Aromatic Residues Located Close to the Active Center Are Essential for the Catalytic Reaction of Flap Endonuclease-1 from Hyperthermophilic Archaeon Pyrococcus horikoshii

Eriko Matsui, Junko Abe, Hideshi Yokoyama, and Ikuo Matsui†

From the Biological Information Research Center, National Institute of Advanced Industrial Science and Technology, Higashi 1-1-1, Tsukuba, Ibaraki 305-8566, Japan

Received for publication, December 15, 2003, and in revised form, January 23, 2004
Published, JBC Papers in Press, January 23, 2004, DOI 10.1074/jbc.M313695200

Flap endonuclease-1 (FEN-1) has important roles in DNA replication and repair. In this study, the kinetic parameters of mutants at highly conserved aromatic residues, Tyr33, Phe79, and Phe278-Phe279, were examined. The substitution of these aromatic residues with alanine led to a large reduction in $k_{cat}$ values, although the mutants retained $K_m$ values similar to that of the wild-type enzyme. Notably, the $k_{cat}$ of Y33A and F79A decreased 333-fold and 71-fold, respectively, compared with that of the wild-type enzyme. The aromatic residues Tyr33 and Phe79 might play important roles in fixing the template strand and the downstream strand, respectively, in close proximity to the active center to achieve the productive transient state leading to the hydrolysis.

Flap endonuclease-1 (FEN-1)$^\dagger$ has important roles in DNA replication, repair, and recombination. It belongs to a family of structure-specific nucleases, which are conserved from archaea to eukaryotes and share homology with the 5′-nuclease domain associated with DNA polymerase I in prokaryotes and 5′→3′ exonuclease of bacteriophages (1–5).

In DNA replication, FEN-1 removes the RNA primers during the maturation of the Okazaki fragment (6), in conjunction with Dna2 having an endonuclease activity and a helicase activity (7). For DNA repair, FEN-1 removes damaged nucleotides in long patch base excision repair (8) and is required for non-homologous end joining of double-stranded DNA breaks (9).

FEN-1 possesses 5′-flap endonuclease and 5′→3′ exonuclease activities (10, 11). The flap endonuclease activity has been shown to require the upstream primer, which fills up the junction portion, and expanding the 3′ of the upstream primer known as a double flap structure elevates the activity (12–14). The 5′→3′ exonuclease activity digested the double-stranded DNA containing the nick, gap, and 5′-recess-end, and the activity was elevated by expanding the 3′-end of the upstream primer in the nick portion (1, 14).

Three crystal structures of FEN-1 have been reported exclusively in thermophilic archaea (15–17). The molecular structure of the members of the FEN-1 family, T5 exonuclease, T4 RNase H, and the exonuclease domain of Taq polymerase were also reported (18–20). All these structures have a conserved helical arch located above the globular domain that contains the active site, which is thought to recognize the 5′-end of the flap strand, tracking the length of the tail and cleaving near the junction between double-stranded and single-stranded DNA (21).

Recently, DNA binding sites of FEN-1 in human, archaea, and T5 bacteriophage were identified using site-direct mutagenesis (12, 13, 17, and 22–27), and two DNA binding models were postulated based on the identified binding sites and the molecular structure (22, 25). Several residues identified as DNA binding sites were located on the large loop (12, 17, 25, and 26), one of them was in contact with the bottom of the flap strand at the junction between the single- and double-stranded DNA (25). Two binding models for Pyrococcus furiosus and T5 bacteriophage FEN-1 showed that the helix-hairpin-helix region interacted with the downstream duplex DNA, and the flap strand interacted with the helical arch of FEN-1. However, the DNA binding mechanism is still unclear, because the number of DNA binding sites identified was too small to understand the substrate recognition.

We previously investigated the substrate specificity of phFEN-1 in detail using 35 different substrates (1). The substrate specificity was similar to that of eukaryotic FEN-1, except that phFEN-1 could cleave a flap strand having a double strand, suggesting that phFEN-1 might recognize strongly the junction portion of the flap strand rather than the 5′-end. Furthermore, the crystal structure of phFEN-1 was solved, and the large loop and small loop 2 (Fig. 2) were suggested to be very flexible by superimposing three kinds of structures from archaean FEN-1s (17), indicating that the structures of two loops might change remarkably for binding to the substrate.
An Essential Role for Aromatic Residues of phFEN-1 in Catalysis

DNA. The four DNA binding sites on the large loop and small loops were identified by site-directed mutagenesis (17). These consist of basic residues presumably bound to phosphate moieties of the DNA strand. We successfully elucidated the functions of the four DNA binding sites that form productive ES complexes for exo- and endo-activities, probably by bending the substrates using site-directed mutagenesis and the PCR method. The wild type gradient of 1M NaCl in a 50 mM Tris-HCl (pH 8.0) buffer. The recombinant HiTrap SP column (Amersham Biosciences) and eluted with a linear.

The measurement of the recognition sites was performed using substrates substituted with 2′-O-methyl at the 2′ position in the deoxyribose. The oligonucleotides having deoxycytidine substituted with 2′-O-methyl at each position and labeled at the 5′ terminus with FAM and designed to have the same sequence as the products. The enzyme amounts of WT and alanine mutants were from 0.1 to 100 ng, and the substrate amounts were from 3 to 5 pmol. The reaction time was from 5 to 10 min. The other reaction conditions were the same as for the kinetics analysis.

The measurement of the recognition sites was performed using substrates substituted with 2′-O-methyl at the 2′ position in the deoxyribose. The oligonucleotides having deoxycytidine substituted with 2′-O-methyl at each position and labeled at the 5′ terminus with FAM and designed to have the same sequence as the products. The enzyme amounts of WT and alanine mutants were from 0.1 to 100 ng, and the substrate amounts were from 3 to 5 pmol. The reaction time was from 5 to 10 min. The other reaction conditions were the same as for the kinetics analysis.

RESULTS

Purification of Mutant Protein—The residues corresponding to Tyr, Ph33, Ph79, and Ph279 of phFEN-1 are conserved among FEN-1s from eukaryotes and archaea, although the residues corresponding to Ph279 are variable within hydrophobic residues as shown below in Fig. 2A. They were located close to the active center as shown in Fig. 2 (B and C). To analyze the function of these aromatic residues, they were replaced with alanine, a hydrophobic residue, and other aromatic residues. Twenty mutant genes were constructed by site-directed mutagenesis. The expression level of each mutant enzyme in E. coli cells was almost the same as that of the wild type. These mutants were purified using the same procedure as was used for the wild type. The electrophoretic mobility of each mutant protein on SDS-gel was the same as that of the wild type protein.

Ty33, Ph35, Ph79, and Ph279 Recognize Nick and 5′-Ress-end Substrates for Exo-activity—The nick and 5′-recess-end substrates were used for a kinetic analysis of exo-activity as shown below in Fig. 3. The highly conserved residue Ty33 is located in the N-terminal region of FEN-1s from eukaryotes and archaea as shown in Fig. 2A. The residue is present close to magnesium ion 1 bound to the catalytic residues (Asp77, Asp80, Glu152, and Glu154) and faces the active center as shown in Fig. 2 (B and C). For the exo-activity against the 5′-recess-end substrate, the kcat values of Y33A and Y33L decreased 333-fold and 1180-fold, respectively, compared with that of the wild type enzyme (WT), although the kcat values of Y33A and Y33L decreased 53-fold and 79-fold, respectively, compared with that of the wild type enzyme (WT). The kcat values of the mutant altered at 33rd position changed slightly compared with that of WT. For the exo-activity against the nick substrate, the kcat values of Y33A and Y33L decreased 53-fold and 134-fold, respectively, compared with that of WT, indicating that the kcat values of Y33A and Y33L for the nick substrate are 6 and 9 times higher than those for the 5′-recess-end substrate, respectively, although the only structural difference between the nick and 5′-recess-end substrates is the presence of a upstream strand as shown in Fig. 1. The kcat values of Y33F, Y33W, and Y33H for the nick substrate were recovered to 29–200% of WT. The Km values of the mutants altered at the 33rd position varied moderately, but not significantly, compared with that of WT. These results clearly indicated that
The residue Phe<sup>79</sup> is conserved among FEN-1s from eukaryotes and archaea and is located also to magnesium ion 1 like Tyr<sup>33</sup> as described above (Fig. 2). The <i>k</i><sub>c</sub> and <i>K</i><sub>m</sub> of F35A and F35L showed no significant decrease compared with those of WT, however, the <i>k</i><sub>c</sub> value of F35Y decreased about 17-fold and 24-fold with the nick and 5'-recess-end substrates, respectively. The <i>K</i><sub>m</sub> values of F35Y were about 4- and 3-fold higher than those of WT with the nick and 5'-recess-end substrates, respectively. These results suggested that the 35th position preferred hydrophobic residues to polar residues, because the hydroxyl group of tyrosine showed inhibitory effects on both binding and catalysis for these substrates.

The conserved Phe<sup>79</sup> residue is adjacent to a catalytic residue, Asp<sup>35</sup>, as shown in Fig. 2 (A and B). For the 5'-recess-end substrate, the <i>k</i><sub>c</sub> value of F79A decreased 71-fold compared with that of WT, and the <i>k</i><sub>c</sub> value of F79L was restored to 20% of the WT value as shown in Fig. 3. For the nick substrate, the <i>k</i><sub>c</sub> value of F79A decreased 25-fold compared with that of WT, and the <i>k</i><sub>c</sub> value of F79L was restored to 20% of that of WT. The <i>k</i><sub>c</sub> values of F79Y and F79W for both substrates were restored to almost the same level as those of WT. The <i>k</i><sub>c</sub> value of F79H for the 5'-recess-end substrate was decreased to ~50% of the WT value, and the <i>k</i><sub>c</sub> value of F79H for the nick substrate was seven times lower than that of WT. The <i>K</i><sub>m</sub> value of F79H for the 5'-recess-end substrate was 13-fold higher than that of WT, whereas the <i>K</i><sub>m</sub> value of F79H for the nick substrate was ~2 times higher than that of WT. The charged imidazole group of histidine inhibited the catalytic activity. These results suggested that the function of Phe<sup>79</sup> could be satisfied only by non-charged aromatic residues.

The aromatic cluster Phe<sup>278</sup>-Phe<sup>279</sup> is located in the vicinity of magnesium ion 2 according to the molecular structure of pHFEN-1 (Fig. 2C). Phe<sup>278</sup> is conserved among FEN-1s from eukaryotes and archaea, although Phe<sup>278</sup> is variable within hydrophobic residues as shown in Fig. 2A. The <i>k</i><sub>c</sub> value of F278Y/F279Y was restored to around 70% of that of WT with both substrates, whereas the <i>k</i><sub>c</sub> values of F278A/F279A, F278L/F279L, and F278W/F279W were decreased ~10-20% with both substrates as shown in Fig. 3. Notably, the <i>k</i><sub>c</sub> value of F278H/F279H decreased dramatically, 83-fold for the 5'-recess-end substrate and 150-fold for the nick substrate compared with those of WT. For both substrates, the <i>K</i><sub>m</sub> values of F278L/F279L, F278Y/F279Y, and F278W/F279W were lower than those of WT, although the <i>K</i><sub>m</sub> value of F278A/F279A was severalfold higher than that of WT. The <i>K</i><sub>m</sub> value of F278H/F279H for the 5'-recess-end substrate was elevated 5 times compared with that of WT, whereas for the nick substrate the <i>K</i><sub>m</sub> value of F278H/F279H was ~60% of that of WT. These results indicated that both Phe<sup>278</sup> and Phe<sup>279</sup> could be substituted by tyrosine but not by histidine.

**The Residues Tyr<sup>33</sup>, Phe<sup>35</sup>, and Phe<sup>79</sup> and the Aromatic Cluster Phe<sup>278</sup>-Phe<sup>279</sup> Make Minor Contributions to the Recognition of the Double-flap Substrate, but Major Contributions to That of the Flap and Pseudo-Y Substrate for Endo-activity**—The double flap substrate shown in Fig. 1 was used to determine the kinetic parameters for endo-activity. As summarized in Fig. 3, the <i>K</i><sub>m</sub> value of Y33L, F79A, and F278H/F279H increased 4-, 7-, and 4-fold, respectively, and their <i>k</i><sub>c</sub>/<i>K</i><sub>m</sub> values decreased 5-, 6-, and 14-fold, respectively, compared with that of WT. As mentioned above, in the hydrolysis of both the 5'-recess-end and nick substrates, Y33L, F79A, and F278H/F279H represent the mutants with the most severely affected <i>k</i><sub>c</sub>/<i>K</i><sub>m</sub> values, decreased 2535-, 58-, and 454-fold for the 5'-recess-end substrate, and 270-, 75-, and 80-fold for the nick substrate, respectively, compared with that of WT. Hence, the small reduction rates of the mutants, Y33L, F79A, and F278H/F279H for the <i>k</i><sub>c</sub>/<i>K</i><sub>m</sub> values of the double flap substrate indicated that the residues Tyr<sup>33</sup>, Phe<sup>35</sup>, and Phe<sup>79</sup> and the aromatic cluster Phe<sup>278</sup>-Phe<sup>279</sup> made minor contributions to recognizing the double-flap substrate.

Furthermore, to investigate the function of Tyr<sup>33</sup>, Phe<sup>79</sup>, and Phe<sup>278</sup>-Phe<sup>279</sup> in the endo-type hydrolysis, the kinetic parameters of mutants were determined using the single flap and pseudo-Y substrates without the 3' projection of the upstream strand (Fig. 1). The results are summarized in Fig. 4. The <i>k</i><sub>c</sub> value of Y33A and Y33L for the single flap substrate decreased 30- and 433-fold, respectively, and the <i>k</i><sub>c</sub> of Y33A and Y33L for the pseudo-Y substrate decreased 485- and 3233-fold, respectively, compared with that of WT, although the <i>K</i><sub>m</sub> values of Y33F for the single flap and pseudo-Y substrates were restored to 38 and 20% of the value of WT, respectively. However, the <i>K</i><sub>m</sub> values of Y33A, Y33L, and Y33F for both the single flap and pseudo-Y substrates were 18–200% of the WT value. The <i>K</i><sub>m</sub> of the mutants altered at the 33rd position varied moderately, but not significantly, compared with that of WT. These high reduction rates for the <i>k</i><sub>c</sub>/<i>K</i><sub>m</sub> values of endo-activity against both the single flap and pseudo-Y substrates were similar to those for the recess-end and nick substrates for exo-activity. These results clearly indicated that Tyr<sup>33</sup> could be substituted with an aromatic residue to maintain high <i>k</i><sub>c</sub> values in both exo- and endo-type reactions.

For the single flap and pseudo-Y substrates, the <i>k</i><sub>c</sub> of F79A decreased 31- and 37-fold, respectively, compared with that of WT, and the <i>k</i><sub>c</sub> of F79L decreased 17 and 7% as shown in Fig. 4. The <i>k</i><sub>c</sub> values of F79Y for both substrates were restored to almost the same level as those of WT. The <i>K</i><sub>m</sub> of F79L for both substrates was varied moderately, but not significantly, compared with that of WT, whereas the <i>K</i><sub>m</sub> value of F79A for the substrates was ~3-4 times higher than that of WT. These results suggested that the function of Phe<sup>79</sup> could be satisfied by an aromatic residue, with high <i>k</i><sub>c</sub> values retained for both exo- and endo-type reactions.

For both substrates, the <i>k</i><sub>c</sub>/<i>K</i><sub>m</sub> of F278A/F279A decreased ~20-fold, compared with that of WT, whereas the <i>k</i><sub>c</sub>/<i>K</i><sub>m</sub> of F278L/F279L recovered to around 15% of the value for WT. On the other hand, the <i>k</i><sub>c</sub>/<i>K</i><sub>m</sub> values of F278H/F279Y were similar to those of WT, suggesting that the cluster Phe<sup>278</sup>-Phe<sup>279</sup> could be replaced with tyrosine to maintain the high <i>k</i><sub>c</sub> values in both the exo- and endo-type reactions.

**Tyr<sup>33</sup>, Phe<sup>35</sup>, and Phe<sup>278</sup>-Phe<sup>279</sup> Contribute Greatly to Recognizing Substrates without the 3' Projection of Upstream Strand but Play Minor Roles in Recognizing Substrates with the 3' Projection of the Upstream Strand**—To determine the effects of an expanding upstream strand in the 3' direction, the nick substrate with the 3' protrusion of the upstream strand (Fig. 1) was used for a kinetic analysis of the exo-activity. The alanine mutants (Y33A, F79A, and F278A/F279A) were compared with
WT. Except for the $K_m$ value of F79A, which increased 4-fold compared with that of WT, the $K_m$, $k_{cat}$, and $k_{cat}/K_m$ of these alanine mutants did not show a significant difference from those of WT (data not shown). The kinetic profiles of the alanine mutants for the nick substrate with the 3$'/H11032$ protrusion of the upstream strand were similar to those for the double flap substrate with the 3$'/H11032$ protrusion of the upstream strand as shown in Fig. 3. Taking into account all of the kinetics parameters described above, it was concluded that Tyr33, Phe79, and Phe278-Phe279 play significant roles in the catalysis of the substrates without the 3$'/H11032$ projection of the upstream strand (the nick, 5$'/H11032$-recess-end, single flap, and pseudo-Y substrates), whereas they are minor determinants in the catalytic reaction of the double flap and nick substrates with the 3$'$ projection of the upstream strand.

**An Essential Role for Aromatic Residues of phFEN-1 in Catalysis**

**Fig. 2.** Sequence alignment and superimposition of molecular structures among the FEN-1 family, and mutation sites on the phFEN-1 molecule. A, sequence alignment among the FEN-1 family. The sequences are as follows: phFEN-1, P. horikoshii FEN-1 (1); mjFEN-1, Methanococcus jannaschii FEN-1 (1); spRAD2, Schizosaccharomyces pombe RAD2 (1); hFEN, human FEN-1 (1); T5 Exo., T5 exonuclease (20); T7 Exo., T7 exonuclease (20); E. coli Pol. 1, 5$'$-exonuclease domain of E. coli polymerase 1 (20); Taq. Pol. 1, 5$'$-exonuclease domain of Thermus aquaticus polymerase 1 (20). An open box denotes identical amino acids. Asp27 (in green) and Asp80 (in green) are active residues. The red letters indicate the positions of the site-directed mutagenesis and the conserved amino acids. B, the superimposition of the molecular structures for the FEN-1 family. The structures are shown in stereo view, phFEN-1, pink (PDB code, 1MC8); T5 Exo., light blue (1EXN); Taq. Pol. 1, blue (1TAQ); mjFEN-1, green (1A77). Magnesium ions 1 and 2 are colored in red and orange, respectively. The figure was created using MOLSCRIPT (40) and Raster3D (41). C, the location of mutations on the phFEN-1 structure. The numbers indicate the mutation sites. Magnesium ions 1 and 2 are colored in red and orange, respectively. Small loop 1 (amino acids 39–55), small loop 2 (amino acids 187–206), and the large loop (amino acids 80–128) are colored in yellow, green, and pink, respectively. The molecular structure of phFEN-1 was taken from PDB code, 1MC8. The figures were created using MOLSCRIPT (40) and Raster3D (41).
Comparison of the $K_m$, $k_{cat}$, and $k_{cat}/K_m$ values among WT and the mutants using the nick and 5’-recess-end substrates for exo-activity, and the double flap substrate for endo-activity. The assay conditions were described under “Experimental Procedures.” The protein amounts for WT and the mutants were 0.025–100 ng, and substrate concentrations were 2 nM to 2 M. The columns representing the values of WT and the mutants for the aromatic residues, Tyr$^{33}$, Phe$^{35}$, Phe$^{79}$, and Phe$^{278}$-Phe$^{279}$ are colored red, yellow, green, blue, and light brown, respectively.
were between 25 and 300% of those for WT as shown in Fig. 3. With these equations and the kinetic parameters shown in Fig. 3 and 4, $K_j$ (the binding constant) and $k_{cat}/K_m$ (identical to the $k_{cat}/K_m$ value) for all the substrates were evaluated, and compared among alanine mutant and WT enzymes as shown in Fig. 5. For the substrates without the 3'-projection of the upstream strand (the nick, 5'-recess-end, single flap, and pseudo-Y substrates), $K_p$ values were reduced remarkably by mutation at the three substrate binding sites (Tyr 33, Phe 79, and Phe 278-Phe 279), because the $K_j$ was postulated as invariant, whereas, for the double flap and nick substrates with the 3'-protrusion of the upstream strand, the $K_p$ values were not markedly changed by the mutations. Furthermore, the $K_j$ values for all the substrates were not significantly changed by the mutations. These results strongly suggest that, for the substrates without the 3'-projection of the upstream strand, the residues Tyr 33, Phe 79, and Phe 278-Phe 279 might contribute to transforming the ES complexes into the productive transient states rather than to forming merely the initial ES complexes, because the $K_p$ values for the substrates without the 3'-projection were drastically changed, whereas the $K_j$ values for the substrates were not significantly reduced by the mutations. On the other hand, the three sites probably play minor roles to form the productive intermediates for the double flap and nick substrates with the 3'-protrusion, because the $K_j$ values for the substrates with the 3'-projection were moderately maintained even with the mutations.

**phFEN-1 Recognizes Strongly Both Strands Covering a Few Nucleotides Adjacent to the Cleavage Positions of the Nick and 5'-Recess-end Substrates—**The cleavage positions on the 5'-end-labeled substrates with a fluorescent group (FAM) were investigated as shown in Fig. 6A. Oligonucleotide markers with the same base sequence as the products were used to negate the difference in electrophoretical mobility due to each specific sequence. For the nick substrate, a major 1-mer product (95%) and a minor 3-mer (5%) product were produced as shown in Fig. 6A, lane 1, indicating that the cleavage positions were 1 base and 3 bases inside from the 5'-end of the downstream strand. For the 5'-recess-end substrate, a major 3-mer product and a trace 1-mer product were detected as shown in Fig. 6A, lane 2, indicating that the cleavage position was 3 bases inside from the 5'-end of the downstream strand. Thus, cleavage positions differed between the nick and 5'-recess-end substrates. For the pseudo-Y substrate having a 4-mer projection of the flap strand, only a 7-mer product was detected as shown in Fig. 6A, lane 6, indicating that the pseudo-Y substrate was cleaved 3-mer inside of the duplex end, and that the cleavage position was the same as that of the 5'-recess-end substrate. For the single flap substrate having a 4-mer projection of the flap strand, 4-mer (66%), 5-mer (23%), and 7-mer (11%) products were detected as shown in Fig. 6A, lane 7, indicating that the cleavage positions were the duplex junction, and 1-mer and 3-mer inside of the duplex end. The single flap substrate having a 19-mer projection of the flap strand was cleaved at the duplex junction, and 1-mer and 3-mer inside of the duplex end in the same way as the single flap substrate having a 4-mer projection of the flap strand, indicating that the cleavage manner is not related to the length of the flap strand (data not shown). For the double flap substrate having a 19-mer protrusion of the flap...
An Essential Role for Aromatic Residues of phFEN-1 in Catalysis

 FIG. 5. Comparison of the $K_f$ and $K_m$ values among WT and alanine mutants using the substrates with and without the 3′ projection of the upstream strand for endo- and exo-activities. The $K_f$ and $K_m$ values were evaluated from the $K_m$ and $k_{cat}$ values according to the equations described under "Results."

Aromatic Residues Located Close to the Active Center Are Essential for the Catalytic Reaction of FEN-1 Probably through Stacking Interactions with the Substrates—The activities of phFEN-1s were measured using substrates with a highly fluorescent dye, fluorescein (FAM), at the 5′-end, because the catalytic activities of phFEN-1 against fluorescent substrates are the same as those against radiolabeled substrates as reported previously (17). In this report, we compared the catalytic activities of mutants altered at three sites (Tyr33, Phe79, and Phe278-Phe279) for both fluorescent and radiolabeled substrates; however, no detectable difference between the substrates was found for any mutant (data not shown). Furthermore, the cleavage positions on the nick, single flap, and pseudo-Y substrates were compared between the fluorescent and radiolabeled substrates. The results revealed that the digestion points on the fluorescent substrates were the same as those of the radiolabeled ones, although the digestion point on the radiolabeled recess-end substrate was shifted 1-mer toward the 5′-end, compared with that of the fluorescent one (data not shown). Thus, the fluorescent substrates were used to measure the kinetic parameters of mutant enzymes due to their ease of handling.

The molecular structure of FEN-1 from archaea has been reported (15–17), and recently several DNA binding sites were found in the molecules (12–13, 17, and 22–27). The aromatic amino acids were reported to form a stacking interaction with single-stranded DNA in a helicase and a DNA-binding protein, replication protein A (34–36). Interactions with bases of double-stranded DNA were found in the hydrolysis reaction of resolvases and MutS proteins belonging to nuclease (37–39). The residues Tyr33 and Phe79 in the vicinity of the active center of the phFEN-1 molecule could be substituted with aromatic amino acids to maintain high $k_{cat}$ values in both exo- and endo-type reactions as shown in Figs. 3 and 4. However, the replacement of aromatic residues with alanine (disappearance of aromatic groups) at the 33rd and 79th positions of phFEN-1 resulted in a decrease in the $k_{cat}$ value of 333- and 71-fold for the 5′-recess-end substrate, and 53- and 25-fold for the nick substrate, respectively, compared with WT, whereas the $K_m$ of the mutants varied moderately, not significantly, compared with that of WT. Furthermore, the change to an aliphatic hydrophobic residue, leucine, at the 33rd and 79th positions induced a remarkable decrease in $k_{cat}$ for the substrates without the 3′ protrusion of the upstream strand, compared with that of WT, whereas the $K_m$ values of the leucine mutants did not change significantly. The results indicated significant roles for the aromatic groups of Tyr33 and Phe79 probably through stacking interactions with the substrates in the catalytic reaction. The aromatic cluster Phe278-Phe279 showed a more limited specificity for the aromatic groups of Tyr33 and Phe79 probably through stacking interactions with the substrates in the catalytic reaction.

As shown in Fig. 5, for the substrates without the 3′ projection of the upstream strand (the nick, 5′-recess-end, single flap, double flap, and pseudo-Y substrates), a significant decrease was observed following substitutions at the 3rd and 4th positions of strand C and at the 26th and 27th positions of strand A as shown in Fig. 6B (part I). For the 5′-recess-end substrate, a significant decrease in $k_{cat}$ was observed following substitutions at the 3rd and 4th positions of strand C and at the 26th and 27th positions of strand A as shown in Fig. 6B (part II). Because phFEN-1 cleaved the nick substrate 1-mer and 3-mer inside of the 5′-end of strand C, and cleaved the 5′-recess-end substrate 3-mer inside of the 5′-end of strand C as mentioned in the former section, the results of the substitution experiments indicated that phFEN-1 recognized both strands covering a few nucleotides adjacent to the cleavage positions of the nick and 5′-recess-end substrates.

DISCUSSION

The results of the substitution experiments indicated that phFEN-1 recognized both strands covering a few nucleotides adjacent to the cleavage positions of the nick and 5′-recess-end substrates.
An Essential Role for Aromatic Residues of pHFEN-1 in Catalysis

Fig. 6. The cleavage positions and recognition sites of the substrates. A, measurement of the product size. The assay conditions were described under “Experimental Procedures” using the WT enzyme. The amount of WT was 1–10 ng, and the amount of substrates was 3–5 pmol.

The structure of each substrate is shown above the corresponding lanes. Lanes 1, 2, 6, and 7 correspond to the products from the nick substrate, the 5'-recess-end substrate, the pseudo-Y substrate with a 4-mer flap strand, and the single flap substrate with a 4-mer flap strand, respectively. Lane 12 corresponds to the products from the double flap substrate with a 1-mer 3'-end projection of the upstream strand and with a 19-mer-flap strand. Lane 14 corresponds to the products from the nick substrate having a 1-mer 3'-end projection of the upstream strand. Lanes 3–5, 8–11, 13, and 15–17 contain 1-mer, 2-mer, 3-mer, 4-mer, 5-mer, 6-mer, 7-mer, 20-mer, 1-mer, 2-mer, and 3-mer oligonucleotide markers labeled with FAM, respectively. These oligonucleotides had the same sequence as the cleaved products. The positions of FAM are shown by an asterisk. The recognition sites in the nick and 5'-recess-end substrates for the pHFEN-1 molecule. The assay conditions were described under “Experimental Procedures.” In B: part I, nick substrates with FAM labeling at the 5'-end of strand C and with the 2'-O-methyl substitution of the deoxyribose moiety were used. The abbreviations, C2–C5, correspond to the substituted deoxyriboses located at the 2nd through 5th positions from the 5'-end of strand C. The abbreviations, A24–A28, correspond to the substituted deoxyriboses located at the 24th through 28th positions from the 5'-end of strand A. The relative activities toward the substituted substrates were calculated with the activity for the substrate without the 2'-O-methyl substitution as 100%. The red lines on strand C show the cleavage positions evaluated with the result in panel A, lane 1. Part II, the 5'-recess-end substrates were used. C2–C5 and A24–A25 correspond to the same substitutions in the nick substrate of B (part I). The red line on strand C shows the cleavage position evaluated with the result in panel A, lane 2.

and pseudo-Y substrates), the $K_p$ values were reduced remarkably by mutation at the three substrate binding sites (Tyr23, Phe79, and Phe278–Phe279), because the $K_{out}$ was postulated as invariant, whereas, for the double flap and nick substrates with the 3' protrusion of the upstream strand, the $K_p$ values were not drastically changed by the mutations. Furthermore, the $K_p$ values for all substrates were not significantly changed by the mutations. These results suggest that Tyr23, Phe79, and Phe278–Phe279 might play essential roles in forming the transient productive ES complex via multiple stacking interactions with the substrates without the 3' projection of the upstream strand using these central aromatic residues located close to the active site, because the $K_p$ values for the substrates without the 3' projection were drastically changed, whereas the $K_p$ values for the substrates were not significantly reduced by the mutations. On the other hand, the three sites probably play minor roles in forming the productive intermediates for the double flap and nick substrates with the 3' protrusion, because the $K_p$ values for the substrates with the 3' projection were moderately maintained even with the mutations as shown in Fig. 5.

As shown in Fig. 2A, the aromatic groups at the 33rd and 79th positions of pHFEN-1 are conserved completely not only among FEN-1s but also among the family enzymes containing T5 and T7 exonuclease, and the exonuclease domains of E. coli polymerase 1 and Taq polymerase 1. In the superimposition of the molecular structures shown in Fig. 2B, these two aromatic residues are kept at the same positions and in a similar direction. Interestingly, as shown in Fig. 2B, the aromatic ring of the Phe112 residue for the T5 exonuclease occupies the same position as that of the Phe79 residue of pHFEN-1, although the two residues Phe112 and Phe79 are derived from distinct portions of the superimposed structures. These results suggested that the cooperative stacking interactions with the substrates with the conserved aromatic residues close to the active center might work commonly as a key determinant in the hydrolysis reactions of the family enzymes.

**The Stacking Interactions of the Residues Tyr23 and Phe79 Might Play Important Roles in Fixing the Template Strand and the Downstream Strand, Respectively**—According to the molecular structure (17), the kinetic parameters and the substrate digestion patterns as summarized in Figs. 3–6, a possible model of the binding of pHFEN-1 with the nick substrate is proposed in Fig. 7. The large loop (81–128) colored in pink (Fig. 2C) was removed from the structure to reduce steric hindrance between the loop and substrate, and to keep the active center accessible to the substrate, because the large loop was demonstrated to be very flexible and to hide the active center (17). For the orientation of the nick substrate to the active cleft, the reported DNA binding models of FEN-1s was referred to (22, 25). The downstream duplex DNA was arranged to bind to the HhH motif (25), and the upstream duplex was designed to bind
An Essential Role for Aromatic Residues of phFEN-1 in Catalysis

...and is able to undergo the stacking interaction with the base flipping out. In the modeled structure (Fig. 7), the second phosphate moiety from the 5'-end of the downstream strand is suitable for forming a hydrogen bond with the active magnesium ion 1, suggesting that the phosphoester linkage should be cleaved. The manner in which the substrate was recognized in the modeled structure might be elucidated well by the following experimental results: 1) the nick substrate was cleaved at the 1st nucleotide from the 5'-end of the downstream strand (Fig. 6A); 2) the 3rd and 4th nucleotides from the 5'-end of the downstream strand were specifically recognized by the phFEN-1 molecule (Fig. 6B (part I)); 3) the 26th, 27th, and 28th bases from the 5'-end of the template strand were strongly recognized by the phFEN-1 molecule (Fig. 6B (part I)).

For the other substrates (the 5'-recess-end, pseudo-Y, and single flap substrates), the same mechanism of binding as that of the nick substrate might occur, although the cleavage positions on the substrates might be adjusted depending on the specific binding of each strand to these central aromatic residues. For the pseudo-Y and single flap substrates, the flap strands might pass through the large loop via interaction with the Phe79 residue.

In the preceding report (17) we identified four DNA binding sites (Arg40-Arg62, Arg84, Arg118-Lys119 and Lys193-Arg194, Lys195) located on a flexible loop at the surface of the molecular structure. Using these DNA binding sites far apart from the active center, phFEN-1 might bend the downstream strands to the active center located interior of the molecule. Simultaneously, the stacking interactions of Tyr33 and Phe79 adjacent to the active center might play important roles in fixing the hinge portion of the template strand and the 5'-end of the downstream strand as shown in the modeled complex. The stacking interactions might cause the partial unwinding of the 5'-end of the downstream strand from the template strand to form the productive transient state leading to the hydrolysis.

Acknowledgments—We acknowledge the assistance of Emiko Yamamoto and Miho Nishio. We also thank Yulong Shen, Yuji Urushibata, and Xiao-Feng Tang, for helpful discussion.
REFERENCES
1. Matsui, E., Kawasaki, S., Ishida, H., Ishikawa, K., Kosugi, Y., Kikuchi, H., Kawarabayashi, Y., and Matsui, I. (1999) J. Biol. Chem. 274, 18297–18309
2. Kaiser, M. W., Lyaminicheva, N., Ma, W., Miller, C., Neri, B., Forl, L., and Lyaminichev, V. I. (1999) J. Biol. Chem. 274, 21387–21394
3. Henfield, D. J., Frank, G., Weng, Y., Tainer, J. A., and Shen, B. (1998) J. Biol. Chem. 273, 27154–27161
4. Lieber, M. R. (1997) BioEssays 19, 233–240
5. Shen, B., Qiu, J., Henfield, D., and Tainer, J. A. (1998) Trends Biochem. Sci. 23, 171–173
6. Bambara, R. A., Murante, R. S., and Henrickson, L. A. (1997) J. Biol. Chem. 272, 4647–4650
7. Bae, S. H., Bae, K. H., Kim, J. A., and Seo, Y. S. (2001) Nature 412, 456–461
8. Klugland, A., and Lindahl, T. (1997) EMBO J. 16, 3341–3348
9. Wu, X., Wilson, T. E., and Lieber, M. R. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 1353–1358
10. Harrington, J. J., and Lieber, M. R. (1994) EMBO J. 13, 1235–1246
11. Harrington, J. J., and Lieber, M. R. (1994) Genes Dev. 8, 1344–1355
12. Storici, F., Neenge, G., Ferrari, E., Gardemin, A. D., Hubscher, U., and Rossen, M. A. (2002) EMBO J. 21, 5930–5942
13. Qiu, J., Bimston, D. N., Partikian, A., and Shen, B. (2002) J. Biol. Chem. 277, 24659–24666
14. Kao, H. I., Henrickson, L. A., Liu, Y., and Bambara, R. A. (2002) J. Biol. Chem. 277, 14379–14389
15. Henfield, D. J., Mol, C. D., Shen, B. H., and Tainer, J. A. (1998) Cell 95, 135–146
16. Hwang, K. Y., Baeck, K., Kim, H. Y., and Cho, Y. (1998) Nat. Struct. Biol. 5, 707–713
17. Matsui, E., Musti, K. V., Abe, J., Yamashita, K., Matsui, I., and Harakata, K. (2002) J. Biol. Chem. 277, 37840–37847
18. Ceska, T. A., Sayers, J. R., Stier, G., and Suck, D. (1996) Nature 382, 90–93
19. Kim, Y., Eom, S. H., Wang, J., Lee, D. S., Sub, S. W., and Steitz, T. A. (1995) Nature 376, 612–616
20. Mueser, T. C., Nossal, N. G., and Hyde, C. C. (1996) Cell 85, 1101–1112
21. Murante, R. S., Rust, L., and Bambara, R. A. (1995) J. Biol. Chem. 270, 30377–30383
22. Dervan, P., Feng, M., Patel, D., Graubay, J. A., Artymiuk, P. J., Ceska, T. A., and Sayers, J. B. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 8542–8547
23. Stucki, M., Jonsson, Z. O., and Hubscher, U. (2001) J. Biol. Chem. 276, 7843–7849
24. Shen, B., Nolan, J. P., Sklar, L. A., and Park, M. S. (1996) J. Biol. Chem. 271, 9173–9176
25. Allawi, H. T., Kaiser, M. W., Oufridev, A. V., Ma, W. P., Broggaard, A. E., Case, D. A., Neri, B. P., and Lyaminichev, V. I. (2003) J. Mol. Biol. 328, 537–554
26. Frank, G., Qiu, J., Somsouk, M., Weng, Y., Somsouk, L., Nolan, J. P., and Shen, B. (1998) J. Biol. Chem. 273, 33064–33072
27. Garforth, S. J., Ceska, T. A., Suck, D., and Sayers, J. R. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 38–43
28. Sakoda, M., and Hiromi, K. (1976) J. Biochem. 80, 547–555
29. Hiromi, K. (1970) Biochem. Biophys. Res. Commun. 40, 1–6
30. Thom, J., Mather, C., and Spradlin, J. (1970) Biochemistry 9, 1768–1775
31. Matsui, I., Ishikawa, K., Miyairi, S., Fukui, S., and Honda, K. (1992) Biochemistry 31, 5232–5236
32. Matsui, I., Ishikawa, K., Matsui, E., Miyairi, S., Fukui, S., and Honda, K. (1991) J. Biochem. (Tokyo) 109, 566–569
33. Matsui, I., and Svensson, B. (1997) J. Biol. Chem. 272, 22456–22463
34. Raghunathan, S., Kozlov, A. G., Lomah, T. M., and Waksman, G. (2000) Nature Struct. Biol. 7, 648–652
35. Bochkarev, A., Pfeuzte, P., Edwards, A. M., and Frappier, L. (1997) Nature 385, 176–181
36. Dillingham, M. S., Sultanas, P., Wiley, P., Webb, M. R., and Wigray, D. B. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 8381–8387
37. Lamers, L. H., Perrakis, A., Enzlin, J. H., Winterwerp, H. H. K, Wind, N., and Sixma, T. K. (2000) Nature 407, 711–717
38. Moldov, G., C, B, Hase, P, and Yang, W. (2000) Nature 407, 703–710
39. Yoshikawa, M., Iwasaki, H., and Shinagawa, H. (2001) J. Biol. Chem. 276, 10432–10436
40. Krasil, P. J. (1991) J. Appl. Crystallogr. 24, 945–949
41. Merritt, E. A., and Murphy, M. E. P. (1994) Acta Crystallogr. Sect. D 50, 869–873
42. Sanyo, R. M., Prasad, R., Wilson, H. S., Knaus, J., and Pelletier, H. (1997) Biochemistry 36, 11205–11215
Aromatic Residues Located Close to the Active Center Are Essential for the Catalytic Reaction of Flap Endonuclease-1 from Hyperthermophilic Archaeon *Pyrococcus horikoshii*

Eriko Matsui, Junko Abe, Hideshi Yokoyama and Ikuo Matsui

*J. Biol. Chem.* 2004, 279:16687-16696.
doi: 10.1074/jbc.M313695200 originally published online January 23, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M313695200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 42 references, 19 of which can be accessed free at http://www.jbc.org/content/279/16/16687.full.html#ref-list-1