 4C). The optimal pH and the effect of divalent cations on Hjc were very similar to those for *E. coli* RuvC. However, the optimal concentration of KCl in the reaction buffer was 200 mM (Fig. 4D), which inhibits the cleavage activity of RuvC by >80% (13,14). NaCl had the same effect as KCl on Hjc activity (data not shown). The optimal temperature of the cleavage reaction on this substrate was 70–75°C (Fig. 4E). It is possible that the synthetic Holliday junction used in the assay was denatured to inappropriate substrate structures above 80°C, because Hjc was very heat stable. Incubation of 20 µM Hjc protein at 95°C for 10 min did not affect its activity. The activity decreased to ~60% after 2 h at 95°C (Fig. 4F). When Hjc was incubated at 90°C for 16 h no decrease in activity was observed (data not shown). Therefore, Holliday junction substrates with much longer arms and higher GC content would be more suitable to determine the true optimal temperature of the cleavage reaction by Hjc.

**Specific binding of Hjc to the four-way DNA junction**

It is well known that Holliday junction resolvases preferably bind to a four-way junction rather than to duplex DNA (6). The DNA binding activity of Hjc was investigated by a gel retardation assay using a 32P-labeled synthetic four-way junction, 4Jh. As shown in Figure 5A, a discretely retarded band was observed.
when Hjc protein was present. The shifted band decreased when an increasing amount of the non-labeled four-way junction was added as competitor. When the non-labeled duplex DNA was added, no effect was observed, even in the presence of a 100-fold excess. These results show that Hjc has specific binding ability to four-way junctions. The amount of DNA–Hjc complex increased as the protein concentration was increased (Fig. 5B). From this experiment the apparent dissociation constant ($K_d$) with the corresponding substrates was calculated to be 60 nM, a value that is comparable to those of the other Holliday junction resolvases reported to date (15–18).

**Binding mode of Hjc to its substrate**

We reported previously that a sedimentation equilibrium analysis revealed that Hjc forms a homodimer in solution (9). To determine the quaternary structures of Hjc bound to its substrate, we constructed a system to produce these proteins as N-terminal fusions with MBP. The highly purified MBP–Hjc fusion protein (Fig. 6A) bound to the four-way junction with a little weaker affinity than that of Hjc (data not shown). The fusion protein could be digested by Factor Xa at the Ile-Glu-Gly-Arg site between MBP and Hjc. The fusion protein was subjected to digestion with increasing amounts of Factor Xa before binding to the substrate four-way junction, as shown earlier (19,20). The gel retardation pattern showed three kinds of DNA–protein complexes (Fig. 6B). Two of the three bands corresponded to the sizes of DNA–MBP–Hjc and DNA–Hjc, respectively. One more band between these two should correspond to a complex of the heterodimer (MBP–Hjc and Hjc) and DNA produced by partial digestion with Factor Xa. These results support the hypothesis that Hjc binds to the DNA substrate as a homodimer.

**Sensitivity of Hjc to the junction conformation**

The structure of the Holliday junction is sensitive to the presence of divalent metal ions, which facilitate their folding onto a
stacked X-structure. The stacked X-structure has two-fold symmetrical arms in which two strands are continuous B-form DNA while the other two complementary strands are sharply bent and pass from one helix to the other (21,22). To investigate the strand selectivity of cleavage by Hjc, two synthetic Holliday junctions, J1 and J3, with well-characterized structures (23) were used for the assays. In the fixed junction J1 the b and r strands are continuous and h and x are the exchanging strands. On the other hand, in the fixed J3 h and x are continuous and b and r are exchanging. Hjc cleaved both J1 and J3 efficiently. To avoid a temperature effect on the conformation of the junctions, we used 37°C in this experiment to maintain the reported structure (23). A clear selectivity was observed in J1 cleavage. Hjc predominantly cleaved the b and r (continuous) strands in junction J1, while the four strands in J3 were cleaved almost equally (Fig. 7). The lack of cleavage selectivity of Hjc in J3 is similar to the characteristics of the S.solfataricus Hjc homolog recently reported (11), although it is not known how the S.solfataricus Hjc cleaves the J1 junction.

Searching for Hje-like activity in P.furiosus

Kvaratskhelia and White demonstrated that there are two peaks of junction resolving activity in a cation exchange chromatogram of a S.solfataricus crude cell extract (11). The distinct difference in the two activities is their sensitivity to junction conformation. Hje has a very strong preference for cleavage of the continuous strand. On the other hand, Hjc cleaves all four strands equally (11). It would be very interesting to know if there are two different junction resolving activities, like S.solfataricus Hjc and Hje, generally present in Archaea. Using the same chromatographic conditions (cation exchange), a crude cell extract of P.furiosus was fractionated. The cleavage assay showed only one active fraction, which eluted at 1.2 M NaCl in the chromatogram (Fig. 8). In the case of S.solfataricus two activities were eluted, at NaCl concentrations of 0.41 and 0.75 M, respectively (11). Some activity passed through the column in our case, however, western blot analysis using an anti-Hjc antibody showed that the Hjc band was clearly detected in both active fractions (data not shown). We used an anion exchange column to fractionate the crude cell extract and, again, one
active peak eluted at 0.7 M NaCl, besides the flow-through fraction (Fig. 8). From these results it seems likely that the junction resolving activity in the P. furiosus cell extract is only derived from Hjc protein. Separation of the activity into two fractions, the flow-through and column-bound fractions, is possibly due to a difference between DNA (or RNA)-bound Hjc and free Hjc in the cell extract.

DISCUSSION

Highly purified Hjc enzyme cleaved four-way junctions with high selectivity. It cleaved the three-way junction with much less efficiency and loop-structured DNA with even less efficiency, only when an increased amount of enzyme was used, like the ‘star activity’ of restriction endonucleases (24). Unlike bacteriophage junction resolvases, and like cellular enzymes, Hjc does not cleave linear duplex DNA containing one base mismatch. From these results Hjc seems to have a high level of selectivity for the Holliday junction, like cellular junction-resolving enzymes, such as RuvC and CCE1. In addition, Hjc was shown to bind to the Holliday junction as a dimer in this work. It has been one of the common features that all junction resolving enzymes studied to date bind to the junction DNA in dimeric form (25–29), and Hjc is no exception.

The sequence dependency for junction cleavage is another important property of junction resolvases to be investigated. The bacteriophage enzymes exhibit rather low sequence dependence. In contrast, the cellular resolvases have some degree of sequence specificity for cleavage (26,28,30). In our previous report Hjc predominantly cleaved at one site of the synthetic Holliday junction with a homologous sequence in the core, which causes the junction center to be mobile (9). Moreover, preliminary results using the RecA-mediated recombination intermediate with natural DNA, reporting the sequence specificity of E. coli RuvC cleavage (30), showed that Hjc has some sequence preference for cleavage sites and, furthermore, that the cleavage pattern of Hjc was different from that of RuvC (K.Komori and Y.Ishino, unpublished results). The sequence preferences for binding and cleavage sites of Hjc remain to be precisely analyzed.

From the results on strand selectivity for cleavage in the junction presented here we could not conclude whether Hjc preferably cleaves the continuous strands rather than the exchange strands. A distinct selectivity for the continuous strand was observed when junction J1 was used as the substrate. However, all four strands were cleaved almost equally in the case of junction J3. We do not yet know the strength of the Hjc sequence preference for cleavage. Sequence preference may compete with conformation preference in the cleavage assay used in this study. Further analyses using different substrates, such as the tethered Holliday junctions used for other junction resolvases (18,31,32), will be helpful to determine the properties more precisely. We have used several different synthetic four-way junctions for the cleavage reaction of Hjc thus far and have found that Hjc always cleaves symmetrically 3 nt on the 3′-side of the junction center (data not shown). This is one of the remarkable features of this enzyme, which should be considered when determining the cleavage specificity.

It is a very interesting issue as to how many junction resolvases are present in archaeal organisms. In this study we could not find a second junction resolvase activity in P. furiosus. One of the possible predictions is that the crenarchaeotes, including S. solfataricus, have Hjc and Hje, whereas the euryarchaeotes, such as P. furiosus, lack Hje. Actually, within the domain Archaea there are many distinct differences between the two subdomains. However, we only carried out cleavage activity assays of the P. furiosus cell extract fractions with one set of reaction conditions, which were optimized for Hjc. Pyrococcus furiosus may have another junction resolvase that can be detected under different reaction conditions. It is also possible that a level of Hje activity that was lower than the detection limit is present within P. furiosus, as compared with that of S. solfataricus. Further analyses, including cloning the gene corresponding to Hje from Sulfolobus spp., will be necessary for the progress of this interesting subject, as the authors who discovered Hje pointed out (11).

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