Site-directed Mutagenesis of the Yeast Resolving Enzyme Cce1 Reveals Catalytic Residues and Relationship with the Intron-splicing Factor Mrs1*

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The Holliday junction-resolving enzyme Cce1 is a magnesium-dependent endonuclease, responsible for the resolution of recombining mitochondrial DNA molecules in Saccharomyces cerevisiae. We have identified a homologue of Cce1 from Candida albicans and used a multiple sequence alignment to predict residues important for junction binding and catalysis. Twelve site-directed mutants have been constructed, expressed, purified, and characterized. Using this approach, we have identified basic residues with putative roles in both DNA recognition and catalysis and acidic residues that have a purely catalytic role. We have shown directly by isothermal titration calorimetry that a group of acidic residues vital for catalytic activity in Cce1 act as ligands for the catalytic magnesium ions. Sequence similarities between the Cce1 proteins and the group I intron splicing factor Mrs1 suggest the latter may also possess a binding site for magnesium, with a putative role in stabilization of RNA tertiary structure or catalysis of the splicing reaction.

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EXPERIMENTAL PROCEDURES

Identification of the Cce1 Homologue of C. albicans—Sequence data for C. albicans was obtained from the Stanford DNA Sequencing and Technology Center site on the World Wide Web. Sequencing of C. albicans was accomplished with the support of the NIDR and the Burroughs Wellcome Fund.

Oligonucleotides—Junction 1 is a fixed four-way junction with 20-base pair arms, described previously (5). Junction Z1 is a fixed four-way junction with 15-base pair arms, the h strand containing a consensus site for cleavage by Cce1, described previously (8).

Oligonucleotide Synthesis and Assembly of DNA Junctions—Oligonucleotides were synthesized and DNA junctions were assembled as described previously (5).

Protein Purification—Native recombinant Cce1 was expressed and purified to near homogeneity as described previously (6).

Single Turnover Kinetic Analysis—Determination of the first order rate constants of junction cleavage were carried out by first incubating 8 \times 10^{-4} M 5'-32P-labeled junction J1 (or junction Z1 for some mutants) with 8 \times 10^{-7} M Cce1 dimer in binding buffer (20 mM Tris, pH 8.0, 200 mM NaCl, 0.2 mM dithiothreitol, 1 mM EDTA, 0.1 mg/ml bovine serum albumin). Samples were pre-equilibrated at 37 °C, and reactions were initiated by the addition of MgCl2 to a final concentration of 15 mM. At set time points, an aliquot of the reaction mix was removed, and the reaction was stopped by the addition of an equal volume of formamide loading buffer (95% /3H formamide, 50 mM EDTA, pH 8.0, 0.1% bromophenol blue, 0.1% xylene cyanol FF) and heating at 80 °C for 2 min, followed by storage on ice. Reaction products were analyzed by denaturing gel electrophoresis and phosphor imaging as described previously (8). All experiments were carried out in triplicate, and, where not specifically stated, S.E. values were typically less than 5%. For mutants E145Q, D293N, and D294N, activity was also assayed using the four-way junction Z1, which is cleaved approximately 20-fold more quickly than J1 by wild-type Cce1. Use of junction Z1 provided a more sensitive assay for residual enzyme activity, allowing detection of up to a 60,000-fold reduction in activity compared with the wild-type enzyme.

Measurement of Equilibrium Dissociation Constants—Equilibrium
dissociation constants were determined by gel electrophoretic retardation analysis and quantified by phosphor imaging, and the data were fitted to an equation for the binding model, as described in Ref. 8.

Site-directed Mutagenesis—Site-directed mutagenesis of CCE1 was carried out in plasmid pUC119-CCE1 (5) using the QuikChange protocol (Stratagene). After mutagenesis, DNA sequencing was used to confirm that no spurious mutations had been introduced. The CCE1 mutants were subcloned into pET19b, expressed, and purified as for wild-type Cce1.

Isothermal Titration Calorimetry—ITC experiments were carried out at 25 °C using VP-ITC titration calorimeter (MicroCal, Northampton, MA). All solutions were degassed before the titrations. Cce1 samples were extensively dialyzed against 20 mM Tris-HCl buffer, pH 8.0, containing 200 mM NaCl, and MgCl₂ solutions were prepared in the same buffer. Titration was carried out using a 370-μl syringe with stirring at 400 rpm. Each titration consisted of a preliminary 1-μl injection followed by 20–30 subsequent 10-μl injections into a cell containing approximately 1.4-ml enzyme solutions. Calorimetric data were analyzed using MicroCal ORIGIN software. All measurements of binding parameters presented are the means of duplicate experiments.

RESULTS

Identification of a Third Sequence for Cce1 from C. albicans—Searches of the unfinished C. albicans genome sequence data (on the World Wide Web) using Saccharomyces cerevisiae Cce1 as a probe revealed a significant match to a predicted open reading frame in contig¹ 4-2987. The Cce1 sequences from S. cerevisiae, S. pombe, and C. albicans share approximately 33% sequence identity. Multiple sequence alignment of the three Cce1 sequences, together with that of Mrs1 from S. cerevisiae, was carried out using the program ClustalX, followed by manual adjustment (Fig. 1). A limited number of residues are conserved in all the Cce1 sequences, and a subset of those are also conserved in Mrs1. These amino acids are likely to play important roles in the structure and function of the proteins.

Site-directed Mutagenesis of S. cerevisiae Cce1—Ten residues in conserved regions of Cce1 were selected for site-directed mutagenesis in order to investigate their importance in four-way DNA junction binding and cleavage. Mutations were introduced using the Stratagene QuikChange system and checked by sequencing of the entire CCE1 coding sequence. The mutated proteins were expressed in E. coli strain BL21 (DE3) using the vector pET19b and purified as described previously for the wild-type enzyme. All the mutants eluted from size exclusion chromatography with a retention time similar to the wild-type enzyme, suggesting that the dimeric quaternary structure of the wild-type enzyme is unaltered in the mutants. For each mutant, catalytic activity was measured using a single turnover kinetic assay, with the four-way junction J1, radioactively labeled on the r strand, as a substrate. For some mutants, the four-way junction Z1, which is cleaved about 20-fold more quickly that J1 (8), was used to provide a more sensitive assay of reduced enzyme activity. Equilibrium dissociation constants were measured by gel electrophoretic retardation analysis in the presence of EDTA. The data for the wild-type and all mutant enzymes is summarized in Table I.

Basic Residues in Cce1—Arg⁴⁶⁰ and Arg⁴⁵⁰ are both conserved in three of the four proteins. The R150A mutant gave the smallest decrease in activity (4-fold) of any of the mutants tested and a moderate decrease in binding affinity, suggesting that it does not play an important role in catalysis or substrate recognition, whereas the equivalent mutation in Arg⁴⁶⁰ resulted in a modest decrease in junction binding affinity but a relatively large effect on catalytic activity, which is 100-fold reduced compared with the wild-type enzyme. Lysine 291 is specific to the junction-resolving enzymes. It appears to have an important role in catalysis, since mutation to an alanine results in a complete loss of detectable activity. The more conservative replacement with an arginine is also catalytically inactive, suggesting that the residue plays a more specific role in catalysis, rather than merely contributing a positive charge.

Indeed, while the K291A mutant still binds four-way DNA junctions relatively well, the K291R mutant displays significantly weaker binding. Possibly, the presence of the more bulky arginine residue is sterically unfavorable in the protein-DNA complex. Arginine 291 is specific to the junction-resolving enzymes. It appears to have an important role in catalysis, since mutation to an alanine results in a complete loss of detectable activity. The more conservative replacement with an arginine is also catalytically inactive, suggesting that the residue plays a more specific role in catalysis, rather than merely contributing a positive charge.

Acidic Residues—Four acidic residues in Cce1 have been mutated to their corresponding amide forms. None of these residues plays an important role in binding the nucleic acid substrate, although probably not in catalysis.
mutations results in significant changes in the equilibrium binding affinity of the enzyme for the four-way junction. Asp292 is not conserved in any of the other sequences, and not surprisingly the D292N mutant retains appreciable catalytic activity. The significant decrease in catalytic activity observed (80-fold) may reflect the fact that this residue is clearly adjacent to the catalytic site, and any mutation at this position might well influence the conformation or overall charge of the active site. Asp293 is conserved in the resolving enzymes but not in Mrs1. The D293N mutant displays a 600-fold decrease in catalytic activity (using junction Z1 as a substrate), suggesting an important role in catalysis, possibly by providing a ligand for one of the catalytic magnesium ions. Two acidic residues (Glu145 and Asp294) are conserved in all four proteins. Mutation of either results in complete loss of activity with the junction J1 substrate. Using the faster cutting junction Z1 enabled the detection of very weak cleavage activity, reduced 60,000-fold.

In common with other resolving enzymes, Holliday junction-resolving enzymes are thought to hydrolyze phosphodiester bonds by means of a metal-activated water molecule. Acidic residues typically function as ligands for catalytic metal ions in nucleases. Mutation of each of three conserved acidic residues (Glu145, Asp293, and Asp294), which are conserved in all three Cce1 homologues, resulted in very large decreases in the catalytic activity without affecting junction binding affinity. Previously, the mutation D226N in S. pombe...
Yde2 (equivalent to D294N in S. cerevisiae Cce1) was shown to reduce drastically catalytic activity without affecting binding specificity (17). Similar phenotypes have been demonstrated for mutations of acidic residues in RuvC (18), T4 endonuclease VII (19–21), T7 endonuclease I (22), RusA (23, 24), and Hjc (25), and in the case of RuvC the acidic residues in question are known to cluster in a metal binding pocket at the active site (18). Isothermal titration calorimetry and EPR have been used to demonstrate that Cce1 binds two magnesium or manganese ions (16) and may thus have a two-metal ion mechanism for hydrolysis of phosphodiester bonds, similar to many nucleases (26). Use of ITC to examine metal ion binding in the Cce1 mutants E145Q, D294N, and Q147A clearly shows that binding is abolished in the former two, but not in the latter, thus providing strong evidence that Glu145 and Asp294 (and probably by implication Asp293) form part of the metal binding pocket in Cce1.

Basic residues in nucleases are required for binding the nucleic acid substrate, either through contacts with the phosphodiester backbone or by contacting specific bases in the major or minor grooves. A second role for basic residues is in catalysis; e.g. the active site residue Lys92 in EcoRV is essential for activity and is thought to stabilize the negative charge developing on the pentavalent phosphorus in the transition state (27). Our mutagenesis studies of conserved basic residues in Cce1 appear to have uncovered examples of residues important in each of these roles. Notably, Arg231 has been demonstrated to play an important role in junction binding, and the R231A mutant can be partially rescued by replacement with a lysine residue. In contrast, Lys291, which is close to the active site residues Asp293 and Asp294, appears to have an important role in catalysis, and substitution with an arginine at this site residues Asp293 and Asp294, appears to have an important role in catalysis, and substitution with an arginine at this position fails to restore activity and is detrimental to binding affinity. The archaeal junction-resolving enzyme Hjc has recently been shown to possess a conserved nuclease domain in each of these roles. Notably, Arg231 has been demonstrated to play an important role in catalysis, and substitution with an arginine at this position fails to restore activity and is detrimental to binding affinity. The archaeal junction-resolving enzyme Hjc has recently been shown to possess a conserved nuclease domain in each of these roles. 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