The Sso7d DNA-binding protein from *Sulfolobus solfataricus* has ribonuclease activity

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Abstract Sso7d is a small, basic, abundant protein from the thermoacidophilic archaeon *Sulfolobus solfataricus*. Previous research has shown that Sso7d can bind double-stranded DNA without sequence specificity by placing its triple-stranded β-sheet across the minor groove. We previously found RNase activity both in preparations of Sso7d purified from its natural source and in recombinant, purified protein expressed in *Escherichia coli*. This paper provides conclusive evidence that supports the assignment of RNase activity to Sso7d, shown by the total absence of activity in the single-point mutants E35L and K12L, despite the preservation of their overall structure under the assay conditions. In keeping with our observation that the residues putatively involved in RNase activity and those playing a role in DNA binding are located on different surfaces of the molecule, the activity was not impaired in the presence of DNA. If a small synthetic RNA was used as a substrate, Sso7d attacked both predicted double- and single-stranded RNA stretches, with no evident preference for specific sequences or individual bases. Apparently, the more readily attacked bonds were those intrinsically more unstable.

Key words: Sso7d; Ribonuclease; DNA-binding protein; Archaeon; *Sulfolobus solfataricus*

1. Introduction

The archaea *Sulfolobus solfataricus* and *S. acidocaldarius* express large amounts of several small, basic DNA-binding proteins, whose molecular masses lie in the range 7–10 kDa [1–3]. The 7-kDa class includes several members, mainly from *S. acidocaldarius*, which are referred to as Sac7a, Sac7b, Sac7c, Sac7d, Sac7e, in increasing order of basicity [2]. In *S. solfataricus*, only one further member was found, referred to as Sso7d [3]. Proteins belonging to the 7-kDa class are extremely similar to one another, with at least 70% identical residues, the most significant differences being restricted to the C-termini [2]. The structure of Sso7d from its natural source was solved by Baumann and coworkers [4] using nuclear magnetic resonance (NMR). They showed that the protein is folded into a compact globular unit consisting of a double-stranded antiparallel β-sheet onto which an orthogonal triple-stranded antiparallel β-sheet is packed, and of a small helical stretch at the C-terminus. Furthermore, the protein displayed a very compact hydrophobic core consisting of side chains at the interface of the two β-sheets, in particular the aromatics Phe5, Phe31 and Tyr33.

In recent years, the structures of Sso7d and Sac7d have been solved in complex with the nucleic acid, either by NMR spectroscopy [5] or by X-ray crystallography [6,7]. These studies showed that the Sso7d and Sac7d protein–DNA complexes are similar to each other in terms of both DNA-binding topology and conformational changes in DNA [5]. Previous studies also showed that Sso7d and Sac7d are able to increase the melting temperature of dsDNA by as much as 40°C [8]. Interestingly, Sso7d is also able to promote the annealing of complementary DNA strands above the melting point of the duplex [9]. Based on these properties, Sso7d and Sac7d are regarded as archaebacterial histones.

In recent years, we isolated and characterized RNases from *S. solfataricus*. We found three such enzymes, referred to as P1, P2 and P3, according to their elution order from a Mono-S column [10]. Surprisingly, P2 proved to be the same molecule as Sso7d. Thus, we recently adopted this latter denomination [11] to conform to the more generally accepted nomenclature. The recombinant protein expressed in *Escherichia coli* also had RNase activity, with a digestion pattern of commercially available tRNAs indistinguishable from that of the natural form [12]. Interestingly, Kulms et al. [13,14] showed that both natural and recombinant Sac7e (formerly referred to as SaRD) also have RNase activity. In spite of these findings, the current evidence in support of the assignment of RNase activity to Sso7d is still regarded as non-conclusive in the relevant literature [7–9,15,16]. Furthermore, Opperman et al. [17] accounted for the enzyme activity found in our Sso7d preparations in terms of RNase A contaminations deriving from the growth medium. In addition, a recent paper reports that chemically synthesized Sso7d was devoid of RNase activity [18]. However, the authors apparently did not make any attempt to promote a proper folding of the protein. Even more intriguingly, Guagliardi et al. [19] also found recently that Sso7d is able to rescue aggregated proteins in an ATP hydro-

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Abbreviations: NOE, nuclear Overhauser enhancement; PAGE, polyacrylamide gel electrophoresis

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lysis-dependent manner, although the residues involved have still to be identified. It is therefore essential to conclusively establish whether it is also endowed with RNase activity, in order to understand the biological role of this small multifunctional protein.

This paper shows that RNase activity is an intrinsic property of Sso7d, as supported by the fact that it was completely abolished by specific single-point mutations, which however did not compromise protein stability. Our results also provide an initial understanding of the catalytic mechanism, although its details have still to be defined.

2. Materials and methods

2.1. Cloning of the Sso7d-encoding gene and production of mutants

Chemical synthesis and expression of a synthetic wild-type Sso7d-encoding gene (*svd*) in *E. coli* was previously reported [12]. The mutant F31A gene and its expression in *E. coli* was obtained by cassette mutagenesis as reported [20]. The same procedure was adopted to produce and express single-point (K12L, K12R, E35L, E35Q) mutant genes, using suitable oligonucleotides. Mutant genes were sequenced, excised with *Hin*dIII and *Xba*I, and transferred into the pT7-7 expression vector.

2.2. Purification of Sso7d and its mutants

E. coli strain BL21(DE3)[pLysE] was transformed with pT7-7 derivative plasmids carrying the wild-type or the different *svd* mutant genes. The recombinant strains were then grown and the expression of the cloned gene induced by isopropyl-β-D-thiogalactopyranoside [12]. Proteins were then purified to electrophoretic homogeneity according to the previously reported procedure [20]. Pure proteins were dissolved in water at a concentration of 10 μg/ml and stored at −20°C. Under these conditions they were indefinitely stable.

2.3. RNase activity assay

RNase activity of wild-type and mutant Sso7d was assayed as previously reported [10] using γ-^{32}P 5′-end-labeled tRNA^{fMet} as a substrate and detecting the cleavage products by denaturing electrophoresis. Apparent K_m values were determined using a modification of a previously reported RNase assay [10]. Briefly, proteins (10 μg/ml) were incubated for suitable times at 60°C in 250-μl mixtures containing 40 mM sodium phosphate, pH 7.2 and variable amounts of baker's yeast tRNA (Roche Diagnostics, Mannheim, Germany), either at 60°C (Sso7d and E35L mutant) or at 37°C (horse heart cytochrome *c* from Roche Diagnostics). The reaction was stopped and the extent of digestion quantified in the supernatants after lanthanum nitrate–perchloric acid precipitation [10]. 1 U enzyme activity is defined as the amount of enzyme that causes an increase in A_{260} of 1 min^{-1} under the adopted conditions. 7 mM diethyl pyrocarbonate (Sigma, St. Louis, MO, USA) was added to all the solutions used for either assay.

2.4. Analysis of cleavage products of CI_{83} RNA incubated in the presence of Sso7d

The specificity of Sso7d was investigated using as a substrate an 83 nt long molecule (CI_{83} RNA), obtained by in vitro T7 RNA polymerase transcription of *Taq*I-digested plasmid pGM680. This plasmid is a pGEM-3Z (Promega, Madison, WI, USA) derivative in which the *cI* gene of phage plasmid P4 (P4 coordinates 8501–8418; GenBank accession number X51522) was cloned immediately downstream of the T7 promoter in such a way that the T7 RNA polymerase transcription start site corresponds to the +1 base of the mature P4 CI RNA. Transcription with T7 polymerase of *Taq*I-digested pGM680 template generates an RNA molecule (CI_{83} RNA) 3 nt longer than the P4 CI RNA [21]. In vitro transcription and γ-^{32}P 5′-end labeling of CI_{83} RNA were carried out using the 'Riboprobe Combination System-Sp6/T7' kit from Promega (Madison, WI, USA). 20 μl of reaction mixture contained 20 μCi of [γ-^{32}P]GTP (20 Ci/mmol) from Amersham Pharmacia (Little Chalfont, UK), 10 nmol each ATP, CTP and UTP, 1 nmol GTP and 0.2 μg template DNA. Radioactivity incorporation, estimated by Cerenkov counting after cold trichloroacetic acid precipitation, was about 1%. CI_{83} RNA was then dialyzed against 10 mM Tris–HCl, pH 7.4, 1 mM EDTA, using a Millipore (Bedford, MA, USA) nitrocellulose membrane filter (pore size 0.025 μm). CI RNA digestion by Sso7d was performed at 60°C in 40 mM potassium phosphate, pH 7.2, in a final volume of 12 μl. After incubation, 3-μl samples were withdrawn and heated at 100°C for 2 min and an equal volume of stop solution (0.3% each bromophenol blue and xylene cyanol; 10 mM EDTA, pH 7.5; 97.5% deionized formamide) was added. Cleavage products were then resolved in polyacrylamide gel electrophoresis (PAGE) carried out in the presence of 7 M urea, using a 10% total gel concentration and a 5% concentration of cross-linker, in 0.1 M Tris–borate, pH 8.4, 2 mM EDTA. Radioactive fragments were detected autoradiographically. Cleavage sites were identified by comparing the digestion pattern to that of a partial alkaline digestion of γ-^{32}P-labeled CI RNA, carried out by incubating 10 ng CI RNA at 90°C in 50 mM sodium carbonate, pH 9.5, in a final volume of 20 μl, and/or by comparison with sequencing reaction products run in the same gel.

2.5. NMR spectroscopy

^{1}H NMR spectra of proteins were collected on an 11.7 T Bruker Advance DMX spectrometer equipped with an SGI INDY computer and a z gradient coil with a proton frequency of 500 MHz. Spectra were recorded with a spectral width of 8096 Hz and 32K data points, at pH 4.5 in H_2O/D_2O (90:10) and referenced to sodium trimethylsilyl[2,2,3,3-$^2H_4$]propionate. Solvent suppression was achieved by presaturating the water resonance. In thermal transition experiments, the temperature range of 300–370 K was explored, keeping the average of the temperature rate change at 5 K/h to ensure thermal equilibrium. The curve fitting of chemical shifts was performed with a logistic function consisting of four standard parameters.

3. Results

This research was performed to obtain conclusive evidence to support or refute the assignment of intrinsic RNase activity to the Sso7d protein. A clear-cut assignment of such a catalytic function might stem from site-directed mutagenesis, in the event single-point mutations would completely abolish or, at least, substantially reduce it. These investigations may also provide insights into the catalytic mechanism of the protein. Based on this rationale, we searched for residues possibly involved in RNase activity. Our choice was oriented by our previous study in which Sso7d was characterized by means of

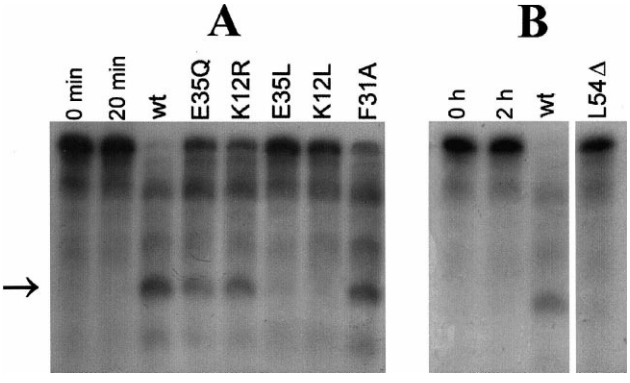

Fig. 1. Hydrolysis of tRNA^{fMet} from *E. coli* by wild-type and mutant Sso7d proteins from *S. solfataricus*. Radioactively 5′-end-labeled RNA (5 μg) was incubated at 60°C for 20 min in 0.1 M potassium phosphate, pH 7.2, in a final volume of 15 μl, in the presence of 3.5 μg of either wild-type (wt) or mutant Sso7d protein, as indicated. The reaction was then stopped and the protein digested by addition of pronase. Cleavage products were resolved by urea/PAGE and detected autoradiographically. 0 min: RNA before incubation; 20 min: RNA incubated for 20 min in the absence of any added protein. The major degradation product of tRNA is indicated by an arrow.

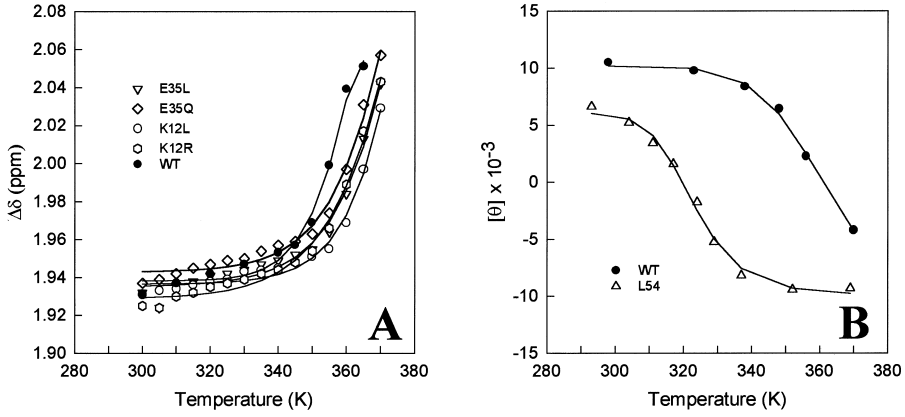

Fig. 2. NMR thermal denaturation profiles of wild-type Sso7d and of some mutants. Profiles were determined by monitoring changes in ^{1}H chemical shifts of the methyl resonances of Met57.

^{1}H NMR and photochemically induced dynamic nuclear polarization spectroscopies [22]. This investigation showed that a photoexcitable flavin dye, whose structure resembles a nucleic acid base, was able to interact with the aromatic cluster via ring-stacking interactions, Phe31 being the most strongly interacting residue. These results suggested, therefore, that a nucleotide involved in the phosphodiester bond to be cleaved could bind to the aromatic cluster. Furthermore, the refined NMR structure of the recombinant Sso7d protein (1JIC) also showed that the residues Lys12 and Glu35 are close to the cluster, exhibit several nuclear Overhauser enhancement (NOE) contacts with it, and, like the cluster itself, are enclosed between the two β-sheets. In particular, the side chain of Glu35 is close to that of Tyr33 and NOEs between the two residues have been detected. Moreover, the chemical shift titration curves of the ionizable groups of Sso7d indicated that Glu35 has an abnormally high pK_a value (greater than 6), and the Glu35 titration can be monitored in the chemical shift changes of Tyr33 ε protons (R. Consonni, unpublished observations). These features suggest a possible involvement of Glu35 and the neighboring Lys12 in catalysis. We therefore produced the mutants K12L, K12R, E35L, and E35Q and assayed their RNase activity towards a commercially available $tRNA^{fMet}$ from *E. coli*. To detect the digestion pattern, the substrate was 5′-end-labeled with [γ-^{32}P]ATP prior to incubation with the individual proteins. Incubation mixtures were then resolved by denaturing electrophoresis and the labeled fragments were detected autoradiographically. Prior to incubation with Sso7d or its mutants, tRNA displayed both the major band and a fainter, faster migrating form (Fig. 1), quite likely a degradation product. After a 20-min incubation at 60°C in the absence of any protein, no further degradation of tRNA could be detected (Fig. 1). In contrast, 20-min incubation of the nucleic acid with wild-type Sso7d resulted in a complete digestion of the major band and the appearance of several fragments. However, no degradation was seen when the mutants K12L or E35L were used, even up to a protein/nucleic acid stoichiometric ratio of approximately 2 (Fig. 1). In particular, a clear-cut indicator of the occurrence of RNase activity, i.e. the major degradation product (indicated by an arrow), was completely absent under these conditions. This conclusively demonstrates that the Sso7d protein is intrinsically endowed with RNase activity. The mutants K12R and E35Q still exhibited catalytic activity, although to a much lesser extent than the wild type (Fig. 1). We also assayed RNase activity of the previously produced mutant F31A to assess the effect of this mutation on protein stability [20]: in keeping with our hypothesis that Phe31 is involved in RNA binding, the ability of this mutant to degrade RNA was significantly impaired but not abolished (Fig. 1).

Thermal denaturation profiles of the mutants K12L, K12R, E35L, and E35Q were determined in NMR by monitoring changes in ^{1}H chemical shifts of the methyl resonances of Met57 (Fig. 2). The profiles show that none of these mutations decreased thermal stability, but all proteins were stable up to at least 360 K. This implies that the effect of the mutations on RNase activity does not result from loss of the overall native conformation. Thus, the residues Lys12 and Glu35

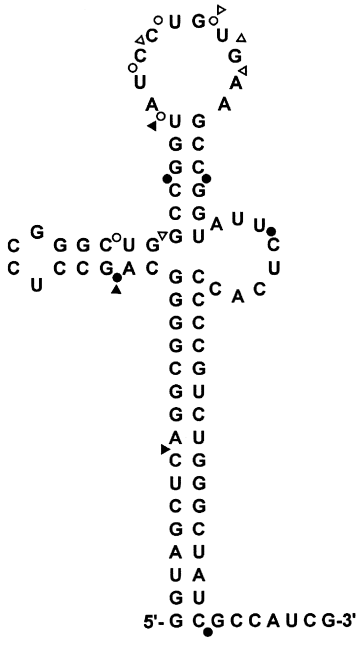

Fig. 3. Primary Sso7d cleavage sites in CI_{83} RNA. The secondary structure of CI_{83} RNA was predicted by MFOLD analysis (CGC software package, see [33]). Triangles and circles indicate Sso7d cleavage sites identified by primer extension and analysis of γ-^{32}P 5′-end-labeled CI_{83} RNA fragments, respectively. Closed symbols identify major cleavage sites, open symbols correspond to fainter signals detected by the above analyses.

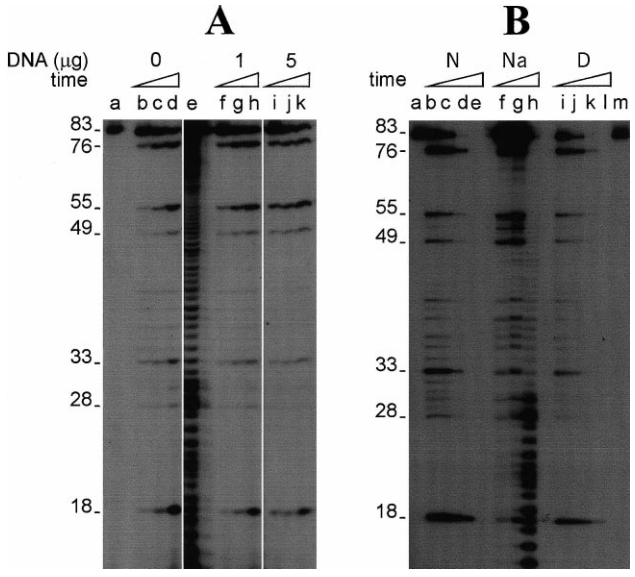

Fig. 4. Cleavage of CI_{83} RNA by Sso7d. γ-^{32}P 5′-end-labeled CI_{83} RNA (8 ng, 16 000 cpm) was incubated at 60°C in 40 mM potassium phosphate, pH 7.2, with Sso7d in a final volume of 12 μl. At different times after incubation, 3-μl samples were withdrawn, heated at 100°C for 2 min and quickly chilled in ice. The cleavage products were resolved on a sequencing urea/PAGE as described in Section 2 and the radioactive fragments were detected autoradiographically. The size of the fragments is indicated, in nucleotides, on the left of each panel. The increased incubation time (specified below) is indicated by a triangle above the lanes. A: CI_{83} RNA digestion was carried out in the presence of 0, 1, or 5 μg of *Hin*fI-digested *E. coli* DNA, as indicated on top. Samples were taken after 10, 20, and 40 min incubation with 1 μg of Sso7d. Lane a, γ-^{32}P 5′-end-labeled CI_{83} RNA (2 ng) incubated in the absence of Sso7d for 40 min; lane e, nucleotide ladder obtained by incubating 12 ng of γ-^{32}P 5′-end-labeled CI_{83} RNA in 50 mM sodium carbonate at 95°C for 6 min. The nucleotide ladder in the highest molecular weight range was read on less exposed autoradiographs and was better resolved by longer runs of electrophoresis. B: Cleavage reaction was performed either with non-denatured RNA substrate (N) or after heating CI_{83} RNA 10 min at 100°C (D) and quick chilling in ice. Samples were taken after 5, 10, 20, and 40 min incubation with 2 μg of Sso7d. Non-denatured and denatured γ-^{32}P 5′-end-labeled CI_{83} RNA (2 ng) incubated in the absence of Sso7d for 40 min were loaded in lanes a and m, respectively. Lanes f, g, h: γ-^{32}P-labeled CI_{83} RNA incubated in 50 mM sodium carbonate at 95°C for 3, 4 and 6 min, respectively.

must be directly involved in the catalytic mechanism. In contrast, the mutation F31A led to a dramatic destabilization, as previously reported [20]; in spite of this, the mutant was mostly in the native conformation at 60°C. So the reduced rate of RNA hydrolysis by this mutant may only marginally be accounted for by its loss of stability. Consistent with this idea, the same mutant also displayed a much lower rate of hydrolysis at 37°C as compared to the wild type (P. Fusi, unpublished observations).

To gain better insight into the specificity of RNA cleavage by Sso7d, we used the small CI_{83} RNA (Fig. 3) as a model substrate. This molecule, produced in vitro (see Section 2), is 3 nt longer at the 3′-end than the natural CI RNA encoded by coliphage P4 (for a review see [23]). In vivo CI RNA is very stable, in agreement with its predicted structure [21,24].

In preliminary experiments, we digested CI_{83} RNA with increasing amounts of purified Sso7d for a constant time and analyzed the digestion products by Northern blot hybridization. In this way we could detect discrete CI_{83} RNA digestion fragments that disappeared at high enzyme concentration, suggesting that the enzyme would first cut CI_{83} RNA endonucleolytically at few preferential sites and then degrade the substrate to oligonucleotides. We then identified the preferential cut sites both by primer extension using a primer complementary to the 3′-end of CI_{83} RNA (S. Serina and G. Dehò, unpublished observations) and by electrophoretic analysis of the digestion products of a 5′-end-labeled CI_{83} RNA substrate. A drawback of the former approach is that cleavage sites within or proximal to the primer cannot be detected.

Fig. 4 illustrates results from a typical experiment using the latter approach. γ-^{32}P 5′-end-labeled CI_{83} RNA was incubated for increasing time periods in the presence of 1 μg (panel A) or 2 μg (panel B) of Sso7d, the products were resolved by PAGE under denaturing conditions and the 5′-labeled fragments visualized by autoradiography. The length of the discrete fragments obtained by digestion was determined by comparison with a nucleotide ladder obtained by alkaline digestion of γ-^{32}P 5′-end-labeled CI_{83} RNA. The data clearly show that the decay of CI_{83} RNA initiates at a few preferential cut sites, summarized in Fig. 3.

Since Sso7d is also a DNA-binding protein [3,5,7], we tested whether DNA would somehow affect the RNase activity of Sso7d by adding *Hin*fI-digested *E. coli* DNA to the reaction mix. On assuming that the DNA–protein dissociation constant is not higher than 10 μM [4] and that a binding site is 8 bp long [7], 1 μg of Sso7d (in 12 μl) would be saturated by about 3 μg of DNA. As shown in Fig. 4A (central and right panels), saturating amounts of DNA did not detectably affect the digestion pattern of CI_{83} RNA by Sso7d. Moreover, the CI_{83} RNA digestion pattern was indistinguishable when either native or heat-denatured RNA was provided (Fig. 4B).

Analysis of preferential cut sites (Fig. 3) did not reveal any striking sequence or structure specificity. It should be noted, however, that spontaneous degradation of CI_{83} RNA upon incubation at 90°C in sodium carbonate, pH 9.5, yielded frag-

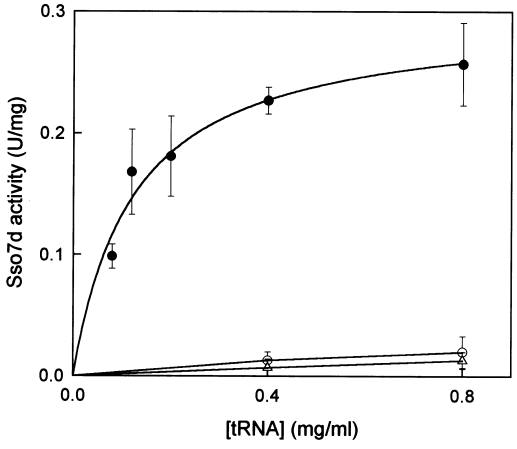

Fig. 5. Dependence of Sso7d RNase activity on tRNA concentration. Proteins (10 μg/ml) were incubated in 250-μl mixtures containing 40 mM sodium phosphate, pH 7.2, and the indicated amounts of baker's yeast tRNA, either at 60°C (Sso7d and E35L mutant) or at 37°C (cytochrome *c*). Activity was determined by measuring A_{260} after lanthanum nitrate–perchloric acid precipitation. Each point represents the mean ± S.E.M. of at least five independent determinations. ●: wild-type Sso7d; ○: E35L mutant; △: horse heart cytochrome *c*. For other details see Section 2.

ments of the same length as those obtained by Sso7d digestion (Fig. 4B), thus suggesting that Sso7d preferential cuts may occur at intrinsically fragile sites in RNA. It should also be noted that in this experiment all signals completely disappeared after a 40-min incubation with 2 μg of enzyme, suggesting a complete degradation of CI_{83} RNA to the level of mononucleotides or short oligonucleotides.

Apparent kinetic parameters were also determined using commercially available baker's yeast tRNA as a substrate. Besides Sso7d, its mutant E35L and cytochrome *c* were also used as negative controls and did not show any significant activity. Sso7d showed a typical saturation profile, with a K_m of 0.12 mg/ml (Fig. 5), corresponding to about 5 μM. Although this figure cannot be regarded as a true K_m value, because of substrate heterogeneity and also the presence of multiple cleavage sites on each tRNA molecule, it is representative of the concentration range in which this protein can effectively cleave RNA substrates.

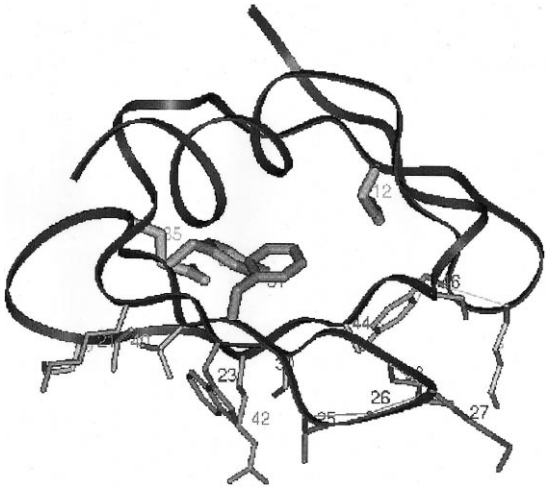

Fig. 6. Ribbon representation of Sso7d protein showing the residues putatively involved in either DNA binding (line style residues) or RNase activity (render style residues).

4. Discussion

The data presented in this paper provide conclusive evidence in support of the assignment of RNase activity to the Sso7d DNA-binding protein. This is mainly substantiated by the complete loss of catalytic activity resulting from the single-point mutations K12L and E35L (Fig. 1). However, one major issue raised by our findings is whether the activity detected in Sso7d is a true catalytic activity or an unspecific one, just resulting from the basic properties of this protein. Several lines of evidence substantiate the idea that the RNase activity found in Sso7d has a physiological role. First, some single-point mutants, albeit more basic than the wild type, are either completely inactive (E35L) or distinctly less active than the wild type (E35Q). Second, the apparent K_m values we determined in this work, as well as other kinetic properties we previously characterized, are quite compatible with a role in its physiological environment: in particular the enzyme showed pH–activity profiles with an optimum in the range 6.7–7.6 and thermostability up to about 85°C [10]. Third, the intracellular amounts of Sso7d in vivo are high enough to justify the rapid hydrolysis of potential RNA substrates, despite the rather low catalytic activity of the archaebacterial RNase compared to other well-known RNases. Actually, Sso7d represents about 4–6% of the total protein content in a *S. solfataricus* crude extract, which corresponds to an intracellular concentration of at least 1 mM. Nevertheless, we found that this protein readily cleaved different RNA molecules at concentrations as low as 30 μM and 60°C.

Thus, Sso7d proves to be a singular protein in several respects: despite its very small size it is a multifunctional molecule, it contains no histidines (unlike most known RNases) and, in addition, to the best of our knowledge it is the smallest enzyme identified so far. Our interest in this protein is also justified by the fact that, to date, very little is known about RNases from archaebacterial sources, the only one extensively characterized being RNase P [25]. Recently, an RNase E-like activity was also observed in the extreme halophile *Haloarcula marismortui* [26]. However, both of these RNases are completely unrelated to Sso7d.

It is interesting to note that the residues putatively involved in RNase activity and those playing a role in DNA binding represent two completely distinct subsets that are found on different surfaces of the molecule (Fig. 6). The former are either enclosed between the two β-sheets or located on the helix; the latter are mostly on the external side of the triple-stranded β-sheet. This corresponds to our observation that the catalytic activity is not affected by the presence of dsDNA (Fig. 4A) and further substantiates our tentative identification of the residues involved in RNase activity.

In the major RNases, general acid–base catalysis is frequently accomplished by two histidine residues [27]. In many eubacterial RNases, one of them may be replaced by an acidic residue [28,29]. However, the lack of any histidine in the protein Sso7d is rather surprising and raises a major issue regarding its precise catalytic mechanism. Whereas one might plausibly assume that Glu35 plays a role in general acid–base catalysis, it is highly unlikely that the same holds true for Lys12, given its high pK_a value. A more reasonable assumption is that the latter residue favors the formation of the reaction intermediates via ionic interactions, i.e. the pentavalent adduct and the subsequent 2′,3′-cyclic phosphodiester, similar to Lys41 in RNase A [27]. Our previous studies showed that Sso7d can hydrolyze 2′,3′-cyclic nucleotides [30]; so, these forms should represent reaction intermediates in the archaebacterial enzyme as well. In any case, if our assumptions prove true, an additional residue directly involved in the acid–base catalysis has still to be identified.

As regards the rules governing Sso7d specificity, we found that the protein attacked CI_{83} RNA both in double- and in single-stranded stretches, with no evident preference for defined sequences or individual bases (Fig. 3). Instead, our results indicate that the intrinsically more unstable bonds are more readily attacked, as shown by the comparison of the enzymatic digestion and spontaneous alkaline hydrolysis. Our results also show that, after prolonged incubation, Sso7d extensively degraded the RNA substrate (Fig. 4B), probably to short oligonucleotides or mononucleotides. Our observations raise the issue of whether Sso7d exhibits any specificity in vivo. Although we do not have any definite answer at present, it should be pointed out that RNAs from *S. solfataricus* have been reported to have exceptionally high modification levels: for instance, in both 16S and 23S rRNAs several residues are methylated at *O*-2′ of ribose, a modifica-

tion that enhances the stability of the polynucleotide chain at high temperatures [31]. These modifications may therefore lead to a more restricted in vivo specificity of Sso7d.

Investigations on proteins belonging to the Sso and Sac families also demonstrate that their amino acid sequences do not show any apparent relationship to any other known protein. We only found some structural resemblance with a class of RNA-binding proteins (such as U1A) in which side chains of conserved aromatics located on a β-sheet surface interact with nucleic acid bases [22,32]. However, sequence analysis strongly suggests that Sso and Sac proteins have evolved both their DNA-binding capacity and, when present, RNase activity, in a completely independent manner. This might reflect the peculiar evolutionary pathways of archaea and/or the requirement of different patterns of high temperature RNA turnover and processing with respect to those found in mesophilic organisms. Our studies on the protein Sso7d might help clarify the pathways of RNA turnover in archaea and thermophilic bacteria. In particular, work is in progress in our laboratories to fully elucidate the cleavage specificity of Sso7d towards endogenous substrates, as well as its precise catalytic mechanism.

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