Crystal Structure of the Archaeal Holliday Junction Resolvase Hjc and Implications for DNA Recognition

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Summary

Background: Homologous recombination is a crucial mechanism in determining genetic diversity and repairing damaged chromosomes. Holliday junction is the universal DNA intermediate whose interaction with proteins is one of the major events in the recombinational process. Hjc is an archaeal endonuclease, which specifically resolves the junction DNA to produce two separate recombinant DNA duplexes. The atomic structure of Hjc should clarify the mechanisms of the specific recognition with Holliday junction and the catalytic reaction.

Results: The crystal structure of Hjc from the hyperthermophilic archaeon Pyrococcus furiosus has been determined at 2.0 Å resolution. The active Hjc molecule forms a homodimer, where an extensive hydrophobic interface tightly assembles two subunits of a single compact domain. The folding of the Hjc subunit is clearly different from any other Holliday junction resolvases thus far known. Instead, it resembles those of type II restriction endonucleases, including the configurations of the active site residues, which constitute the canonical catalytic motifs. The dimeric Hjc molecule displays an extensive basic surface on one side, which contains many conserved amino acids, including those in the active site.

Conclusions: The architectural similarity of Hjc to restriction endonucleases allowed us to construct a putative model of the complex with Holliday junction. This model accounts for how Hjc recognizes and resolves the junction DNA in a specific manner. Mutational and biochemical analyses highlight the importance of some loops and the amino terminal region in interaction with DNA.

Introduction

Holliday junction plays a crucial role in the molecular mechanisms of genetic recombination and double strand break repair [1, 2]. It acts as a central recombination intermediate, where each strand of two DNA duplexes is exchanged to form a joint molecule with a four-way junction at a crossover. The completion of the recombinational/repair process requires Holliday junction–specific endonucleases, which exist ubiquitously from prokaryotes to eukaryotes. To date, various junction resolvases have been identified, such as bacteriophage T4 endonuclease VII, T7 endonuclease I, RusA and RuvC in eubacteria, Hjc in archaea, Cce1 in yeast mitochondria, and recently found A22 in vaccinia virus [3, 4]. Junction resolution activities also have been detected in nuclear extracts from yeast and mammalian cells; however, neither the protein nor the gene corresponding to the activity has been identified yet [5, 6, 7]. The primary sequences of Holliday junction specific resolvases determined thus far show no significant similarity to one another. The comparisons between the crystal structures of RuvC and endonuclease VII also provide no significant indication that the enzymes share a common ancestor in terms of molecular evolution [8, 9], in spite of their common features of acting as dimers and requiring divalent metals, Mg2+ or Mn2+, for paired nicking activities to the two opposite strands.

Hjc was identified from the hyperthermophilic archaeon Pyrococcus furiosus [10], and all of the archaeal genomes sequenced to date contain the gene encoding an Hjc-like sequence [10, 11]. The activity of this resolvase with the smallest molecular mass was indeed highly specific to the topology of Holliday junction [10, 12]. The enzyme also catalyzes the cleavage of a Holliday junction formed by the RecA-mediated strand exchange reaction between plasmid DNAs in vitro [10]. Moreover, the junction cleavage activity of Hjc is modulated by the RadB protein, a RecA/Rad51 family protein in P. furiosus, in an ATP-dependent manner [13]. These results strongly suggest that the role of Hjc in the archaeal cells is to complete the homologous recombination process through the cleavage of Holliday junction. The enzyme introduces symmetrical nicks into phosphodiester bonds between the third and fourth nucleotide on the 3’ side of the junction [10, 12, 14].

To understand the molecular basis for the mechanisms of Holliday junction recognition and cleavage, we have crystallized Hjc from P. furiosus and determined the structure at 2.0 Å resolution. Its dimeric architecture and active site conformation are highly similar to those of type II restriction enzymes. In combination with mutational and biochemical analyses, the Hjc structure allows the construction of a putative complex model with Holliday junction.

Results and Discussion

Overall Structure

The asymmetric unit of the crystal contains two dimeric Hjc molecules (Figure 1a). A single dimeric molecule is related by an internal two-fold axis, and two dimers are connected by a second, noncrystallographic two-fold axis. The two dimeric molecules are packed closely to

Key words: Archaea; DNA repair; Holliday junction; homologous recombination; X-ray crystallography
Structure

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Figure 2. Hjc Dimer Interface
Close-up stereo view of the dimer interface, highlighted by hydrophobic side chains. The two-fold axis runs longitudinally on the page plane.

Figure 1. Structure of Hjc Resolvase
(a) Stereo diagram of two dimeric Hjc molecules in an asymmetric unit. Each molecule is colored differently. Bound sulfate molecules are colored yellow.
(b) Ribbon diagram of the single Hjc subunit in the same orientation as in (a).
(c) Topology diagram of the Hjc subunit. β strands are represented as arrows, and α helices are represented as rectangles. Short helical turns are represented as small squares.
(d) Sequence alignment of Hjc from archaea. Identical residues are shown in orange letters, and conservative substitutions are in pink. Black boxes represent sites of the mutations. Asterisks indicate residues important for endonuclease activity, and sharps indicate residues important for dimerization.

Figure 2. Hjc Dimer Interface
Close-up stereo view of the dimer interface, highlighted by hydrophobic side chains. The two-fold axis runs longitudinally on the page plane.

Each other through two symmetric contacts, each of which involves the N-terminal α1 helix, β2, and β3. Notably, each contact is reinforced by a sulfate ion, which scaffolds the conformation of the N terminus, as described later, and this ion appeared to be essential for successful crystallization. The four subunits in the asymmetric unit show essentially identical structures with an rmsd value of 0.86 Å on average, except for the N terminus region coordinating the sulfate ion. The Hjc subunit consists of a single compact domain with an α/β structure, where the central β sheet is buttressed by two major α helices, α1 and α2 (Figures 1b–1d). In addition to this sheet consisting of strands β1, β2, β3, β5, and β6, two strands, β4 and β7, form an extra sheet and, together with the carboxyl terminal α3 helix, lie on the opposite side of the dimer interface.

Dimer Contact
The dimer interface, with an area of 1700 Å², is mainly composed of the β1 and β2 strands and the α2 helix. The contact involves hydrophobic interactions between Val24 in one subunit and Val35, Phe68, and Phe72 in the other noncrystallographic symmetry (ncs)-related subunit (Figures 1c, 1d, and 2). These four residues from each subunit form a central hydrophobic core in the interface. The two side chains of ncs-related Tyr42 also form a π–π interaction. The structural features of this interface are consistent with the result, indicating that mutations at Phe68 or Phe72 affected Hjc dimer formation [15].

Similarity to Resolvase/Restriction Endonuclease
Thus far, several three-dimensional structures have been reported with respect to the Holliday junction resolvase and some recombinases. The architectures of bacteriophage T4 endonuclease VII (T4 endoVII) [8] and E. coli RuvC [9], both of which form a dimer, are strikingly different from each other. T4 endoVII folds into a unique elongated dimeric architecture in which a domain is swapped between two subunits (Figure 3a). Its subunit structure, predominantly composed of α helices and two short β strands, shows some similarity to colicin E7 [16]. The two ncs-related subunits contact each other through the interface, which involves each pair of α helices, stabilized by hydrophobic interactions. On the other hand, RuvC contains a five-stranded β sheet with α helices on both sides (Figure 3b). This folding topology is similar to those of RNase H1, HIV integrase, and Mu transposase, which contain catalytic centers formed by four conserved acidic residues [17, 18]. The extensive interface of the RuvC dimer involves hydrophobic and some polar interactions, mostly contributed by a major α helix of each subunit. Although Hjc contains the five-stranded β sheet, as found in RuvC, its topology is different (Figure 3c). It is also obvious that Hjc differs from
Crystal Structure of Hjc

Figure 3. Comparison of Three Holliday Junction Resolvases
(a) T4 Endonuclease VII (1en7).
(b) RuvC (1hjr).
(c) Hjc.
Two orthogonal views are shown for each subunit colored differently. Putative active site residues are highlighted by their side chains.

T4 endoVII, which lacks a central major $\beta$ sheet. Furthermore, the Hjc dimer interface is dominated by hydrophobic interactions, which are contributed by a part of the major $\beta$ sheet, in contrast with the major roles of $\alpha$ helices in the interfaces of other enzymes.

Both a visual inspection and a three-dimensional homology search on the DALI server [19] revealed similarity in the 3-D structures between Hjc and type II restriction endonucleases. The internal framework of each subunit superimposes well between the two enzymes (Figure 4a). A pairwise least square superposition of the core region (1-90) of Hjc with each restriction enzyme provides average rmsd values of 2.5 Å over 83 residues of FokI, 2.8 Å over 80 residues of EcoRV, and 2.9 Å over 87 residues of BglII.

The structural similarity between Hjc and restriction endonucleases extends to the active sites (Figure 4b). In fact, the superposition of Hjc (pink) onto the three restriction endonucleases, FokI (red), EcoRV (yellow), and BglII (blue), reveals the striking spatial coincidence of Glu9, Asp33, Glu46, and Lys48 in Hjc with three acidic residues and one lysine residue, respectively, all of which constitute the canonical catalytic motifs of type II restriction endonucleases. It should be noted that this resemblance in the active site structures also contrasts with the similarity between the type II restriction endonucleases and the very short patch repair (Vsr) endonuclease, where some residues within the catalytic motif are replaced by histidine [20].

Previous combined approaches of mutational analyses and sequence comparisons have suggested that Hjc contains motifs related to type II restriction endonucleases and that Glu9, Asp33, Glu46, and Lys48 are actually involved in catalysis [15, 16, 21]. The corresponding residues in *Sulfobolus solfatarius* Hjc (Glu12, Asp42, Glu55, and Lys57) are also proven to be essential for the catalysis and allowed modeling of a putative Hjc structure [14]. In terms of the active site arrangement and subunit structure prediction, these studies are in good agreement with the findings from the crystal structure presented in this study.

Figure 4. Alignment of Hjc to Three Type II Restriction Endonucleases
(a) Ribbon diagram of Hjc (gray), FokI (red, 2fok), EcoRV (yellow, 1rva), and BglII (blue, 1dmu). Only the homologous regions and side chains that are important for activity are depicted.
(b) Using the same color scheme as in (a), side chains of the catalytic residues are overlaid on Hjc structure.
Figure 5. Surface Representation of Hjc
(a) Electrostatic surface of the Hjc dimer shown in two orientations generated by GRASP [42]. It is colored according to the local electrostatic potential range from \(-15 \text{kT}\) to \(+15 \text{kT}\) (red) to \(+15 \text{kT}\) (blue).
(b) Surface of Hjc as in (a), representing amino acid conservation. Coloring scheme represents the level of amino acid similarity observed on the eight aligned sequences of Hjc. The colors range from 0% (white) to 50% (yellow) and 100% (red) sequence identity.

DNA Binding Interface
Hjc is a highly basic protein with a calculated pI value of 10.5. Forty-five percent of the Hjc dimer surface is covered by positive charges, which are primarily concentrated on one side of the Hjc dimer (Figure 5a). This biased charge distribution implies that the positively charged surface is involved in Holliday junction binding. The basic interface is also abundant in many conserved amino acids, including catalytically important charged residues [15] (Figure 5b). In spite of the different folding, a similar bipolar charge distribution was observed in T4 endoVII and RuvC, both of which contain catalytic residues within their positive surfaces. The spacing (25–35 Å) of the two catalytic sites within the dimers of T4 endoVII (Figure 3a) and RuvC (Figure 3b) is also close to that in the Hjc dimer. Furthermore, in contrast to restriction endonucleases, the positive surfaces of the junction resolvases, including Hjc, appear to be flatter and more extensive. This feature may be required to make intimate contact with the junction DNA.

Model of the Hjc–Holliday Junction Complex
The previous biochemical analyses of P. furiosus Hjc have revealed that it specifically binds to Holliday junction DNA and cleaves at the third and fourth nucleotides on the 3' side of the junction [10, 12, 14]. Strand selectivity in the cleavage of Holliday junction also showed that Hjc prefers continuous strands to crossover strands [12].

The crystal structure of BglII bound to DNA containing its specific recognition sequence was previously determined [22]. The superposition of the Hjc protein backbone onto that of the BglII–DNA complex implied that two DNA duplexes bound to BglII are arranged so as to form a Holliday junction–like structure. Based on this finding, a model structure of the Hjc–Holliday junction DNA complex has been built (Figure 6). In this model, the DNA structure is more similar to the stacked-X form [23] than to the extended-X form [24], although the orientation of the stacking arms is different. The two cleaved phosphodiester bonds of the two continuous DNA strands in the model are exactly located near the active site of each Hjc subunit. When the extended-X form DNA is docked onto Hjc, these cleaved bonds are farther apart (±33 Å) than the spacing between the two active sites in Hjc dimer. Therefore, it is likely that Holliday junction needs to form a stacked structure upon binding to Hjc. This notion is consistent with the findings from biochemical analysis of S. solfataricus Hjc that, the junction DNA takes symmetry-X form upon binding to the protein, and that several base pairs around the junction center may be disrupted [14]. Our current model exhibits a number of unique features in terms of protein–DNA interactions. For instance, several conserved basic and aromatic residues around the N-terminal end of the α1 helix should be in contact with the minor groove of the DNA, together with two basic residues on the loop between β1 and β2. In addition, several basic residues in the loop between β3 and β4 appear to interact with
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Figure 7. Biochemical Characterization of Hjc Mutant Proteins
(a) Purified proteins (1 μg each) were analyzed by 15% SDS-PAGE. The gel was stained with Coomassie brilliant blue.
(b) Holliday junction cleavage assay for mutant proteins. Wild-type (2, 10, or 100 nM) and mutant Hjc proteins (2, 10, 100 nM, or 4 μM) were incubated with 32P-labeled 4Jh (100 nM) at 60°C for 30 min. The reaction products after phenol extraction were analyzed by 6% PAGE and were detected by autoradiography. Lane (−), no protein as a negative control.
(c) Holliday junction binding activities of mutant proteins. The wild-type and mutant Hjc proteins (20, 50, or 100 nM) were incubated with 32P-labeled 4Jh (10 nM) on ice for 10 min. The products were analyzed by 6% PAGE, followed by autoradiography.

The DNA in the major groove. These conformational views were examined by mutational and biochemical analyses.

Mutational Analysis of Holliday Junction Recognition Sites
To test the proposed Hjc–Holliday junction interaction, three mutant Hjc proteins, ∆5, lacking 5 N-terminal amino acids, K30A/K31A, and K51A/K52A were created by site-directed mutagenesis (Figures 1d and 7a). In the latter two mutants, two consecutive lysine residues on the loop regions between β1 and β2 and between β3 and β4 were replaced by alanine. All of the mutant proteins showed purification behavior similar to that of the wild type, suggesting that they retain the original Hjc conformation (data not shown). These mutant proteins were used for the junction cleavage assay (Figure 7b). As compared to the wild type, the activity of K51A/K52A was decreased by 90%. The K30A/K31A and ∆5 mutants could not cleave the junction under the same conditions. K30A/K31A displayed a trace of activity only under extreme conditions such as incubation at 60°C overnight in the presence of an excess amount of the mutant protein (data not shown). The ∆5 mutant could not cleave the junction even under these extreme conditions (data not shown).

Next, the mixtures of these mutant proteins with a synthetic Holliday junction were analyzed by a gel retardation assay to observe the Hjc–Holliday junction complex (Figure 7c). The K30A/K31A and K51A/K52A mutants exhibited half of the full binding activity. The ∆5 mutant almost completely lost the binding activity, while the same mixture treated with glutaraldehyde exhibited similar band shift to that observed in the wild type protein–junction DNA complex (data not shown). Taken together, the results indicate that the N-terminal moiety of the α1 helix is crucial for the Hjc–Holliday junction complex formation and the loop region between β1 and β2 subsidiarily contributes to DNA binding, which may be important to correctly position DNA to the active site. Interestingly, the sulfate ion, bound to the N terminus of the α1 helix in our crystal structure (Figure 8), could be replaced by a phosphate ion (data not shown), and thus the position of this sulfate ion may mark one of the DNA binding sites in Hjc. Actually, the sulfate ion lies very close to a phosphate backbone in our model. A previous mutational analysis demonstrated that Phe72 is important not only for dimerization but also for Holliday junction–specific cleavage [15], suggesting a strong coupling between the correct dimeric structure and junction DNA recognition. The Hjc structure indeed suggests that mutations at Phe72 in the dimer interface change the spacing of the termini of the two α1 helices compared to the wild type, the activity of K51A/K52A was decreased by 90%. The K30A/K31A and ∆5 mutants could not cleave the junction under the same conditions. K30A/K31A displayed a trace of activity only under extreme conditions such as incubation at 60°C overnight in the presence of an excess amount of the mutant protein (data not shown). The ∆5 mutant could not cleave the junction even under these extreme conditions (data not shown).

Link Between Holliday Junction Resolvase and Restriction Endonuclease
The restriction endonuclease–like fold has been found in a number of proteins such as λ-exonuclease [25], archaeal tRNA splicing endonuclease [26], Ysr mismatch endonuclease [20], MutH mismatch endonuclease [27], and TnsA transposase [28]. Our Hjc structure demonstrates that this fold is expanded to a Holliday junction resolvase that is presumably involved in archaeal homologous recombination. On the other hand, RuvC resolvase was found to adopt the integrase-like fold, while T4 endoVII folds into a unique architecture that is different from both endonuclease- and integrase-like folds. It is thus implied that these three types of enzymes may have all evolved from different origins. Intriguingly, the Tn7 transposition enzymes form a heterodimer of TnsA

Figure 8. Refined Model of the Sulfate Binding Site in Hjc and the Corresponding Electron Density from a Simulated Annealing Omit Map
Two sulfate ions and five residues at the N terminus of the sulfate binding site could be replaced by a phosphate ion (data not shown), and thus the position of this sulfate ion may mark one of the DNA binding sites in Hjc. Actually, the sulfate ion lies very close to a phosphate backbone in our model. A previous mutational analysis demonstrated that Phe72 is important not only for dimerization but also for Holliday junction–specific cleavage [15], suggesting a strong coupling between the correct dimeric structure and junction DNA recognition. The Hjc structure indeed suggests that mutations at Phe72 in the dimer interface change the spacing of the termini of the two α1 helices (Figures 2 and 6).
endonucleases, including the active site residues that
were calculated using a randomly selected 5% of the data set that revealed that the fold is similar to that of type II restriction endonuclease I from T7 bacteriophage (T7 free structure provides important insights into the classification of the Holliday junction resolvase in addition to the overall recognition scheme with the junction DNA).

Table 2. Refinement Statistics

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<tr>
<td>SPRing8</td>
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<tr>
<td>BL24XU</td>
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<td>BL40B2</td>
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| Wavelength (Å) | 0.8340 | 1.0332 | 0.9800 | 0.9793 |
| Resolution (Å) | 2.0 | 2.7 | 3.0 | 3.0 |
| Completeness (%) | 81.2 (76.4) | 97.5 (91.2) | 98.7 (98.6) | 98.5 (98.2) |
| \( R_{merge} \) | 0.034 (0.149) | 0.037 (0.151) | 0.051 (0.146) | 0.055 (0.155) |
| \( \chi^2 \) | 1.15/1.35 | 1.15/1.35 | 1.15/1.35 | 1.15/1.35 |
| Unique Reflections | 29,280 | 14,961 | 10,866 | 10,842 |
| Phasing power (centric/acentric) | 1.9/2.6 | 2.2/2.4 | 0.56/0.57 | 0.66/0.65 |
| Phasing power (centric/acentric) | 1.9/2.6 | 2.2/2.4 | 0.56/0.57 | 0.66/0.65 |
| FOM* (centric/acentric) | 0.37/0.40 |

* Values in parentheses refer to statistics in the highest resolution shell (2.07–2.00 Å for native, 2.80–2.70 Å for L1 and 3.11–3.00 Å for L2 and L3).

and TnsB, whose folds are similar to those of restriction endonucleases and integrases, respectively [28, 29]. These individual proteins can cleave DNA without the formation of the fully functional heterodimer. Furthermore, although endonuclease-like TnsA hardly possesses sequence preference, integrase-like TnsB displays strong sequence specificity. Consistent with this, RuvC and Cce1 resolvases, which both belong to the integrase family, are also known to possess considerable sequence preference [30, 31]. Conversely, Hjc, with its endonuclease-like fold, appears to recognize Holliday junction structure and does so without sequence preference, as found in TnsA ([14]; K. K. et al., unpublished data).

During the preparation of this manuscript, Hadden et al. had reported the crystal structure of Holliday junction resolvase endonuclease I from T7 bacteriophage (T7 endoI) [32]. The dimer of T7 endoI forms a dumbbell-like architecture, where each domain is comprised of elements from both subunits. The single domain folds into architecture similar to that of the restriction endonuclease, and the active site residues, which are conserved by two subunits, assemble to form the same canonical catalytic motif. The architectural resemblance between T7 endoI and Hjc is obvious from visual inspection. T7 endoI is also known to cleave Holliday junction in a structure-specific manner [33]. These findings strongly suggest that both enzymes use the same catalytic mechanism.

The Holliday junction is known to adopt various conformations, which depend upon divalent cations and binding proteins [34]. A crystallographic study revealed that the Holliday junction alone adopts the stacked-X form in the presence of divalent cations [23]. On the other hand, the junction DNA was found to adopt the extended-X form upon binding to RuvA and Cre recombinase [24, 35, 36, 37]. Although more precise descriptions about the mechanisms of junction DNA recognition and cleavage by Hjc would require an atomic resolution structure of the Hjc-DNA complex, the present DNA-free structure provides important insights into the classification of the Holliday junction resolvase in addition to the overall recognition scheme with the junction DNA.

### Biological Implications

Holliday junction is a universal DNA intermediate formed during homologous recombination. The 3-D structural information of Holliday junction recognition with various proteins is crucial for full understanding of the recombination process. Holliday junction resolvases show no amino acid sequence similarity to each other, although they possess similar biochemical properties such as dimerization and requirement of divalent metal ions for the activity. Some of the resolvase are known to cleave DNA with sequence preferences, while others recognize particular conformation of junction DNA. Various crystal structures of resolvases show that each protein differs in its fold, suggesting that they evolved convergently to recognize the junction. Hjc, the archaeal Holliday junction resolvase, recognizes and cleaves the junction DNA through recognition with the particular 3-D structure but not the base sequence. The atomic structure of Hjc revealed that the fold is similar to that of type II restriction endonucleases, including the active site residues that...
form the canonical catalytic motif. This fold is utilized by many nucleases other than the restriction endonuclease itself. It seems that each protein evolved from a common origin and suited itself for specific targets. This structural study will contribute to the further understanding of the recognition mechanism of Holliday junction resolution.

Experimental Procedures

Preparation of Hjc Proteins

The Hjc protein was purified as described previously [10]. Selenomethionine-containing Hjc was prepared using the methionine auxotrophic strain B834(DE3). Seleno- DL-methionine was added to the media, and protein expression was induced. The selenomethionine-containing Hjc was purified using the same protocol as for the native Hjc protein. The purified Hjc protein was stored in buffer containing 20 mM Tris-HCl (pH 7.5), 1 mM EDTA, and 10% glycerol. The protein was concentrated by Centricon-10 ultrafiltration (Millipore) to 20 mg ml⁻¹. The incorporation of selenomethionine was confirmed by MALDI-TOF mass spectroscopy (Perceptive) (data not shown).

Crystalization of Hjc

Hjc was crystalized at 20°C by the microbatch method with a sili-
cone oil overlay. Good quality crystals were grown when equal amounts of the protein solution and a solution containing 100 mM Tris-HCl (pH 7.0), 1 mM EDTA, 27.5% (w/v) PEG4000, 50 mM (NH₃)₂SO₄, and 10% glycerol were mixed. Monoclinic crystals appeared overnight and were grown for an additional 2 days. The crystal belongs to the space group P2₁, with unit cell dimensions a = 37.1 Å, b = 119.3 Å, c = 63.8 Å, and β = 91.5°, and contains four molecules of Hjc protein in asymmetric unit.

Data Collection and Phasing

Crystals were harvested in harvesting buffer containing 100 mM Tris-HCl (pH 7.0), 30% PEG4000, 50 mM (NH₃)₂SO₄, 50 mM NaCl, and 10% glycerol. X-ray diffraction data were collected at 1.0332 Å using the microbatch method with a sili-
cone oil overlay. Good quality crystals were grown when equal amounts of the protein solution and a solution containing 100 mM Tris-HCl (pH 7.0), 1 mM EDTA, 27.5% (w/v) PEG4000, 50 mM (NH₃)₂SO₄, and 10% glycerol were mixed. Monoclinic crystals appeared overnight and were grown for an additional 2 days. The crystal belongs to the space group P2₁, with unit cell dimensions a = 37.1 Å, b = 119.3 Å, c = 63.8 Å, and β = 91.5°, and contains four molecules of Hjc protein in asymmetric unit.

The initial phases were estimated by MAD data using the seleno-
methionine data. Seven of the eight selenium sites were found using the direct method program SnB v2.0 [39]. The heavy atom para-
meters were refined by SHARP [40], and the one remaining site was determined from a difference Fourier map. The initial model was built using the program QUANTA version 1998 (MSI). The orientations of the four ncs symmetry molecules were first calculated from the selenium positions and later by the protein models. All data between 20 and 3.0 Å resolution were included in simulated annealing refine-
ment with bulk solvent correction using the CNS program [41]. Five percent of the reflections were kept separate to monitor Rfree, and were not used in the refinement. A strict ncs restraint (300 kcal mol⁻¹Å⁻²) and overall B factor refinement were applied in the initial stage of the refinement. When the R factor reached 0.25 (Rfree = 0.30), the protein model was used to refine the native data. After several rounds of manual rebuilding and refinement, the individual B factors were refined along with the addition of sulfate ions and solvent molecules (Figure 8). The final refinement statistics are shown in Table 2. The current model contains regions of residues 1–116 for two molecules in the asymmetric unit and 1–114 and 1–120 for the other two molecules, respectively. No electron density for two loop regions (83–86 and 96–98) were defined in one molecule among four molecules in an asymmetric unit. In some molecules, several side chains (Lys4, Lys30, Lys59, Lys60, Lys95, and Phe99) were disordered and eliminated from the refinement.

Site-Specific Mutagenesis

A PCR-mediated mutagenesis was performed using the Quick Change site-directed mutagenesis kit (Stratagene). The plasmid pFUHJ2, for the expression of the hjc gene in E. coli [10], was directly mutagenized according to the manufacturer’s instructions, with some modifications. The primers used for the K30A/K31A mutation were 5'-TCGCCAGGAGGCCCAGTACGTTAGT-3' and its complement sequence and 5'-GTCAGGTTAACGCGCAGAT CATTGTAC-3' and its complement sequence for the K57A/K58A mutation. The resultant plasmids were designated as pHUJ301 and pHUJ512, respectively. The truncated hjc gene (5, five amino acid deletion of the N terminus of Hjc) was amplified by PCR directly from the genomic DNA of P. furiosus under the same conditions as described previously [10]. The primers were 5'-CGTGATATGCA GGCAGAGAAGATGATTAT-3' and 5'-GCACGAGGAATCTCTTAT CATGATTCCCTCCTCAAC-3' for forward and reverse, respectively. The PCR product was digested with Ndel/EcoRV and was inserted into the Ndel/blunt-ended pET21a. The resultant plasmid was designated as pDELS. The nucleotide sequences of the hjc mutant genes were confirmed by DNA sequencing (ABI Prism 310 Genetic ana-
lyzer, PE Applied Biosystems).

Purification of Mutant Hjc Proteins

E. coli JM109 (DE3) carrying pHUJ301, pHUJ512, or pDELS was grown at 37°C with shaking in 200 ml L broth containing 20 mg ampicillin. The induction of the hjc gene expression and the purification of the mutant proteins were performed under the same condi-
tions as described previously [10], except that the Mono Q and heparin chromatography steps were omitted from the purification procedure.

Binding and Cleavage Assays

The gel-retardation assay and the Holliday junction cleavage assay using the synthetic Holliday junction, 4Jh and 4Jb, were performed as described earlier [12].

Accession Numbers

The atomic coordinates and the structure factors of Hjc have been deposited in the Protein Data Bank (entry code 1GEF).

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