A Novel Family of Sequence-specific Endoribonucleases Associated with the Clustered Regularly Interspaced Short Palindromic Repeats

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Clustered regularly interspaced short palindromic repeats (CRISPRs) together with the associated CAS proteins protect microbial cells from invasion by foreign genetic elements using presently unknown molecular mechanisms. All CRISPR systems contain proteins of the CAS2 family, suggesting that these uncharacterized proteins play a central role in this process. Here we show that the CAS2 proteins represent a novel family of endoribonucleases. Six purified CAS2 proteins from diverse organisms cleaved single-stranded RNAs preferentially within U-rich regions. A representative CAS2 enzyme, SSO1404 from *Sulfolobus solfataricus*, cleaved the phosphodiester linkage on 3′-phosphate-and 5′-hydroxyl-terminated oligonucleotides. The crystal structure of SSO1404 was solved at 1.6 Å resolution revealing the first ribonuclease with a ferredoxin-like fold. Mutagenesis of SSO1404 identified six residues (Tyr-9, Asp-10, Arg-17, Arg-19, Arg-31, and Phe-37) that are important for enzymatic activity and suggested that Asp-10 might be the principal catalytic residue. Thus, CAS2 proteins are sequence-specific endoribonucleases, and we propose that their role in the CRISPR-mediated anti-phage defense might involve degradation of phage or cellular mRNAs.

Numerous prokaryotic genomes contain structures known as clustered regularly interspaced short palindromic repeats (CRISPRs), composed of 25–50-bp direct repeats separated by intervening sequence spacers (or inserts) of similar length (1–3). CRISPRs represent the most widely distributed family of repeats in prokaryotes and are found in a great variety of bacteria and archaea, including many pathogens (such as Mycobacterium tuberculosis, Streptococcus, Yersinia, Neisseria, and Corynebacterium) (4–8). Most organisms possess multiple CRISPR clusters (2–20) that form tandem arrays containing from 4 to over 100 units (a repeat and a spacer) (1, 9–11). Sequence analysis of the CRISPR spacers in various organisms revealed that some of the spacer sequences are homologous (up to 100% sequence identity) to sequences in phage genomes, plasmids, or in the chromosome and have either sense or antisense orientations (5, 12–14). Remarkably, phages and plasmids fail to infect the specific strains carrying the cognate spacers, implying a relationship between CRISPRs and immunity against specific foreign DNA. Recent experiments on *Streptococcus thermophilus* have directly demonstrated that, after a phage challenge, this bacterium adds new CRISPR spacers derived from the phage genome (15, 16). Removal or addition of the phage-specific spacers correlated with the cell resistance to the respective phage implying that resistance specificity is determined by the sequence identity between the phage genome and spacers (15).

On the chromosome, CRISPR loci are flanked by a large number of *cas* (CRISPR-associated) genes encoding uncharacterized proteins. A comprehensive bioinformatic analysis of the CAS system in sequenced genomes resulted in a refined classification with 25 gene families and at least nine types of the *cas* operon organization (9, 17). Eight CAS protein families have been predicted to possess nuclease activity; nine families have been characterized as putative RNA-binding proteins (RAMP-domain proteins), and two families have been predicted to pos-

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† The on-line version of this article (available at http://www.jbc.org/) contains supplemental Table 1, Fig. 1, and additional references.

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3 The abbreviations used are: CRISPR, clustered regularly interspaced short palindromic repeats; CAS, CRISPR-associated; PAA, polyacrylamide; PAGE, polyacrylamide gel electrophoresis; PNK, polynucleotide kinase; ssRNA, single-stranded RNA; TA, toxin-antitoxin; nt, nucleotide; r.m.s.d., root mean square deviation; MES, 4-morpholineethanesulfonic acid; CAPS, 3-(cyclohexylamino)propanesulfonic acid.
that the CRISPRs and CRISPR spacer sequences to phage genes, led to the hypothesis
analysis, combined with the data on the homology of some
sess helicase and DNA/RNA polymerase activity (17). This
We show that CAS2 proteins are endoribonucleases that are
specific to single-stranded (ss)RNAs and preferentially cleave
them within U-rich regions. The crystal structure of a represen-
tative CAS2 protein, SSO1404 from Sulfolobus solfataricus,
was solved to a 1.6 Å resolution and revealed a ferredoxin-like
fold with the double split β-α-β motif, as well as the putative
active site.

**EXPERIMENTAL PROCEDURES**

**Protein Overexpression, Purification, and Site-directed Mutagenesis**—The cloning of the genes encoding SSO1404 and other CAS2 proteins (SSO8090, TM1796, AF1876, MTH1083, and NE0845) into the modified pET15b was carried out as described previously (20). The proteins were expressed as a fusion with an N-terminal His
tag in Escherichia coli strain BL21 (DE3) and purified to more than 95% homogeneity using metal-chelate affinity chromatography on nickel affinity resin and gel filtration on a Superdex 200 26/60 column (Amersham Biosciences) as described before (20, 21). Site-directed mutagenesis of SSO1404 was performed as described previously (21) using a protocol based on the QuikChange site-di-
rected mutagenesis kit (Stratagene).

**Preparation of RNA Substrates**—The short RNA substrates (Table 1) were purchased from IDT. The oligonucleotides were
5′-end-labeled with [γ-32P]ATP (6,000 Ci/mmol; Amersham Biosciences) and T4 polynucleotide kinase (PNK) (Fermentas) and then purified by denaturing PAGE (15% polyacrylamide, 8 M urea gel). The labeled oligonucleotides were eluted from the gel, precipitated with 2% LiClO4 in acetone, washed with acetone, dried, and dissolved in diethyl pyrocarbonate-treated Milli-Q water. The long RNA substrates were synthesized using the Ambion T7 RNA polymerase MAXiScript transcription kit.

**TABLE 1**

| Substrate | Nucleotide sequence (5′ → 3′) and cleavage sites* |
|-----------|--------------------------------------------------|
| RNA1 (24 nt) | UUUCUAU | UCCUUGUAGAUAUAC |
| RNA2 (25 nt) | UCCCCAA | U | UCCUUUGUAGAUAUAC |
| RNA3 (38 nt) | UUGGAAG | U | AG | UG | UAAAGAUCUUAUAGCAGAUAUA |
| RNA4 (41 nt) | GUCUCA | CC | C | C | UCCUGGCUUUAUAGCAGAUAUA |
| RNA5 (39 nt) | AAGAAGC | U | U | UCCUUUGUAGAUAUAC |
| RNA6 (39 nt) | CUGUCU | CC | AUAU | U | U | AU | AAUAACCUUUAUGAUA |
| RNA7 (36 nt) | GUCUUCU | CC | AUAU | U | U | AAUAACCUUUAUGAUA |
| RNA8 (30 nt) | GAUCACAAGU | CC | ACCGUUAUGAUA |
| RNA9 (30 nt) | CGGUCUCC | U | UCCAAGGUAAUCUUGAAAG |
| RNA10 (30 nt) | AGUCGGCAACACCC | U | UCCGGAACACCC |
| RNA11 (35 nt) | AUCUA | U | U | UCCGGAACACCC |
| RNA12 (32 nt) | CAUUC | U | UACCUC | UCCUUGUAGAUAUAC |
| RNA13 (32 nt) | AUCUA | U | U | UCCGGAACACCC |
| RNA14 (36 nt) | GCUCUUCUGA | CC | gccacGcUuUccUGCC |
| RNA15 (37 nt) | AGAAAGU | AGCUCAG | GcUuUccUGCC |
| RNA16 (36 nt) | ACGAGAGG | U | U | UACGCUUUAUGGACGUUA |
| RNA17 (36 nt) | GUCUA | UUGGA | U | U | UCCGGAACACCC |
| RNA18 (16 nt) | CGGUCUCC | U | UCCAAGGUAAUCUUGAAAG |
| RNA19 (16 nt) | CAUUCUCU | UUACCUU |
| RNA20 (16 nt) | GUCUCUCU | UUUCACCA |
| RNA21 (13 nt) | UACGCU | UUCUCA |
| RNA22 (10 nt) | UACGCU | UUCU |
| RNA23 (8 nt) | GU | UUCUCU |
| RNA24 (6 nt) | GUUUC |
| RNA25 (30 nt) | UUUCUAU | CC | CI | CCCUUGAAUUGAUA |
| RNA26 (40 nt) | UUCCUCA | U | U | UCCUUGUAGAUAUAC |

* Cleavage sites are shown by arrows (boldface arrows indicate the preferred cleavage sites). The substrates correspond to CRISPR DNA sequences from the several organisms. The numbers of nucleotides are given in parentheses. The cleavage sites are shown by arrows (boldface arrows indicate the preferred cleavage sites). The substrates correspond to CRISPR DNA sequences from the several organisms.

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**CAS2 Endoribonuclease**
For the synthesis of the mouse β-actin mRNA (304 nt) fragment, pTRI-actin-mouse DNA (MAXIscript kit, Ambion) was used as a template. To produce long CRISPR RNA substrates, a DNA template was prepared by cloning the 1,292-bp-long 5′-fragment of the S. solfataricus CRISPR cluster-2 DNA into the pBluescript SK+ vector (Stratagene). During the transcription reaction (MAXIscript kit, Ambion), this DNA template (linearized by EcoRV) directed the synthesis of a 270-nt-long RNA substrate containing the CRISPR cluster-2 upstream region, repeat-1, spacer-1, repeat-2, and a 27-nt fragment of spacer-2. Long RNA substrates were uniformly labeled during transcription using [α-32P]UTP (3,000 Ci/mmol). The labeled RNA substrates were purified by denaturing 8% PAA, 8 M urea gel, eluted, ethanol-precipitated, and stored at −70 °C. Double-stranded RNA substrates were prepared by the incubation of equimolar amounts (10 μM) of the 5′-32P-labeled sense and unlabeled antisense RNA oligoribonucleotides (RNA5, RNA25, and RNA26) in 50 mM Tris-HCl (pH 7.0), 100 mM KCl at 90 °C for 1 min and slow cooling at room temperature for 1.5 h. The formation of RNA duplexes was verified by electrophoresis in 10% nondenaturing PAA gels.

**Enzymatic Assays**—The reaction mixture for RNase assays (10 μl) contained 0.1 μM [32P]RNA, 50 mM Tris–HCl (pH 8.5), 100 mM KCl, 5 mM MgCl2, 1 mM dithiothreitol, and 0.01–0.038 μg of enzyme. The pH dependence of SSO1404 was characterized using three buffers: MES-K (pH 5.5 to 6.5), Tris-HCl (pH 7.0 to 9.0), and CAPS-K (pH 9.4 to 11.0). The reaction mixture was incubated at 37 °C for the indicated period of time and quenched by the addition of equal volume of formamide loading buffer (80% formamide, 0.025% bromophenol blue, 0.025% xylene cyanol, and 10 mM EDTA (pH 8.0)). The reaction products were resolved by electrophoresis in 15% PAA, 8M urea gels using TBE (10 mM Tris borate (pH 8.3), and 2 mM EDTA) as a running buffer. As nucleotide size markers, an imidazole ladder or RNase T1, respectively, was used (22, 23). For the analysis of the RNA product 5′-end, after RNase reaction, RNA products were precipitated by 2% LiClO4, washed by acetone, dissolved in Milli-Q water, and phosphorylated with [γ-32P]ATP and T4 PNK using conditions for forward or phosphate exchange reaction according to the manufacturer’s protocol (Fermentas). After the PNK reaction, RNA products were analyzed using denaturing 15% PAA, 8 M urea gels as described above for RNAse assays. The reaction mixtures for DNase assays (40 μl) contained 50 mM HEPES-K buffer (pH 7.5), 100 mM KCl, 5 mM MgCl2, 1 mM dithiothreitol, 0.3 μg of λ DNA (double-stranded DNA) or 0.75 μg of M13 DNA (ssDNA), and 1–4 μg of enzyme. After 1 h of incubation at 37 °C, the reactions were quenched by the addition of 6× DNA loading dye (Fermentas) and analyzed on EtBr-stained 1% agarose gels.

**Protein Crystallization and Structure Determination**—SSO1404 crystals were grown using the hanging drop vapor diffusion method with the drops containing a mixture of 2 μl of 10 mg/ml purified selenomethionine-incorporated SSO1404 protein and 2 μl of reservoir buffer (0.2 M NaI, 20% w/v PEG 3350, and 2% v/v isopropyl alcohol). For diffraction studies, the crystals were stabilized with the crystallization buffer supplemented with 20% ethylene glycol as a cryoprotectant and flash-frozen in liquid nitrogen. A single crystal of selenomethionine-incorporated SSO1404 was used to collect diffraction data at beamline 19-BM of the Structural Biology Center of the Advanced Photon Source (24) and was maintained at a temperature of 100 K. A single-wavelength anomalous diffraction dataset was collected at a wavelength of 0.979 Å. Crystallographic data collection and model refinement statistics are summarized in Table 2. Reflection data were collected, indexed, integrated, and scaled with HKL-3000 (25).

A two-site selenium substructure was determined; the structure was phased by single-wavelength anomalous diffraction, and an initial model was built. All structure solution and initial model building was performed by HKL-3000, which is integrated with SHELXD, SHELXE, MLPHARE, DM, O, COOT, RESOLVE, and ARP/wARP (25–32). The initial model was improved by iterative cycles of manual rebuilding in COOT, followed by maximum likelihood refinement with REFMACS (33). In later stages of refinement, a multigroup TLS model generated by the TLSMD web server was used to further improve the model. The final model was validated using Molprobity (34), SFCHECK (35), and PROCHECK. The atomic coordinates and structure factors for SSO1404 have been deposited in the Protein Data Bank with the accession code 2i8e.

**RESULTS AND DISCUSSION**

**Enzymatic Activity of CAS2 Proteins**—To characterize the biochemical activity of CAS2 proteins, we cloned and purified six members of this family from different organisms as follows: SSO1404 and SSO8090 from S. solfataricus, AF1876 from Archaeoglobus fulgidus, TM1796 from Thermotoga maritima, MTH1083 from Methanobacterium thermoautotrophicum, and NE0845 from Nitrosomonas europaea. Given the prediction that CAS2 proteins might possess nuclease activity (17),

| TABLE 2 |
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| **Crystallographic data collection and refinement statistics** |
| **Data collection** | **SSO1404 (Protein Data Bank 2i8e)** |
| Resolution range (Å) | 50–1.59 (1.65–1.59) |
| Wavelength (Å) | 0.9794 |
| Space group | P61 |
| Unit cell parameters | a = b = 64.3 Å, c = 39.5 Å, α = β = 90°, γ = 120° |
| No. of reflections | 12,619 (1219) |
| Rmerge (%) | 0.052 (0.309) |
| Rmerge-avg (%) | 0.038 (0.293) |
| Mean I/σI | 63.8 (8.2) |
| Completeness (%) | 99.5 (96.2) |
| Redundancy | 11.7 (10.3) |
| Wilson B factor (Å²) | 27.3 |

* Rmerge-avg is calculated for unmerged anomalous pairs.
the purified proteins were tested for the presence of DNase or RNase activity against single-stranded (ss) DNA (M13 DNA), double-stranded (ds) DNA (λ DNA), or ssRNA as follows: RNA5, RNA6, and RNA14 (Table 1), which are identical in sequence to the sense strands of the CRISPR repeats or spacers from *S. solfataricus*, *A. fulgidus*, or *T. maritima* (36 or 39 nt). No nuclease activity was found against either of the DNA substrates, but all proteins degraded the ssRNAs (Fig. 1). With the tested ssRNA substrates, the CAS2 proteins generated a limited number (one to five) of products of various lengths (7–29 nt) indicating that they cleave ssRNAs endonucleolytically. A similar but not identical pattern of products was observed, and most cleavage sites contained one or two U (Fig. 1). This observation suggests that the CAS2 ribonucleases recognize similar RNA sequences but also display some difference in substrate preference. SSO1404 also showed detectable cleavage of a long model RNA substrate (the 304-nt transcript of the 5'-fragment of the mouse β-actin gene) and generated several products of various lengths (18–200 nt), but the activity was lower than that against short oligoribonucleotides (data not shown). Thus, the CAS2 family proteins are ssRNA-specific endoribonucleases.

**Reaction Requirements and RNA Cleavage Products of SSO1404**—SSO1404 exhibited RNase activity over a broad pH range (7.0–10.0) with maximum activity at pH 8.5–9.0 (Fig. 2A). Very little cleavage of RNA5 by SSO1404 (between U20 and C21) was observed in the absence of both monovalent and divalent cations, as well as in the presence of Na⁺, K⁺, or Mn²⁺ (Fig. 2B). Cleavage between U9 and U10 was greatly enhanced by the addition of Mg²⁺. The Mg²⁺-dependent activity was further stimulated by K⁺, whereas Na⁺ had inhibitory effect. Thus, SSO1404 requires Mg²⁺ (5 mM), K⁺ (100 mM), and pH 8.5–9.0 for optimal cleavage of ssRNA.

RNases can be subdivided into two groups depending on the position of the cleavage of the phosphodiester linkage (36). Enzymes of the first class cleave on the 3’-side (producing a 5’-phosphorylated second product) and include
numerous intracellular endo- and exoribonucleases. The enzymes of the second class (e.g. RNase A, RNase T1, and barnases) cleave the linkage on the 5’-side releasing products containing a cyclic 2’,3’-phosphodiester bond. The products of ssRNA cleavage by a representative CAS2 protein, SSO1404, were characterized using the T4 PNK-catalyzed reactions of RNA phosphorylation (at 5’-hydroxy termini) and the phosphate exchange between the 5’-phosphate groups of the oligonucleotide substrate and ATP (37). After hydrolysis of the RNA5 substrate by SSO1404, the reaction products were incubated with PNK and [γ-32P]ATP and analyzed by denaturing PAGE and autoradiography (Fig. 2C). Incubation with PNK did not produce a labeled 3’-end product (no second band on the gel), and the gel showed only one labeled band corresponding to the 5’-end product (Fig. 2C, lane 2). This suggests that the 3’-end product is phosphorylated on its 5’-end. The presence of this 3’-end product in the reaction mixture with SSO1404 was confirmed using the PNK-catalyzed phosphate exchange reaction between the RNA 5’-phosphate and [γ-32P]ATP in the presence of ADP excess (38). This experiment produced two labeled products on the gel with the expected length of 9 and 30 nt (Fig. 2C, lane 3). Thus, SSO1404 and apparently other CAS2 enzymes are the 5’-phosphomonoester-producing endoribonucleases.

**Endoribonuclease Activity of SSO1404 against Long CRISPR Substrates**—Previous analyses of small RNAs produced in *S. solfataricus* and *A. fulgidus* revealed that the long CRISPR transcripts are processed into fragments with the size of one repeat and one spacer (62–75 nt) (39, 40). It has been proposed that these short CRISPR RNA substrates are produced by an unknown *cas* RNAse that would cleave within the repeat sequence producing intact spacers flanked with the repeat fragments (3). The demonstration of ssRNase activity of SSO1404 suggested the possibility that CAS2 could be the RNAse that is responsible for processing CRISPR RNAs. Thus, we tested SSO1404 for the ability to cleave the 270-nucleotide-long CRISPR transcript of the *S. solfataricus* CRISPR cluster-2. The uniformly labeled long CRISPR RNA substrate was prepared using T7 RNA polymerase and the 270-bp DNA fragment of the 5’-end of the *S. solfataricus* CRISPR cluster-2 containing the upstream region, repeat-1, spacer-1, repeat-2, and a 27-nt fragment of spacer-2 as the template. If SSO1404 were a CRISPR-processing endoribonuclease, it would be expected to cleave this substrate within the repeat sequences and produce a minimal product containing one spacer flanked by two repeat halves (63–66 nt-long), as well as a series of products containing two or more repeat + spacer units. SSO1404 showed detectable endoribonuclease activity with this long CRISPR RNA substrate, but it produced products that were smaller than expected (23, 27, and 45 nt long), as well as several longer fragments (65, 75, 95, 110, and 160 nt long) (Fig. 3A). This finding indicates that SSO1404 does not have the expected specificity toward CRISPR transcripts and thus is unlikely to be the CRISPR-processing endoribonuclease.

**SSO1404 Cleaves ssRNAs Preferentially within U-rich Regions**—The substrate specificity of SSO1404 was further characterized using an extended set of shorter synthetic ssRNA substrates, as well as several dsRNAs. The RNA1 to RNA5 substrates were identical in sequence to the sense strands of repeat-1 (24 nt), repeat-2 (25 nt), and three spacers (1–3) of the *S. solfataricus* CRISPR cluster-2 (Table 1). SSO1404 cleaved all these substrates, but the highest activity was observed with RNA5, which was preferentially cleaved between U9 and U10 (Fig. 3B). The hydrolysis of this substrate proceeded to near completion with the formation of one main labeled product.

Eight ssRNA substrates (RNA7 to RNA10 and RNA14 to RNA17) corresponded to repeats and spacers from other CRISPR-containing organisms (*S. thermophilus*; *T. maritima*, and *Methanococcus jannaschii*), whereas RNA11, RNA12, and RNA13 were scrambled RNA substrates (Table 1). SSO1404 demonstrated endonucleolytic activity toward most of these substrates with the highest activity toward RNA9, RNA10, and RNA13 (Fig. 3C). The analysis of the cleavage sites showed that SSO1404 preferred U-rich sequences, and in many substrates (e.g. RNA9, RNA10, RNA12, and RNA13) the cleavage site was located between two Us (Table 1). Examination of the computer-predicted secondary structures of the cleavable RNA substrates revealed that SSO1404 cleaves RNA substrates in predicted single-stranded regions (Fig. 3). No cleavage was observed with the dsRNA substrates prepared by annealing ssRNA substrates (RNA5, RNA25, and RNA26) with their complementary RNA chains and containing blunt ends or a two-nucleotide 5’-overhang (not shown). Thus, SSO1404 is a single-stranded RNA-specific endoribonuclease.

Although several substrates shown in Fig. 3 (RNA2, RNA5, and RNA12) contain a potential SSO1404 cleavage site within the predicted loops, the enzyme cleaved these substrates only in close proximity to the 5’- or 3’-ends suggesting that it requires the presence of a free RNA end. To determine whether a stem-loop is required as an entry site for SSO1404, we tested its activity against three truncated oligoribonucleotides (16 nt) that lack the potential to form stem-loops and contain the SSO1404 cleavage site from RNA5 (RNA18), RNA11 (RNA19), or RNA9 (RNA20). SSO1404 efficiently cleaved all three substrates at the same position as in the corresponding longer substrates (Table 1) indicating that the primary sequences of these short oligoribonucleotides were sufficient to direct site-specific cleavage by SSO1404, and no specific secondary structure was required. To determine the minimal size of the SSO1404 substrate, the 39-nt-long RNA5 was further trimmed to 16-nt RNA18, 13-nt RNA21, 10-nt RNA22, 8-nt RNA23, and 6-nt RNA24 (Table 1). SSO1404 efficiently cleaved the four longer substrates (RNA5, RNA18, RNA21, and RNA22) but showed no activity against RNA23 (8 nt) and RNA24 (6 nt) (Fig. 4). Thus, oligoribonucleotides as short as 10 nt can serve as SSO1404 substrates.

An ssRNA endoribonuclease activity with a preference for U-rich regions also has been observed in the abortive phage infection determinant AbiB from *Lactococcus lactis* and in mRNA interferases MazF-mt1 and MazF-mt6 from *M. tuberculosis* (41, 42). The *L. lactis* AbiB prevented growth of the sensitive phage bIL170 through the selective degradation of phage mRNAs by endonucleolytic cleavage at U/U, A/U, and U/A sites (41). The mRNA interferases are toxin components of chromosomal TA modules that are abundant in free-living prokaryotes and induce reversible cell cycle arrest or programmed cell death in response to starvation or other
primary structures and the positions of the SSO1404 cleavage sites in selected RNA substrates.

SSO1404 dimer, two monomers are joined by their products of the RNA5 cleavage by RNase T1 and 2M imidazole (pH 7.0), in the presence of SSO1404 for (15 min, lane 1) or (30 min, lane 3) or in the absence (lanes 2, 4, 6, 8, 10, and 12) of SSO1404 (30 μg/ml). Reaction products were processed and visualized as described under “Experimental Procedures.” Lanes T1 and Im represent the products of the RNA5 cleavage by RNase T1 and 2M imidazole (pH 7.0), respectively.

stress conditions (43–46). These enzymes have different mRNA cleavage specificities, and their expression in the cell causes the effective inhibition of protein synthesis leading to temporal cell growth arrest. In this regard, it might be relevant that U-rich and AU-rich regions have been identified upstream of the Shine-Dalgarno sequence in prokaryotic mRNAs and shown to enhance translation (47, 48). The CAS2 proteins are unrelated to mRNA interferases or AbiB but, as mentioned above, appear to be homologous to VapD, a component of a distinct class of TA systems. To elucidate the molecular basis for the ssRNase activity of CAS2, we determined the crystal structure of SSO1404 at 1.6 Å resolution. The structure demonstrated that SSO1404 is a homodimer (Fig. 5A). This is consistent with the results of our gel filtration experiments, which showed that the native protein has a molecular mass of 23.7 ± 0.6 kDa (predicted molecular mass 11.9 kDa). In the SSO1404 dimer, two monomers are joined by their β-sheets, and the α-helices are exposed on the surface. In the SSO1404 monomer, the four β-strands form an antiparallel β-sheet, and the α-helices are packed on one side, and the overall structure may be described as a double-split β-α-β fold. In addition, the last β-strand (β5) of each monomer interacts with the β-sheet of the other monomer creating two-joint, five-strand, antiparallel β-sheets (Fig. 5A).

A search for structural homologs of SSO1404 using the Dali (49) and secondary-structure matching (50) programs (ProFunc data base (51) identified two uncharacterized proteins, TT1823 from Thermus thermophilus (Protein Data Bank code 1zpw, secondary-structure matching Z-score = 7.7, r.m.s.d. = 1.3 Å) and PF1117 from Pyrococcus furiosus (Protein Data Bank code 2i0x, secondary-structure matching Z-score = 7.0, r.m.s.d. = 1.7 Å) (Fig. 5, B–D). Like SSO1404, these two proteins belong to the CAS2 family (COG1343) (17). In addition, the Dali search recognized structures of domain III of the translation elongation factor G (EF-G) from T. thermophilus (Protein Data Bank code 1fnm) and elongation factor 2 from yeast (Protein Data Bank code 1n0v, Z-score = 6.1, r.m.s.d. = 2.3 Å) as significantly similar to SSO1404. All these structures have the double-split β-α-β fold, which is also observed in many ribosomal and other RNA-binding proteins (52). However, the ribosomal proteins and elongation factors do not have the conserved residues that are important for the RNase activity of SSO1404 (Tyr-9, Asp-10, Arg-17, Arg-19, Arg-31, and Phe-37) (see below).

Both Dali and VAST searches also recognized various proteins with the ferredoxin-like fold as being structural relatives of each of the three CAS2 structures with similar, moderate scores and r.m.s.d. values (supplemental Table 1), suggesting that CAS2 proteins comprise a new superfamily within the ferredoxin-like fold. Indeed, this is how the available CAS2 structures have been classified in the SCOP data base. The ferredoxin-like domains consist of one of the most populated protein folds, with numerous structural and functional derivatives (53). In particular, this fold is present in numerous RNA-binding proteins, including the RNA-binding domain, the anticodon-binding domain of PheRS, ribosomal proteins S6 and S10, and also the prominent components of the CAS system, the RAMP superfamily proteins, which possess a duplicated ferredoxin-like domain (17, 54). However, to our knowledge, no nucleases with the ferredoxin-like fold have been characterized with the possible exception of the IS200 transposase of S. solfataricus (SSO1474, Protein Data Bank code 2f5g). Like many ribosomal proteins (55), SSO1404 exposes several aromatic and hydrophobic residues to the solvent (Ile-32, Tyr-34, Ile-75, Ile-84, Val-85, Ile-86, Phe-80, and Tyr-88), and some of these residues are conserved in large subsets of the CAS2 family proteins (supplemental Fig. 1), suggesting their involvement in the interaction with the bases of RNA. The extended loop α2-β4 of SSO1404 also could be a candidate for interaction with RNA.

FIGURE 3. RNase activity of SSO1404 with CRISPR-related substrates. A, cleavage of the 5′-fragment (270 nt) of the S. solfataricus CRISPR cluster-2 containing the cluster-2 upstream region, repeat-1, spacer-1, repeat-2, and a 27-nt fragment of spacer-2. Autoradiograph of the 8% PAA, 8M urea gel showing the cleavage of the uniformly labeled [32P]RNA (0.1 μM) by SSO1404 (30 μg/ml). RNA was incubated in the absence of SSO1404 (30 min, lane 1) or in the presence of SSO1404 for (15 min, lane 2) or (30 min, lane 3). B, RNA1 to RNA5; C, RNA6 to RNA9; D, RNA10 to RNA12. The right part of the of B–D shows predicted secondary structures and the positions of the SSO1404 cleavage sites in selected RNA substrates. Im, imidazole.

FIGURE 4. Ribonuclease activity of SSO1404 against a series of the truncated RNA5 substrates. A, autoradiograph of the 15% PAA, 8 M urea gel showing the activity of SSO1404 against the RNA5 substrate and its five truncated derivatives (the substrate lengths are shown on the right). B, sequences of the substrates used in the experiment and the positions of the cleavage sites of SSO1404. RNA substrates (0.1 μg/ml) were incubated for 15 min (37 °C) in the absence (lanes 1, 3, 5, 7, 9, and 11) or in the presence (lanes 2, 4, 6, 8, 10, and 12) of SSO1404 (30 μg/ml). Reaction products were processed and visualized as described under “Experimental Procedures.” Lanes T1 and Im represent the products of the RNA5 cleavage by RNase T1 and 2M imidazole (pH 7.0), respectively.
Mutational Analysis of SSO1404 and the Potential Catalytic Site—The structure of the SSO1404 dimer revealed several major cavities or grooves located at the interface of the two monomers, which might represent potential catalytic sites (Fig. 6A). The most prominent cavity consists of the deep cleft between two monomers accommodating the side chains of conserved Asp-10, which is bracketed by two α2-β4 loops (Fig. 6A). To identify the active site residues involved in RNA cleavage, we performed alanine replacement mutagenesis of the conserved residues of SSO1404. A multiple sequence alignment of CAS2 proteins (17) identified several conserved amino acids (Fig. 7; supplemental Fig. 1). The single conserved aspartate, Asp-10, was of special interest as the putative principal catalytic residue. In the SSO1404 structure, Asp-10 is located at the end of the first β-strand and is preceded by the conserved tyrosine Tyr-9. Several other partially conserved residues are spatially close to Asp-10, including Arg-17, Arg-19, and Arg-31. Alanine replacement mutagenesis of SSO1404 revealed that Asp-10, Arg-31, and Phe-37 are strictly required for the catalytic activity, whereas Y9A, R17A, and R19A showed very low activity (Fig. 8). The T12A and D65A mutants showed an ~2-fold drop in activity, whereas the other mutant proteins exhibited slightly reduced (Q33A, Y34A, and S35A) or wild-type level (D13A, D14A, N18A, and R67A) activity.

In the SSO1404 structure, five residues that are important for activity (Tyr-9, Asp-10, Arg-17, Arg-31, and Phe-37) are located in the long cavity formed by the α1 helix on one side, the β2 and β3 strands on the other side, and the β1 strand at the bottom (Fig. 6B). SSO1404 requires a divalent metal cation for activity, Mg$^{2+}$ being the optimal metal (Fig. 2B). In most known RNases, two or three conserved acidic residues are critical for catalysis and are involved in the coordination of one or two metal cations (Mg$^{2+}$ or Mn$^{2+}$), which activate a nucleophilic water molecule for hydrolysis of the phosphodiester bond or stabilize the transition state in cleavage reactions (56). The structures of SSO1404 and two other CAS2 proteins (TT1823 and PF1117) revealed no metal atoms bound to the protein. Nevertheless, given that SSO1404 required the addition of Mg$^{2+}$ or Mn$^{2+}$ for activity and the conserved Asp-10 was critical for catalysis, we suggest that this residue is involved in the coordination of the metal cation in SSO1404 and other CAS2 proteins. Interestingly, the side chains of Asp-10 in the two monomers are oriented toward the active site of the other monomer and are positioned 6.5 Å apart (Fig. 6B) suggesting that these residues do not form two independent active sites. In addition, these aspartates are located inside a deep, narrow cleft making them inaccessible for direct interaction with the RNA substrate (Fig. 6A). Therefore, we propose that the side chains of the two Asp-10 bind one Mg$^{2+}$ atom, which might coordinate a nucleophilic water molecule. As shown here (Fig. 2C), SSO1404 generates products terminating in a 3'-hydroxyl and a 5'-phosphate suggesting that, like in the E. coli RNase E, the reaction involves an attack of a water molecule on the susceptible phosphodiester bond followed by scission of the 3'-oxygen–phosphorus bond (57). Therefore, the catalytic mechanism of SSO1404 probably involves the in-line nucleophilic attack on the scissile phosphate by an activated hydroxyl group generated from a water molecule that is coordinated to magnesium.

The SSO1404 D65A protein had a 2-fold reduced activity (Fig. 8). This conserved aspartate is located on the long α2-β4 loop (Fig. 6, A and B) that varies in length among CAS2 proteins and accommodates several partially conserved charged residues (Glu-64, Asp-65, Glu-66, and Arg-67 in SSO1404) (Fig. 7). Based on the structure of the SSO1404 dimer (Fig. 6, A and B), we suggest that the α2-β4 loops are responsible for the recognition of RNA substrates and might determine the substrate selectivity of different CAS2 proteins. We propose that like the E. coli RNase E S1 domain (58), the SSO1404 α2-β4 loop might function to clamp down the RNA substrate in the active site orienting its phosphate backbone for nucleophilic attack by the magnesium-coordinated activated water molecule.
Conclusions—This work is the first step toward a comprehensive biochemical and structural characterization of CAS proteins. Recent bioinformatic analyses suggest that the CRISPR and CAS proteins might use several dissimilar mechanisms to abrogate phage infection and that at least one of these mechanisms could resemble eukaryotic RNA interference (3, 17). This study shows that SSO1404 and other CAS2 proteins represent a novel family of endoribonucleases, which possess a ferredoxin-like fold and are specific to ssRNA substrates. SSO1404 cleaves ssRNAs preferentially within U-rich regions and, in this respect, resembles the phage abortive infection endoribonuclease AbiB from _L. lactis_ (41). A similar mechanism of selective degradation of phage transcripts might be proposed for SSO1404 and other CAS2 proteins as well. Under this hypothesis, CAS2 would be the functional analog of the eukaryotic slicer nuclease, a function that is performed by the PIWI domains that are unrelated to CAS2 (59). Considering the apparent mechanism of CRISPR-associated anti-phage defense that involves integration of sequences homologous to fragments of phage mRNAs into the CRISPR loci, the CAS2 endoribonuclease activity might contribute to this process as well. Another possible role of CAS2 proteins in anti-phage defense might be associated with the global inhibition of translation by mRNA cleavage, a mechanism that has been proposed for RelBE and several other TA systems (MazEF, PemIK, and ChpBIK) that contain RNase components (mRNA interferases) (45, 60, 61). These TA systems play important roles in stress response to nutritional limitations or DNA damage, and their expression results in growth arrest or programmed cell death (43–45). Moreover, it has been shown that the _E. coli_ MazEF TA module prevents multiplication of the phage P1 by promoting cell death (62). A similar mechanism of anti-phage response appears to be a possibility.
for CAS2, especially in light of the previously described relationship with VapD, an uncharacterized protein that is functionally linked to the VapBC toxin-antitoxin system (17). S. solfatarius and many other CRISPR-containing organisms have at least two CAS2 proteins that might have different sequence specificities and could target distinct sets of mRNAs.

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