Dehydrogenases from All Three Domains of Life Cleave RNA*

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Specific interactions of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) with RNA have been reported both in vitro and in vivo. We show that eukaryotic and bacterial GAPDH and two proteins from the hyperthermophilic archaean Sulfolobus solfataricus, which are annotated as dehydrogenases, cleave RNA producing similar degradation patterns. RNA cleavage is most efficient at 60 °C, at MgCl₂ concentrations up to 5 mM, and takes place between pyrimidine and adenosine. The RNase active center of the putative aspartate semialdehyde dehydrogenase from S. solfataricus is located within the N-terminal 73 amino acids, which comprise the first mononucleotide-binding site of the predicted Rossmann fold. Thus, RNA cleavage has to be taken into account in the ongoing discussion of the possible biological function of RNA binding by dehydrogenases.

It is known that dehydrogenases and other metabolic enzymes can bind RNA. In numerous studies on RNA binding, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was identified as the major RNA-binding protein. It was shown that human and/or rabbit muscle GAPDH selectively binds tRNA (1), AU-rich sequences at the 3′-untranslated region conferring instability of the corresponding mRNAs (2), hammerhead ribozyme (3), and viral cis-acting regulatory RNA elements (4–7). The selectivity of GAPDH binding to RNA was demonstrated in vitro and in vivo (4, 8, 9). Furthermore, one of the proteins that specifically binds to a small stable RNA from Mycoplasma capricolum was identified as bacterial GAPDH (10). On the other hand, human GAPDH was also identified as a major protein that binds to single-stranded DNA (ssDNA) and oligodeoxynucleotides containing the TAAAT motif (11, 12).

Recently it was proposed that the NAD-binding structure (Rossmann fold) of GAPDH represents a novel RNA binding domain that provides a molecular basis for RNA recognition by dehydrogenases and other metabolic enzymes (13). Baker et al. (14) provided another line of evidence for the relationship between dehydrogenases and enzymes interacting with RNA. They revealed that spinach CSP41, an mRNA-binding protein and ribonuclease (15), is homologous to nucleotide-sugar epimerases and hydroxysteroid dehydrogenases and proposed that these enzymes share a common ancestor.

We are interested in the identification of archaeal endoribonucleases and therefore monitored protein fractions from the hyperthermophilic archaean Sulfolobus solfataricus using RNase activity assays. Two major proteins were copurified in a cell fraction with RNase activity. Surprisingly, they were identified as hypothetical dehydrogenases. During their biochemical characterization as endonucleases, we found that eukaryotic and bacterial GAPDH enzymes also possess RNase activity. Our results show for the first time that the key glycolytic enzyme GAPDH, which possesses RNA-binding capability, also acts as RNase. The finding that two archaeal proteins annotated as dehydrogenases, as well as eukaryotic and bacterial GAPDH, efficiently cleave RNA suggests that dehydrogenases and dehydrogenase-related proteins of all three life domains may be involved in RNA turnover.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Culture Conditions—S. solfataricus strain P2 was maintained in 100 ml of liquid cultures at 75 °C. Per 1 liter of culture medium we added 1 g of yeast extract, 1 g of casamino acids, 3.1 g of KH₂PO₄, 2.5 g of (NH₄)₂SO₄, 0.2 g of MgSO₄ × 7H₂O, 0.25 g of CaCl₂ × 2H₂O, 0.1 ml of the following solutions: 1.8% MnCl₂ × 4H₂O and 4.5% Na₂B₄O₇ × 10H₂O, and 10 µl of each of the following solutions: 2.2% ZnSO₄ × 7H₂O, 0.5% CuCl₂ × 2H₂O, 0.3% NaMoO₄ × 2H₂O, 0.15% CoCl₂ × 6H₂O. The medium was adjusted to a pH of 4.2–4.4 and autoclaved, and 10 µl of sterile filtered 0.3% VOSO₄ × 2H₂O per 1 liter was added. In a fermenter, 10 liters cultures were grown for 5 days at 70 °C, pH of 4.2–4.4, and an air supply of 10 liters min⁻¹. Escherichia coli M15 (REP4) cells were grown on standard I medium (Difco) at ampicillin and kanamycin concentrations as specified by Qiagen (Qiagen).

Partial Purification and Identification of the S. solfataricus Asd-1 and Acd-5 Proteins—S. solfataricus cells (60 g) were resuspended in extraction buffer (16) and sonified. The cytoplasmatic fraction was precipitated with ammonium sulfate and dialyzed against TEG buffer (40 mM Tris, pH 8.0, 0.1 mM EDTA, 5% glycerol). The chromatography steps were performed using the fast protein liquid chromatography System (Amersham Biosciences). The protein fractions containing endoribonuclease activity were pooled and applied on the next column. The following columns were used: HiTrap heparin column (Amersham Biosciences), cationexchanger (BioRad Econo-Pac S-cartridge), anionexchanger (BioRad Econo-Pac Q-cartridge), HiLoad™ Superdex 200 size exclusion chromatography column (Amersham Biosciences). Then, the endonuclease containing pool was applied on preparative 8% native PAGE, 0.25-cm slices were cut, and the proteins recovered by diffusion. Two major proteins were detected in the fractions with the highest RNase activity. They were transferred to Immobilon-polyvinylidene difluoride membrane (Millipore) and sequenced by Edman degradation in a pulsed-liquid sequencer (Applied Biosystems, Inc., model 477A/120A).

Construction and Purification of Recombinant Proteins—For amplification of the S. solfataricus asd-1 gene and its parts (short N-terminal part, amino acids 2–73; N-terminal half, amino acids 2–205; C-terminal half, amino acids 206–350), the following primers were used (restriction sites underlined): ssAsd-1se Bam (5′-GGGGATCCCGTATAAGATAAAAGTTTCG-3′) and ssAsd-1as Hind (5′-CCCAAGCTTTTAGAAATATTA-3′) this paper is available on line at http://www.jbc.org

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‡ The abbreviations used are: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ssDNA, single stranded DNA; BSA, bovine serum albumin; dsDNA, double stranded DNA.

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FIG. 1. Purification of \textit{S. solfataricus} proteins, which are annotated as dehydrogenases and show \textit{RNA} activity. \textit{A}, silver stained SDS-PAGE showing the fractions of \textit{S. solfataricus} proteins after native polyacrylamide electrophoresis. \textit{GF}, protein fraction showing specific \textit{RNA} activity after the size exclusion chromatography (gel filtration) purification step. \textit{Lanes 1–8}, slices from which the proteins separated by the native PAGE were recovered by diffusion. The relative cleavage activity with the N26 substrate is shown above the panel. The electrophoretic behavior of the protein marker is marked at the left side of the panel. The two arrows on the right side of the panel show the proteins identified by N-terminal sequencing. \textit{B}, silver stained SDS-PAGE showing the cloned, overexpressed, and purified \textit{S. solfataricus} proteins His\textsubscript{6}-Asd-1 (lane 1) and His\textsubscript{6}-Acd-5 (lane 5), \textit{M}, protein marker. \textit{C}, cleavage of the N26 transcript by different protein fractions: lane 1, His\textsubscript{6}-Acd-5; lane 2, His\textsubscript{6}-Asd-1; lanes 3 and 4, \textit{S. solfataricus} protein fraction after anion exchange chromatography; lane 5, \textit{RNA} III from \textit{E. coli}. \textit{Ø}, negative control, \textit{RNA} substrate incubated with buffer. The assays in the lanes 1–4 and the negative control were performed for 20 min at 70 °C, the \textit{RNA} III assay for 3 min at 37 °C. The presence or absence of \textit{MgCl\textsubscript{2}} is indicated above the panel.

CCCTAATCTTCT-3\textsuperscript{‘} for the full-length \textit{asd}-1, \textit{SsAsd}-1espère (5’-ACATGCATTCTGATAAGATAAAAGTTTC-3’ and \textit{SsAsd}-1/73asHind (5’-CCCAAGACTGTAAGTAGAGCTTACACT-3’) for the short N-terminal part, \textit{SsAsd}-1espère and \textit{SsAsd}-1/205asHind (5’-CCCAAGCTTTCCTTTTGAGACGCTT-3’) for the N-terminal half, and \textit{SsAsd}-1/206-6essePh (5’-ACATGTACAGTAGAAGAAGGTCTATTAAAGA-3’) and \textit{SsAsd}-1asHind (5’-CCCAAGCTTTCCTTTTGAGACGCTT-3’) were used for amplification of the \textit{S. solfataricus} asd-5 gene (\textit{Sph}1 and \textit{Hind}III restriction sites are underlined). As templates we used genomic DNA from \textit{S. solfataricus} P2 isolated by the method of Ausubel et al. (17). PCR was performed at an annealing temperature of 44 °C. The resulting PCR products were cloned into the pQE30 hexahistidine tag (His\textsubscript{6}) vector, and propagated in \textit{E. coli} M15 (REP4) cells. Overexpression at 37 °C was confirmed by N-terminal sequencing. The resulting \textit{PCR} products were cloned into the pQE30 hexahistidine tag (His\textsubscript{6}) vector, and propagated in \textit{E. coli} M15 (REP4) cells. Overexpression at 37 °C was confirmed by N-terminal sequencing. The purified proteins were analyzed by SDS-PAGE and silver staining.

\textbf{RESULTS}

\textit{In Vitro Transcription of RNAs and Enzymatic Assays—\textit{In vitro} transcription using \textit{T7} RNA polymerase and purification of 18-20PiUTP-labeled transcripts on denaturing gels were performed as described (18, 19). As templates for the synthesis of the N26 RNA we used an oligonucleotide with annealed 18-mer promoter oligonucleotide (20). Transcripts comprising parts of 23 S RNA of \textit{r-} to bacteria were generated directly from \textit{T7} promoter containing \textit{PCR} products corresponding to \textit{rDNA} positions from 109 to 205 (\textit{E. coli} numbering) (21). The assays were performed in a 10–1 µl reaction volume in TKG buffer (30 mM Tris, pH 7.5, 130 mM KCl, 5% glycerol). The amount of used substrate and enzyme, the addition of \textit{MgCl\textsubscript{2}}, nucleic acids, NADPH, NADH, ATP, or \textit{RNA} A inhibitor \textit{rNasIn} (Promega), the temperature conditions, and the used reaction time are indicated. Rabbit muscle \textit{GAPDH}, ovine serum albumin (BSA), eukaryotic lactate dehydrogen-ase, and \textit{RNA} A were purchased from Sigma. Highly purified \textit{E. coli} and \textit{Bacillus steaothermophilus} \textit{GAPDH} were obtained from S. Boschi-Muller from the laboratory of G. Branlant. Reaction products were heated with formaldehyde containing dye and analyzed on 10% polyacryl-amiurea gel. Bands were detected using a BioRad molecular imaging and the Quantity one (BioRad) software.

\textbf{Mapping of RNA 5’-Ends by Primer Extension Analysis—To determine the exact RNA cleavage sites used by \textit{GAPDH}, \textit{Acd-1}, \textit{Acd-5}, and \textit{RNA} A we performed primer extension analysis. After incubation of \textit{RNA} with the appropriate protein, the cleavage products were phenol-extracted, ethanol-purified, and treated as previously described (21, 22). Radioactively labeled sequencing reactions of the cloned DNA template were loaded on the same gel to map the position of the cleavage sites.

\textbf{Refolding of Proteins in Solution—After separation in SDS-PAGE, the gel was soaked in cold 2 mM KCl. A high amount of polypeptide was visible as a white band, which was excised and electroeluted using BioTrap equipment (Schleicher and Schuell). The polypeptides dissolved in denaturing buffer (20 mM Hepes, pH 7.5, 0.5 mM EDTA, 5 mM \textit{MgCl\textsubscript{2}}, 150 mM NaCl, 6 mM guanidine hydrochloride, 0.1% Nonidet P-40, 3 mM dithiothreitol) were dialyzed overnight at 20 °C against renaturing buffer (the same buffer without guanidine hydrochloride). Thereafter, dialysis against TKG buffer at 4 °C was performed.

\textbf{RESULTS}

\textbf{The Acd-1 and Acd-5 Proteins from \textit{S. solfataricus} Show Identical \textit{RNA} Activity—We are interested in the identification of archaeal endoribonucleases that specifically cleave double-stranded \textit{RNA}. To do this, we monitored protein fractions from the hyperthermophilic archaeon \textit{S. solfataricus} for such activity, using the well characterized small \textit{RNA} III substrate N26 derived from \textit{phage} T7 R1.1 (20). The N26 substrate is a double-stranded \textit{RNA} consisting of 46 bases. The assays were performed at 10 mM \textit{MgCl\textsubscript{2}} and different temperatures. An \textit{RNA} activity that cleaves the N26 transcript at two positions that differ from both \textit{RNA} III processing sites was detected in \textit{S. solfataricus} cell-free extracts. The resulting cleavage pattern was identical at low and high temperatures, and the reaction optimum was reached at 80 °C (not shown). This \textit{RNA} activity was partially purified. In native state the \textit{RNA} activity performed as a 90-kDa protein (not shown). The final protein fractions exhibiting the highest \textit{RNA} activity contained two major protein bands (Fig. 1A, lanes 5–7). They were identified as dehydragenases by N-terminal sequencing and data base search. The 50-kDa band yielded the sequence Ala-Asp-Lys-Ile-Lys-Val-Ser-Leu-Leu-Gly-Ser-Thr-Gly-Met-Val-Gly-Gln-Lys-Met-Val, which matches the hypothetical aspartate semialdehyde dehydragenase (NCBI accession number AAK41162, coded by asd-1), a protein consisting of 349 amino acids and...
in the catalytic site (Cys-135, Gln-162, Arg-267, and His-274) of Acd-5 proteins from dehydrogenases (14). This prompted us to clone the Asd-1 and binding and cleaving enzyme from spinach shows homology to eukaryotic GAPDH can bind RNA (2, 3) and that an RNA Asp, which identifies it as acyl-CoA dehydrogenase (NCBI ac-

Pro-Phe-Lys-Ser-Leu-Glu-Asp-Phe-Lys-Val-Glu-Ile-Thr-Gln-

deptide was observed in reaction mixtures, which always con-
tained 10 mM MgCl₂ and were incubated at various tempera-
tures ranging from 37 °C to 80 °C (not shown). Interestingly, they showed identical cleavage pattern in absence of MgCl₂.
The generated cleavage pattern was different from those pro-
duced by the original S. solfataricus protein fractions at 10 mM MgCl₂ or by E. coli RNase III (Fig. 1C). The original S. solfa-
taricus fractions were also able to cleave N26 in absence of MgCl₂ generating a degradation pattern identical to that pro-
duced by the His₆-tagged proteins under the same reaction condi-
tions (Fig. 1C). This result demonstrates that the RNase activities, which cleave N26 without addition of MgCl₂, were present in the S. solfataricus protein fractions and are not artifacts due to the purification of the overexpressed proteins from E. coli.

In the absence of MgCl₂, the two His₆-tagged proteins and the original S. solfataricus protein fractions degraded different transcripts derived from 23 S rRNA of α-proteobacteria, generating identical cleavage patterns with temperature optimum of 60 °C (Fig. 2A and B). The 23 S rRNA-derived transcripts are highly structured, double-stranded RNAs (21). Interest-

ingly, the original protein fractions as well as the cloned pro-
teins could not cleave these transcripts endonucleolytically at 10 mM MgCl₂ (Fig. 2B). In contrast, the N26 transcript was specifically cleaved at 10 mM MgCl₂ by the S. solfataricus protein fractions (Fig. 1C) with temperature optimum of 80 °C (not shown). These observations suggest that distinct RNase activities were copurified in the protein fractions from S. sol-

with aberrant migration in SDS-PAGE. It shows strong homol-
y dehydrogenase as well as significant similarity at the amino acid level to the corresponding experimentally characterized eu-
karyotic dehydrogenases (52% similarity to the yeast en-
zyme and 41% to that of Arabidopsis thaliana) (23, 24). Addi-
tionally, the S. solfataricus Asd-1 protein possesses the impor-
tant residues involved in the dinucleotide binding site (Gly-
Xaa-Xaa-Gly-Xaa-Xaa-Gly, where Xaa is any amino acid) and in the catalytic site (Cys-135, Gln-162, Arg-267, and His-274) of the E. coli enzyme, which are conserved also in A. thaliana (24, 25).
The 44-kDa band showed the N-terminal sequence Val-Phe-
Pro-Phe-Lys-Ser-Leu-Glu-Asp-Phe-Lys-Val-Glu-Ile-Thr-Gln-
Asp, which identifies it as acyl-CoA dehydrogenase (NCBI ac-
cession number AAK42872, coded by acd-5), a protein of 397
amino acids. It shows homology to acyl-CoA dehydrogenases
from all three life domains.

It is known that different dehydrogenases and especially eu-
karyotic GAPDH can bind RNA (2, 3) and that an RNA
binding and cleaving enzyme from spinach shows homology to
dehydrogenases (14). This prompted us to clone the Asd-1 and Acd-5 proteins from S. solfataricus to test their capability to
degrade RNA. They were overexpressed and purified from E. coli as His₆-tagged proteins (Fig. 1B). No cleavage of the N26 transcript by either protein or by a combination of both pro-
teins was observed in reaction mixtures, which always con-

FIG. 3. The RNase activity is an intrinsic feature of GAPDH. A, cleavage of a transcript (20 nM) derived from the 23 S rRNA of R. leguminosarum by rabbit muscle GAPDH (2 nM of monomer). All assays were performed for 10 min at 37 °C. Lane 1, negative control; lane 2; substrate incubated with enzyme without competitor; in the lanes 3–7 the following substrate competitors were added: lane 3, tRNA; lane 4, ssDNA; lane 5, dsDNA (50 ng μl⁻¹ of each substance); lane 6, 0.5 mM ATP; lane 7, 2 mM ATP; lane 8, RNasin added to the reaction mixture; lane 9, assay with boiled GAPDH (10 min at 100 °C, cooled to room temperature). B, comparison of the RNA cleavage patterns produced by dehydrogenases and RNase A and differentiation between GAPDH and RNase A by addition of tRNA or ssDNA. The assays were performed with the N26 transcript (20 nM) at 37 °C for the time indicated above the panels. The cleavage patterns produced by His₆-Asd-1 and His₆-Acd-5 (200 nM monomer, lanes 1 and 2) are identical. Rabbit muscle GAPDH (200 nM monomer, lanes 3–17 and 33–38) and RNase A (400 femtomolar, lanes 18–26 and 100 femtomolar, lanes 27–38) also produce identical patterns that are different from those generated by Asd-1 and Acd-5. The addition of tRNA or ssDNA (50 ng μl⁻¹ of each) to the reaction mixture causes opposite effects on RNA cleavage by GAPDH and RNase A. Ø, negative control, N26 incubated with buffer for 40 min. C, the His₆-Asd-1 and rabbit muscle GAPDH polypeptides produce distinct RNA cleavage patterns after electrosolution from SDS-PAGE and refolding in solution. The assays were performed at 37 °C with the N26 transcript as substrate. Positive controls: lane 1, incubation with Asd-1 (100 nM monomer) for 10 min; lane 2, incubation with GAPDH (100 nM monomer) for 10 min. Negative control: lane Ø, N26 incubated with buffer for 3 h. Lanes 3, 4, and 5, assays with the refolded Asd-1 for 1, 2, and 3 h, respectively. Lanes 6, 7, 8, assays with the refolded GAPDH for 1, 2, and 3 h, respectively.
fataricus. We further characterized the RNase properties of the cloned Asd-1 and Acd-5 proteins.

Eukaryotic GAPDH Exhibits RNase Activity Which Is Similar but Not Identical to That of the S. solfataricus Asd-1 and Acd-5 Proteins—We decided to include in our studies rabbit muscle GAPDH (Sigma) as a control protein known to be a dehydrogenase capable of binding RNA (3). To our surprise, GAPDH degraded different transcripts derived from 23 S rRNA of α-proteobacteria, generating cleavage patterns very similar to those produced by the cloned Asd-1 and Acd-5 proteins and by the original S. solfataricus fractions in absence of MgCl₂ (Fig. 2). Incubation of these transcripts with BSA or lactate dehydrogenase led to negligible RNA degradation, whereas GAPDH and the Asd-1 and Acd-5 proteins performed strong RNA cleavage (Fig. 2A). The RNase activity of all three proteins was higher at 60 °C than at 37 °C (Fig. 2A). Surprisingly, the Asd-1 and Acd-5 proteins cleaved RNA more efficiently at 60 °C than at 80 °C. As expected, GAPDH did not cleave RNA at 80 °C (Fig. 2B). All three proteins cleaved efficiently RNA at MgCl₂ concentrations up to 5 mM (not shown).

We also tested whether highly purified bacterial GAPDH isolated from E. coli or B. stearothermophilus can cleave RNA. The bacterial enzymes processed a transcript derived from 23 S rRNA of Rhizobium leguminosarum and generated a cleavage pattern identical to the cleavage patterns produced by the Asd-1 and Acd-5 proteins (Fig. 2C).

The assays shown in Fig. 2 were performed for 10 min with an excess of proteins. We also performed assays with decreasing enzyme concentrations. The cloned Asd-1 protein cleaved 90% of a transcript derived from 23 S rRNA of Bartonella henselae, which was present in a 20-fold higher concentration than the enzyme during 4 h of incubation at 60 °C (not shown). Thus, Asd-1 can slowly perform turnover of the RNA substrate. The same applies for the Acd-5 protein. In contrast, we did not observe turnover using GAPDH (not shown), suggesting that GAPDH is not released from the cleaved substrate.

It has been demonstrated previously that the NAD-binding site of GAPDH (Rossmann fold) is part of the RNA-binding domain. In accordance with this assumption, 50 μM ATP, 10 μM NAD⁺, and 10 μM NADH strongly interfere with RNA binding (2). Surprisingly, ATP, NADH, and NADPH in concentrations up to 200 μM did not interfere with the RNA degradation promoted by GAPDH and the two archaeal proteins (not shown). Higher ATP concentrations (0.5-2 mM) inhibited RNA cleavage by all three proteins (for GAPDH see Fig. 3A), which may be due to subunit dissociation (26).

It is known that GAPDH can bind tRNA and ssDNA (1, 11). We tested whether tRNA and DNA interfere with the RNA degradation mediated by GAPDH and the two archaeal proteins. We obtained identical results for all three proteins. In Fig. 3, A and B we show the data for GAPDH. In the presence of tRNA and ssDNA, the cleavage of the transcripts was reduced (Fig. 3A). All three proteins did not cleave the competitive ssDNA (not shown). Addition of double stranded DNA (dsDNA) did not interferewith RNA degradation (Fig. 3A), suggesting that GAPDH and the Asd-1 and Acd-5 proteins bind ssDNA but not dsDNA.

To analyze the possibility that the observed RNase activity is due to a minor contaminant protein in the commercially obtained GAPDH fraction, we used the RNase A specific inhibitor RNasin, which efficiently stopped the RNA degradation (Fig. 3A). The RNase activity of both archaeal proteins was also blocked by RNasin (not shown). In addition, boiled and spontaneously renatured GAPDH fractions still exhibited strong RNase activity, a feature characteristic for RNase A (Fig. 3A). We compared the RNA cleavage patterns produced by suitable dilutions of pure RNase A and by the dehydrogenases. Femtomolar concentrations of RNase A and nanomolar concentrations of GAPDH generated identical cleavage patterns that were slightly different from those produced by the Asd-1 and Acd-5 proteins when the N26 substrate was used (Fig. 3B).

However, the activity of RNase A can be distinguished from the RNA degradation by dehydrogenases by addition of tRNA or ssDNA. Whereas the presence of tRNA or ssDNA inhibits the degradation of the N26 transcript by the dehydrogenases (Fig. 3A, and lanes 3-17 of B), the RNA cleavage by RNase A was enhanced under these conditions (Fig. 3B, lanes 18-32). To avoid the possibility that the excess of GAPDH changes the behavior of contaminant RNase A in the commercially obtained GAPDH fraction, we added pure RNase A in femtomolar concentration to GAPDH in nanomolar concentration and studied the influence of ssDNA addition on the RNA cleavage (Fig. 3B, lanes 33-38). Under these conditions, RNase A still efficiently cleaved RNA, suggesting that the RNase activity of GAPDH is.

FIG. 4. GAPDH and the putative dehydrogenases from S. solfataricus cleave RNA between pyrimidine and adenine. A, primer extension reactions were performed using a transcript (200 nm) derived from 23 S rRNA of B. capsulatus that was incubated with buffer (Ø), RNase A (400 femtomolar, lane 1), rabbit muscle GAPDH (200 nm monomer, lane 2), His₁₅-Asd-1 (200 nm monomer, lane 3), and His₁₅-Acd-5 (200 nm monomer, lane 4) for 20 min at 60 °C. Lanes G, A, T, and C each refer to the corresponding nucleotide of the DNA template (cloned 23 S rDNA region) as determined by sequencing. The most prominent signals corresponding to the detected 5′-ends are marked by arrows. The positions of the cleavage sites in the predicted RNA secondary structure are shown in B.

FIG. 5. The N-terminal part of the S. solfataricus Asd-1 polypeptide contains the RNase active center. A, schematic representation of the Asd-1 deletion derivatives. B, silver-stained SDS-PAGE gels showing the following purified His₁₅-tagged polypeptides: lane 1, full-length Asd-1 protein; lane 2, ΔAsd (amino acids 1–205); lane 3, ΔAsd (amino acids 206–350); lane 4, ΔAcd (amino acids 1–73). Protein molecular mass markers in kilodaltons are indicated on the side of the panels. C, cleavage assay using the N26 transcript and equal amounts of the polypeptides (10 ng μl⁻¹) shown in B. Lane 1, full-length Asd-1 protein; lane 2, ΔAsd (amino acids 1–205); lane 3, ΔAcd (amino acids 206–350); lane 4, ΔAcd (amino acids 1–73). The assay was performed at 60 °C for 40 min. Ø, negative control.
similar to but distinct from that of RNase A. An additional experiment was performed to confirm this assumption. The fractions containing the rabbit muscle GAPDH and Asd-1 proteins were separated in SDS-PAGE. The polypeptides were electrophoresed, subjected to denaturation and refolding in solution, and incubated with the N26 transcript to test their RNase activity. Fig. 3C demonstrates that the RNase activity is an intrinsic property of the GAPDH and Asd-1 polypeptides. In addition to the capability to degrade RNA, the two polypeptides retained their slightly different specificity, producing different cleavage patterns.

**The Dehydrogenases Cleave RNA between Pyrimidine and Adenine**—RNase A is a single strand-specific RNase that preferentially cleaves 3’ to pyrimidine residues (27). To determine the cleavage specificity of rabbit muscle GAPDH and the Asd-1 and Acd-5 proteins, primer extension analysis was performed (Fig. 4). All three proteins were found to cleave between pyrimidine and adenine residues. The Asd-1 and Acd-5 proteins cleaved between C and A, whereas GAPDH cleaved in addition between U and A. The most prominent cleavage sites were localized in loops and bulges of the predicted secondary structure (Fig. 4). In accordance with the above presented data, the cleavage positions determined for GAPDH were similar to those used by RNase A.

**The Ribonuclease Domain of Asd-1 Is Located in the N-Terminal Part of the Protein**—Although the dehydrogenases studied here exhibit strong RNase activity, their primary amino acid sequences do not show significant similarities to any nuclease in the data bases. We performed deletion mutagenesis to localize the Asd-1 domain required for the RNase activity. We were able to overexpress and purify His6-tagged polypeptides consisting of the N-terminal 73 amino acids (comprising the putative first mononucleotide binding domain of the Rossmann fold), the N-terminal 205 amino acids (comprising the complete Rossmann fold), and the C-terminal 145 amino acids of Asd-1 (Fig. 5, A and B). The RNase activity is associated with the N-terminal 73 amino acids as shown by cleavage assays using the N26 substrate in Fig. 5C.

**DISCUSSION**

We found that the well known metabolic enzyme GAPDH and the Asd-1 and Acd-5 proteins of *S. solfataricus* cleave RNA. This is the first report describing RNA degradation by dehydrogenases. Keeping in mind that the Asd-1 protein can perform turnover of the RNA substrate (this study), and that GAPDH specifically interacts with different RNA strands in vivo (4, 8, 9), we think that RNA cleavage by GAPDH and dehydrogenase-like proteins should have physiological relevance. Since GAPDH was identified repeatedly as a major RNA-binding protein, it was proposed that this key metabolic enzyme may function in the RNA transport (1) as RNA chaperone (3) or as regulatory protein binding to viral and cellular non-coding RNA regions (2, 4, 9). Our finding that GAPDH cleaves RNA leads to different interpretation of some of the existing data. For example, it was demonstrated that GAPDH suppresses in vivo the translation of *Hepatitis A* virus proteins (9). Additionally to the proposed suppression mechanism based on GAPDH binding to the internal ribosome entry site, GAPDH may work against viral propagation by RNA cleavage. Similarly, GAPDH may participate in eukaryotic mRNA turnover not only by binding to AU-rich elements of 3’-untranslated mRNA regions (2) but also by cleaving.

We localized the RNase active center of the Asd-1 protein in the 73 N-terminal amino acids. The first 50 N-terminal amino acids harbor the putative first mononucleotide binding domain of the Rossmann fold. Nevertheless, the presence of 50–200 μM ATP had no influence on RNA cleavage by the Asd-1 protein. The NAD-binding fold was identified as a part of the RNA binding domain of GAPDH (2, 13). Surprisingly, the presence of NADH, NADPH, and ATP at concentrations up to 200 μM did not have any influence on RNA cleavage by rabbit muscle GAPDH, whereas the presence of ssDNA inhibits the cleavage reaction. These results suggest that amino acids differ from those involved in dinucleotide or mononucleotide binding are responsible for the RNase activity of GAPDH. Obviously, the RNase active center is blocked by ssDNA binding but not by mononucleotides. The RNA cleavage domain of rabbit muscle GAPDH seems to be more thermostable than its metabolic active site since GAPDH can cleave RNA at 60 °C, a temperature at which its metabolic catalytic site is already denatured (28). It is known that different protein domains may possess different thermal stabilities. Even domains of mesophilic enzymes can be active at temperatures up to 100 °C (29).

The fact that dehydrogenases and dehydrogenase-like proteins from all three domains of life cleave RNA supports the hypothesis that dehydrogenases and RNases have a common evolutionary origin (14) and suggests that the common protein ancestor was present in all three lineages. Multifunctional proteins may be the key to the highly complex networks that maintain the functions and structures in the eukaryotic cells possessing relatively low number of protein-encoding genes (30). It emerges that the networks of protein-protein and protein-nucleic acids interactions are much more complicated than it was assumed. Proteins that were originally characterized to have one defined metabolic function are shown now to participate in a number of other cellular processes. Examples for such eukaryotic multifunctional proteins are the glycolytic enzymes GAPDH and rabbit phosphoglucose isomerase (31, 32). There are also examples for prokaryotic multifunctional glycolytic enzymes: the bacterial enolase is a component of the *E. coli* RNA-degrading complex called degradosome (33). Our finding that archaeal proteins, which are most probably dehydrogenases, cleave RNA supports the view that multifunctionality may be a common feature of proteins in all three domains of life.

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