Sequence-Specific Endoribonuclease Activity of the *Tetrahymena* Ribozyme: Enhanced Cleavage of Certain Oligonucleotide Substrates That Form Mismatched Ribozyme–Substrate Complexes†

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ABSTRACT: A shortened form of the self-splicing intervening sequence RNA of *Tetrahymena* acts as a sequence-specific endoribonuclease. Specificity of cleavage is determined by Watson–Crick base pairing between the active site of the RNA enzyme (ribozyme) and its RNA substrate [Zaug, A. J., Been, M. D., & Cech, T. R. (1986) *Nature (London) 324*, 429–433]. Surprisingly, single-base changes in the substrate RNA 3 nucleotides preceding the cleavage site, giving a mismatched substrate–ribozyme complex, enhance the rate of cleavage. Mismatched substrates show up to a 100-fold increase in k_{cat} and, in some cases, in k_{cat}/K_m. A mismatch introduced by changing a nucleotide in the active site of the ribozyme has a similar effect. Addition of 2.5 M urea or 3.8 M formamide or decreasing the divalent metal ion concentration from 10 to 2 mM reverses the substrate specificity, allowing the ribozyme to discriminate against the mismatched substrate. The effect of urea is to decrease k_{cat} and k_{cat}/K_m for cleavage of the mismatched substrate; K_m is not significantly affected at 0–2.5 M urea. Thus, progressive destabilization of ribozyme–substrate pairing by mismatches or by addition of a denaturant such as urea first increases the rate of cleavage to an optimum value and then decreases the rate.

The nuclear rRNA precursor of *Tetrahymena thermophila* contains a 413-nucleotide intervening sequence (IVS) that is excised from the larger RNA by self-splicing (Kruger et al., 1982). The activity that mediates self-splicing is intrinsic to the IVS portion of the molecule (Zaug et al., 1983, 1984). Shortened forms of the excised IVS RNA have enzymatic activity, catalyzing cleavage–ligation reactions on RNA substrates with multiple turnover (Zaug & Cech, 1986a,b).

Among other activities, this RNA enzyme (ribozyme) can act as a sequence-specific endoribonuclease (Figure 1). The reaction mechanism, in which RNA is cleaved after the sequence CUCU with concomitant addition of guanosine to the 5′ end of the downstream fragment, is an intermolecular version of the first step of pre-rRNA self-splicing (Zaug et al., 1986). Under certain conditions, the ribozyme cleaves single-stranded RNA with greater sequence specificity than any known protein enzyme, prompting us to describe its activity as being like that of an RNA restriction endonuclease.

In our earlier study (Zaug et al., 1986), we reported that site-specific mutations in the active site of the ribozyme altered the sequence specificity of cleavage in the manner expected if the substrate were bound to the ribozyme by Watson–Crick base pairing. Surprisingly, however, high sequence specificity of cleavage was achieved only by the inclusion of urea or formamide in the reaction. The denaturants were added in an attempt to destabilize mismatched ribozyme–substrate complexes. We now explore the effect of such mismatches in more detail. Both the enhanced reactivity of mismatched substrates in the absence of urea and their decreased reactivity in the presence of urea are found to be due to changes in the catalytic rate constant (k_{cat}) and not in K_m. We discuss several models that could explain why k_{cat} is so sensitive to mismatches in the ribozyme–substrate complex.

Our interest in this system is 2-fold. First, the ability to manipulate the substrate and catalytic portions of the molecule separately facilitates the detailed examination of the first step in pre-rRNA self-splicing. We hope to understand catalysis by RNA in the same detail that catalysis by certain protein enzymes is understood. Second, the ribozyme and variants with altered sequence specificity provide a set of sequence-specific endoribonucleases that may be useful reagents for a variety of studies of the molecular biology of RNA.

Materials and Methods

Nucleotides and Enzymes. Unlabeled nucleoside triphosphates were purchased from P-L Biochemicals, labeled nucleoside triphosphates from New England Nuclear, calf intestinal phosphatase from New England Nuclear, T_4 polynucleotide kinase from United States Biochemicals, and restriction endonucleases from New England Biolabs. T_7 RNA polymerase was isolated from *Escherichia coli* strain BL21, containing the plasmid pAR1219 (Davanloo et al., 1984).

L – 21 ScaI RNA Preparation. Plasmid pT7L-21 was cut with *Sca*I restriction endonuclease, extracted with phenol and chloroform, ethanol precipitated, and then resuspended in H_2O to 1 $\mu g/\mu L$. Transcription was done in 2 mL of 40 mM Tris-HCl, pH 7.5, 12 mM $MgCl_2$, 10 mM DTT, 4 mM spermidine, 1 mM each nucleoside triphosphate, 10 μg/mL linearized plasmid, and 200 units of T_7 RNA polymerase/μg of DNA. Incubation was for 1 h at 37 °C. Products were ethanol precipitated and purified by electrophoresis in a 4% polyacrylamide/8 M urea gel. The L – 21 *Sca*I RNA was visualized by UV shadowing, excised, and eluted overnight at 4 °C in 250 mM NaCl, 10 mM Tris-HCl, pH 7.5, and 1 mM EDTA. The gel was removed by centrifugation. The RNA was ethanol precipitated and chromatographed on a Sephadex G-50 column equilibrated in 250 mM NaCl, 10 mM Tris-HCl,

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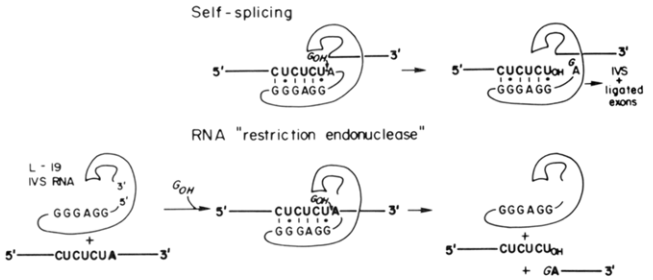

FIGURE 1: Model for the endonuclease action of shortened forms of the IVS RNA of *Tetrahymena* pre-rRNA. The mechanism is an intermolecular version of the first step of pre-rRNA self-splicing. Thin letters and lines, IVS; bold letters and thick lines, exon sequences (top) or substrate RNA (bottom); italicized G, free guanosine nucleotide or nucleoside. Reprinted from Zaug et al. (1986) by permission.

pH 7.5, and 1 mM EDTA. Fractions that contained RNA were pooled and ethanol precipitated. The precipitate was washed with 70% ethanol, dried, and resuspended in H_2O. L − 21 *Sca*I RNA concentration was determined by spectrophotometry. The extinction coefficient, $\epsilon_{260nm} = 3.2 \times 10^6$ M^{-1} cm^{-1}, was determined by taking a sample of L − 21 *Sca*I RNA ($A_{260nm} = 0.534$), hydrolyzing it to completion with a mixture of ribonucleases T_1, T_2, and A, remeasuring the absorbance ($A_{260nm} = 0.753$), and multiplying the ratio of these absorbances by the extinction coefficient calculated for a mixture of free nucleotides in the proportion that they occur in the L − 21 *Sca*I RNA.

Synthesis of Oligoribonucleotide Substrates. Oligoribonucleotides were produced by transcription of synthetic DNA templates as described by Lowary et al. (1986) and Milligan et al. (1987). Transcription solutions (2 mL) contained 12 mM $MgCl_2$, 40 mM Tris-HCl, pH 7.5, 10 mM DTT, 4 mM spermidine, 2 mM each nucleoside triphosphate, 1.5 μM promoter top strand DNA, 1.5 μM promoter template bottom strand DNA, and 100 000 units/mL T_7 RNA polymerase. (The top and bottom strand deoxyoligonucleotides were synthesized on an Applied Biosystems 380B DNA synthesizer, deprotected, ethanol precipitated, and used without further purification.) Incubations were done for 2 h at 37 °C. Products were ethanol precipitated and purified by electrophoresis on 20% polyacrylamide/7 M urea gels. Purification proceeded as described above for L − 21 *Sca*I RNA, except that chromatography was on a Sephadex G-25 column.

Labeling of Oligoribonucleotides. Purified oligonucleotide (100 pmol) was incubated with 8 units of calf intestinal phosphatase in 100 μL of H_2O at 37 °C for 1 h. The solution was extracted with phenol and ether and evaporated to dryness. 5′ end labeling was accomplished using T_4 polynucleotide kinase and [γ-^{32}P]ATP. Labeled RNA was purified by electrophoresis on a 20% polyacrylamide/7 M urea sequencing gel, visualized by autoradiography, excised, and eluted as described above.

Initial Velocity Determination. Reactions (30 μL) contained 0.01 μM L − 21 *Sca*I RNA, 500 μM GTP, 10 mM $MgCl_2$, 50 mM Tris-HCl, pH 7.5, and a mixture of unlabeled and 5′ end-labeled substrate varying in concentration from 0.1 to 4.0 μM. Reactions were initiated by addition of the $MgCl_2$ and proceeded at 50 °C. Portions (3 μL) were removed at times ranging from 1 to 120 min and added to a stop mixture that contained 100 mM EDTA. Samples were subjected to electrphoresis on a 20% polyacrylamide/7 M urea gel. Bands were visualized by autoradiography, cut from the gel, and counted in toluene-based fluor in a Beckman LS7000 scintillation counter. Initial velocities were determined as described by Bass and Cech (1984).

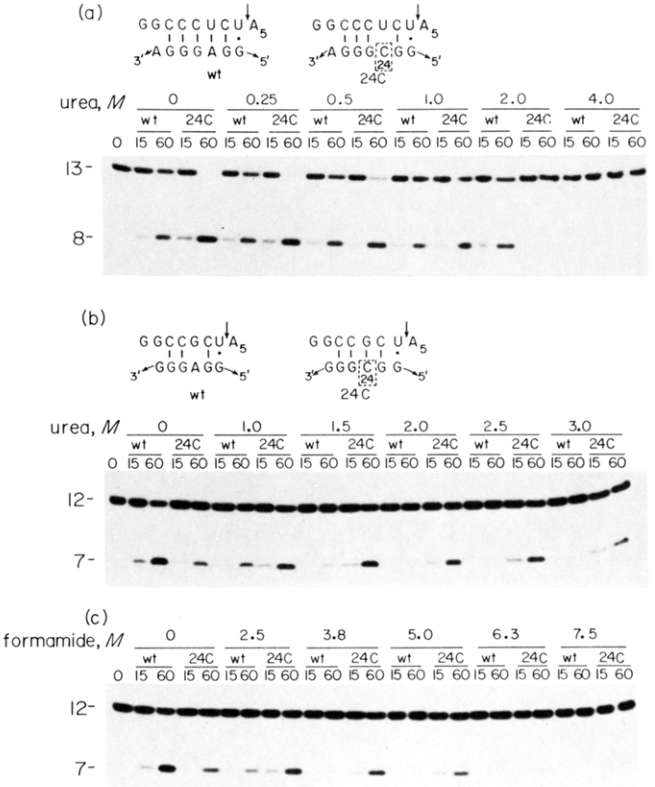

FIGURE 2: Increased specificity of cleavage in the presence of urea or formamide. (a) An oligonucleotide substrate ($GGCCCUCUA_5$) of length 13, ^{32}P labeled at its 5′ end, was incubated with the wild-type (wt) L − 19 IVS_β RNA or the 24C variant, both prepared as described by Zaug et al. (1986). Diagrams show the proposed base-paired complexes between the substrate (top strand) and the active site of the ribozyme (bottom strand). Reactions contained 0.12 μM substrate, 0.05 μM ribozyme, 0.5 mM GTP, 10 mM NaCl, 10 mM $MgCl_2$, 50 mM Tris-HCl, pH 7.5, and the indicated concentration of urea. Incubation was at 50 °C for 15 or 60 min as indicated. Samples were analyzed by electrophoresis in a 20% polyacrylamide/7 M urea gel, an autoradiogram of which is shown. (b) An oligonucleotide substrate ($GGCCGCUA_5$) of length 12 was incubated with the wild-type L − 19 IVS_β RNA or the 24C variant. Conditions as described in (a), except with 1.0 μM substrate and 0.10 μM ribozyme. (c) Same substrate and reaction conditions as in (b), except formamide was used instead of urea. (2.5 M formamide is 10% v/v.) Cleavage by the 24C variant ribozyme surpassed cleavage by the wt ribozyme between 1.0 and 2.0 M formamide (data not shown).

RESULTS

Increased Specificity of Cleavage in the Presence of Urea and Formamide. An oligoribonucleotide substrate containing the recognition sequence CUCU was incubated either with the wild-type L − 19 IVS_β RNA or with the 24C variant ribozyme. As shown in Figure 2a, both ribozymes cleaved the substrate at the same site. It had previously been shown that the cleavage site immediately follows the recognition sequence (Zaug et al., 1986). In the absence of urea, the rate of cleavage was actually faster with the variant ribozyme than with the wild-type ribozyme, despite the fact that the variant cannot form a matched ribozyme–substrate complex. As the concentration of urea was increased, the rate of cleavage by the 24C variant ribozyme steadily decreased, while the rate of cleavage by the wild-type ribozyme stayed approximately constant in the range 0–2 M urea. Thus, around 2 M urea there was optimal discrimination between the substrates that form matched and mismatched ribozyme–substrate complexes (Figures 2 and 3).

An oligoribonucleotide substrate containing a CGCU sequence was similarly incubated with the two ribozymes. In this case, the wild-type ribozyme was expected to give a

Table I: Kinetic Parameters for Cleavage of Oligonucleotide Substrates by L - 21 *Sca*I RNA[a]

substrate	mismatch −5	mismatch −3	k_{cat} (min^{-1})	K_m (μM)	k_{cat}/K_m ($min^{-1}\ \mu M^{-1}$)	$\Delta G°$ (kcal/mol)[b]
GGCCCCCU↓AAAAA		C·A	3.7	0.14	26	−6.7
GGCCCACUAAAAA		A·A	1.7	0.3	6	−6.7
GGCCCGCUAAAAA		G·A	1.0	0.3	3	−6.7
GGCCCUCUAAAAA			0.04 ± 0.01	0.16 ± 0.06	0.3	−10.9
GGCUCCCUAAAAA	U·G	C·A	7.2	6.3	1	−3.6
GGCUCACUAAAAA	U·G	A·A	1.0	3.9	0.3	−3.6
GGCUCGCUAAAAA	U·G	G·A	0.7	1.7	0.4	−3.6
GGCUCUCUAAAAA	U·G		0.15	0.13	1	−7.8

[a] Substrates were incubated with 0.01 μM L-21 *Sca*I RNA under standard reaction conditions at 50 °C in the absence of urea (see Materials and Methods). For GGCCCUCUA$_5$ substrate, three complete kinetic analyses were performed with substrate concentrations spanning the range 0.10–4.0 μM. Listed is the mean ± SD of k_{cat} and K_m from the three Lineweaver–Burk plots. In all other cases, k_{cat} and K_m were estimated from rate measurements done at 0.10 and 1.0 μM substrate. While the hierarchy of k_{cat} and K_m values was reproducible between experiments, the actual values varied by as much as 2-fold. [b] Estimated free energy change for formation of the substrate–ribozyme complex at 50 °C, based on thermodynamic parameters derived from studies of oligoribonucleotides (Freier et al., 1986) and corrected to 50 °C using values of $\Delta H°$ and $\Delta S°$ obtained from D. Turner (personal communication). These parameters assume that all mismatches are equally destabilizing; thermodynamic measurements of the effect of mismatches in DNA indicate that a C·A mismatch is less stable than an A·A or G·A mismatch (Aboul-ela et al., 1985).

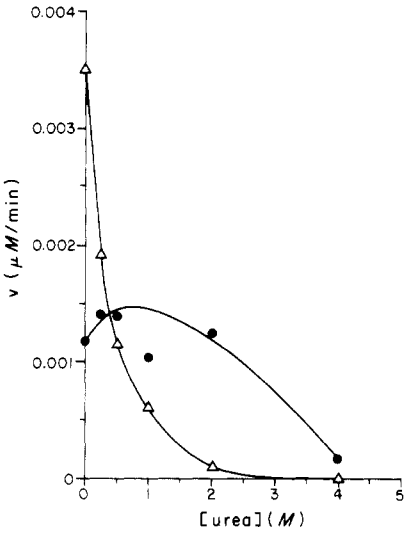

FIGURE 3: Cleavage of oligonucleotide substrate that forms a mismatched ribozyme–substrate complex is very sensitive to urea. Data from 15-min points of Figure 2a were quantitated by liquid scintillation counting of the sliced gel. ●, wt ribozyme. △, 24C variant ribozyme (mismatched complex).

mismatched ribozyme–substrate complex, while the 24C variant ribozyme matched the substrate perfectly. As shown in Figure 2b, the rate of cleavage by the wild-type ribozyme decreased steadily with increasing urea concentration, while the rate of cleavage by the 24C variant actually increased and then remained constant up to 3 M urea, the highest concentration tested. Good discrimination between the matched and mismatched complexes was obtained in the broad range of 1.5–3.0 M urea.

A different RNA denaturant, formamide, also affected the specificity of the cleavage of the CGCU-containing substrate. As shown in Figure 2c, the rate of cleavage by the wild-type ribozyme decreased with increasing formamide concentration, while the rate of cleavage by the 24C variant increased and then decreased at concentrations >5 M formamide. Optimum cleavage by the 24C variant was achieved at 2–4 *M* (data not shown). Thus, the dependence of cleavage rate on denaturant concentration for both enzymes was similar with the two denaturants, 2.5 M formamide having an effect equivalent to that of 1.5 M urea. The relative effects of formamide and urea are consistent with their acting to destablize an RNA duplex. Studies with DNA have shown that 2.5 M formamide (40% v/v) is approximately as destabilizing as 2.1 M urea (Lerman et al., 1984); it is expected that the effect would be somewhat different for RNA·RNA duplexes [see discussion by Casey and Davidson (1977)].

Synthesis of Ribozyme by Direct Transcription. The L - 19 IVS$_\beta$ RNA used as the ribozyme in the experiments described above was made by in vitro transcription of pre-rRNA followed by a cascade of self-reactions mediated by the folded structure of the IVS: splicing, cyclization of the excised IVS, and site-specific hydrolysis at the cyclization junction (Zaug et al., 1984). The L - 19 IVS RNA was then purified from the ligated exons and other reaction products, and its 3′-terminal guanosine was removed by periodate oxidation and β-elimination to give L - 19 IVS$_\beta$ RNA. To facilitate the synthesis of large amounts of ribozyme, a plasmid was constructed (Figure 4) such that the initial product of transcription would be the active ribozyme itself, with no further self-processing required. A phage T$_7$ promoter was juxtaposed to the DNA encoding nucleotide 22 of the IVS, so that the active-site GGAGGG sequence comprises nucleotides 1–6 of the transcript. Truncation of the plasmid with *Sca*I restriction endonuclease and transcription with purified phage T$_7$ RNA polymerase (Davanloo et al., 1984) give the L - 21 *Sca*I RNA.

Transcription of linear DNA templates by T$_7$ RNA polymerase frequently produces RNA with one or more extra nucleotides at its 3′ end beyond those specified by the template (Lowary et al., 1986; Milligan et al., 1987). The 3′ end of the L - 21 *Sca*I RNA was analyzed by 3′ end labeling with [^{32}P]pCp and RNA ligase, followed by complete digestion with RNase T2 and analysis of the nucleoside 3′-phosphate products by thin-layer chromatography. The label was distributed as follows: 47% C, 32% U, 9% G, 12% A. (The last templated nucleotide was predicted to be a U.) Thus, while the L - 21 *Sca*I RNA appears to be homogeneous when analyzed by electrophoresis in a 4% polyacrylamide gel, it has a heterogeneous 3′ end.

The L - 21 *Sca*I RNA differs from the L - 19 IVS$_\beta$ RNA by being 2 nucleotides shorter at its 5′ end and approximately 3–4 nucleotides shorter at its 3′ end. The activity of these two ribozymes is similar. For example, with a GGCCCUCUA$_5$ substrate under standard reaction conditions, the L - 21 *Sca*I RNA has k_{cat}/K_m = 0.2 $min^{-1}\ \mu M^{-1}$ (Table I) compared to values of k_{cat}/K_m in the range of 0.05–0.16 $min^{-1}\ \mu M^{-1}$ for the L - 19 IVS$_\beta$ RNA [Figure 4b,c of Zaug et al. (1986)].

Kinetic Analysis of Oligoribonucleotide Cleavage in the Absence of Urea. We have shown that, in the absence of urea,

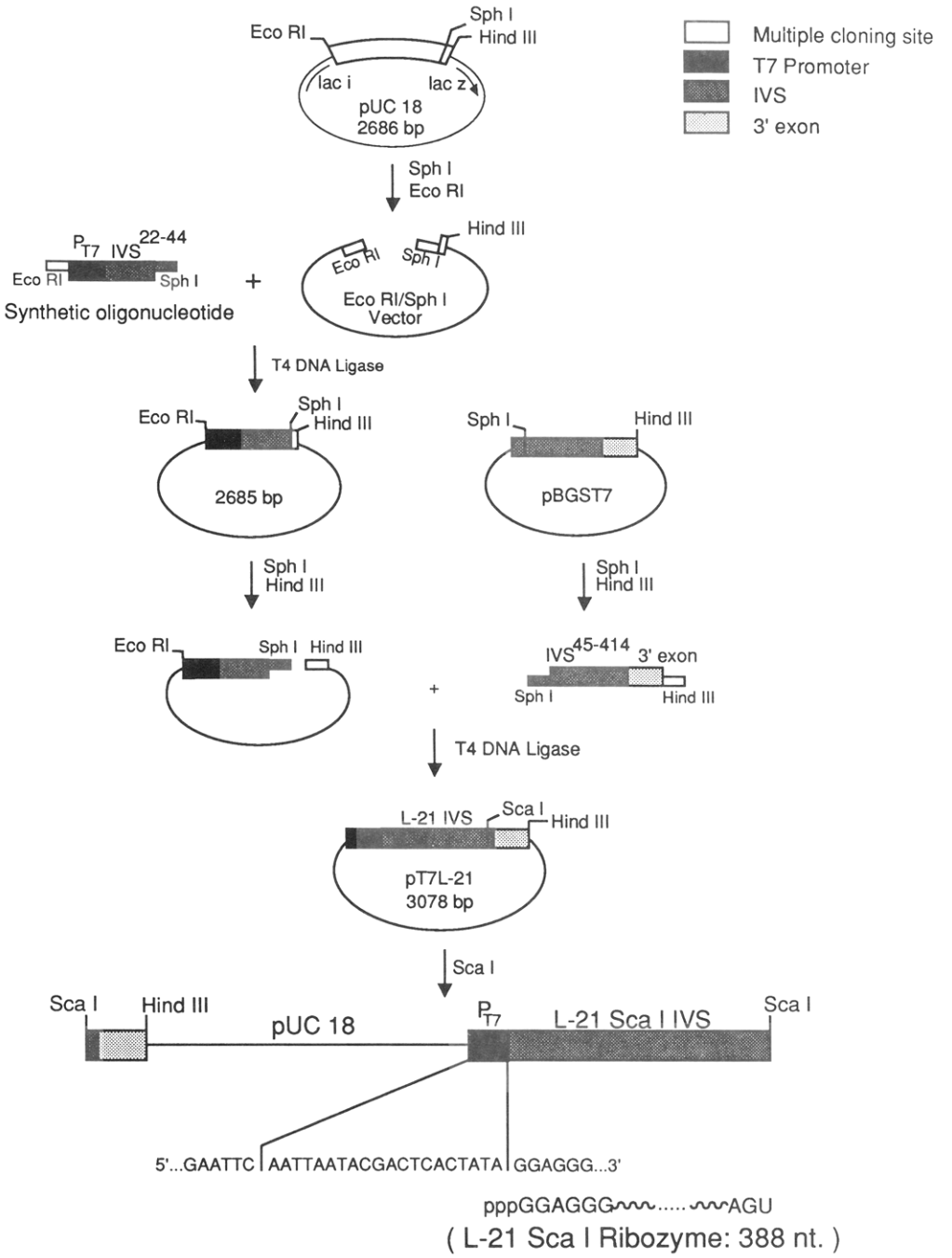

FIGURE 4: Construction of pT7L-21, a plasmid that facilitates synthesis of the wild-type ribozyme (L − 21 *Sca*I RNA). The pUC18 vector was prepared by removing the *Sph*I/*Eco*RI fragment from the multiple cloning site. A synthetic DNA fragment containing an *Eco*RI end with a 5′ overhang, a promoter for bacteriophage T_7 RNA polymerase (Dunn & Studier, 1983), and bases 22–44 of the *Tetrahymena thermophila* rRNA IVS (including the *Sph*I end with its 3′ overhang) was inserted into the vector. This plasmid was cloned and sequenced and then cleaved with *Sph*I and *Hin*dIII. The 395 bp *Sph*I/*Hin*dIII fragment of pBGST7 (Been & Cech, 1986), containing bases 45–414 of the IVS plus 25 bases of the 3′ exon, was inserted to give pT7L-21. pT7L-21 is linearized with *Sca*I restriction endonuclease, truncating the template in the IVS five nucleotides upstream from the 3′ splice site. Transcription with purified T_7 RNA polymerase in vitro then gives the L − 21 *Sca*I RNA, which contains nucleotides 22–409 of the IVS.

certain substrates are cleaved more rapidly by a ribozyme that forms a mismatched ribozyme–substrate complex than by a ribozyme that has perfect complementarity to the substrate (Figure 2). We now show that the same sort of enhanced cleavage is observed when the mismatch is introduced by varying the sequence of the substrates while leaving the ribozyme constant.

We prepared the series of substrates *pGGCCCNCU$^{\downarrow}$A$_5$, where *p indicates the radiolabeled phosphate and N = C, A, G, and U, and incubated them with L − 21 *Sca*I RNA in the presence of 0.5 mM GTP. For N = A, G, and U, the only labeled product was an octanucleotide, the size expected for cleavage at the vertical arrow. For N = C, initial cleavage occurred at the same site; later in the reaction, the octanucleotide was further cleaved to a heptanucleotide, indicating secondary cleavage following the five C residues (data not shown). The k_{cat} for cleavage varied over a 100-fold range as N was changed: C > A ≈ G ≫ U (Table I). The K_m values showed much less variability. The matched substrate (N = U) and the other pyrimidine-containing substrate (N = C) have slightly lower K_m's than the two substrates that would be expected to form purine–purine mismatches with the ribozyme. We also studied the series of substrates *pGGCUCNCU$^{\downarrow}$A$_5$, where N = C, A, G, and U. In all cases,

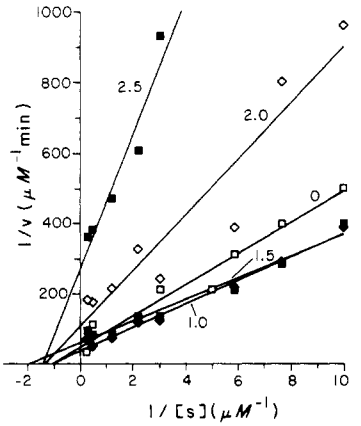

FIGURE 5: Effect of urea concentration on the rate of cleavage of the $GGCCCGCUA_5$ substrate by L - 21 *ScaI* RNA. Ribozyme concentration was 0.01 μM; oligonucleotide substrate concentration ranged from 0.10 to 3.78 μM. Urea concentrations (M) are indicated.

Table II: Urea Affects Cleavage of $GGCCCGCUA_5$ Primarily by Changing k_{cat}[a]

[urea] (M)	K_m (μM)	V_{max} (μM min^{-1})	k_{cat} (min^{-1})	k_{cat}/K_m (μM^{-1} min^{-1})
0	0.89	0.020	2.0	2.2
1.0	0.83	0.025	2.5	3.0
1.5	0.50	0.016	1.6	3.2
2.0	0.74	0.009	0.9	1.2
2.5	0.70	0.004	0.4	0.6

[a] Data from Figure 5. Temperature was 50 °C.

cleavage occurred predominantly at the position indicated by the arrow. The k_{cat} for cleavage varied over a 50-fold range as N was changed; the order C > A ≈ G > U was the same as with the *pGGCCCNCUA$_5$ series (Table I). K_m varied in the order C > A > G ≫ U, similar to k_{cat}.

It is useful to compare each substrate that has a C at position −5 (first four rows of Table I) with the corresponding substrate that has a U at position −5 (last four rows of Table I). The k_{cat} of each GGC*C*CNCUA$_5$ substrate is always similar to that of the corresponding GGC*U*CNCUA$_5$ substrate; most of the comparisons show less than a 2-fold difference, which is not considered significant. The K_m's of the two substrates with N = U (i.e., a matched nucleotide in position −3) were not significantly different. In each case where there was a mismatched nucleotide at position −3 (N = C, A, or G), the K_m was significantly lower with a C at position −5 than with a U at position −5. According to our model (Figure 1), a C at position −5 would form a G·C base pair with the ribozyme, while a U would form a G·U wobble base pair.

Kinetic Analysis of Cleavage in the Presence of Urea. The effect of urea on cleavage was studied in some detail with *pGGCCCGCUA$_5$, a substrate whose cleavage by wild-type ribozyme was expected to be strongly inhibited by higher concentrations of urea (Figure 2b). As shown in Figure 5 and Table II, low concentrations of urea slightly enhanced the cleavage of L - 21 *ScaI* RNA, while 2.0 and 2.5 M urea inhibited cleavage as expected. The effects were almost entirely due to changes in k_{cat}, K_m staying constant at 0.5 ± 0.2 μM. This is not the result expected if K_m simply represented the dissociation constant of the ribozyme–substrate complex; in that case, increasing the concentration of urea would be expected to increase the K_m.

The effect of urea of the initial velocity of six different oligoribonucleotide substrates was determined (Figure 6). A relatively high substrate concentration was chosen so that the

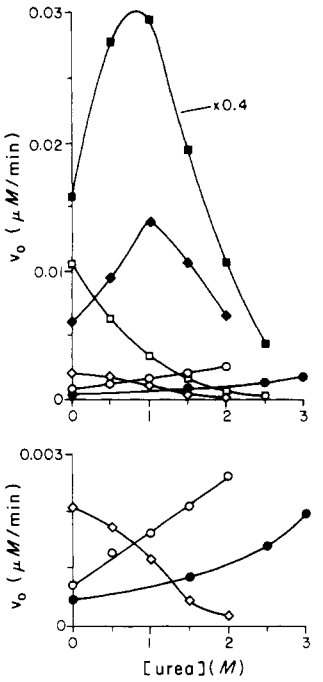

FIGURE 6: Effect of urea concentration on the rate of cleavage of oligonucleotide substrates by the L - 21 *ScaI* RNA. Oligonucleotide substrates contained the recognition sequences CCCCCU (■), CCCGCU (♦), CCCUCU (●), CUCCCU (□), CUCGCU (◊), and CUCUCU (○). In each case, the recognition sequence was preceded by two G residues and followed by five A residues. Reactions contained 0.80 μM oligonucleotide and 0.01 μM L - 21 *ScaI* RNA. All velocities are initial velocities except for the CCCCCU-containing substrate, where cleavage proceeded so rapidly that some of the velocities are based on data points where a large fraction of the substrate was cleaved; in this case, initial velocity is underestimated.

velocity measurements would approximate V_{max}. (The substrate concentration was below K_m for two of the substrates, $GGCUC^C_GCUA_5$, so cleavage rates for these substrates underestimate V_{max}.) The substrates can be divided into three groups on the basis of the response of their cleavage rate to urea. The substrates $GGCUC^C_GCUA_5$ (mismatched at position −3 and wobble base paired at position −5) showed steady reduction in cleavage with increasing urea concentration. The substrates $GGCCC^G_CCUA_5$ (mismatched at position −3 and G·C base paired at position −5) showed an increased rate of cleavage at low urea concentrations followed by a decrease at >1 M urea. The substrates $GGC^U_CCUCUA_5$ (matched at position −3 and either G·U or G·C base paired at position −5) showed increased rate of cleavage with increasing urea concentration over the entire range tested. The best matched of these two substrates, $GGCCCUCUA_5$, showed little change in cleavage rate between 0 and 1.5 M urea, consistent with the previous results with the same substrate and the L - 19 IVS_β ribozyme (Figure 3, closed circles).

Cleavage in Ribozyme Excess. To test whether the enhanced cleavage of mismatched substrates persisted under single-turnover conditions, GGCCC*G*CUA$_5$ and GGCCC*U*CUA$_5$ (0.05 μM) were treated with a 20-fold molar excess of L - 21 *ScaI* RNA (1.00 μM) in the absence of urea. Cleavage of the mismatched substrate was more rapid than cleavage of the matched substrate (data not shown). Although cleavage of the mismatched substrate occurred too quickly to obtain a reliable rate, the rate was at least 8 times faster than that of the matched substrate. A 23-fold rate difference was predicted from the k_{cat} and K_m values of Table I, using the equation $v_0 = k_{cat}[E_0][S_0]/(K_m + [E_0])$ for reaction in enzyme excess. Thus, the results of this experiment gave no indication

of a previously unrecognized rate-limiting step involving product dissociation. The results do not, however, rule out the possibility that some of the rate difference between matched and mismatched substrates might be due to a differential rate of product release.

Cleavage as a Function of Divalent Cation Concentration. Urea and formamide, both of which destabilize RNA duplexes, allow the ribozyme to discriminate against mismatched substrates. To test the generality of this correlation, we examined cleavage of matched ($GGCCC\underline{U}CUA_5$) and mismatched ($GGCCC\underline{G}CUA_5$) substrates by the L − 21 *Sca*I RNA as a function of decreasing Mg^{2+} concentration. Experiments were done in the absence of urea. The initial rate of cleavage of the mismatched substrate exceeded that of the matched substrate at 10 and 5 mM $MgCl_2$, but specificity was reversed at 2 mM $MgCl_2$ (data not shown). Neither substrate was cleaved at 1 mM $MgCl_2$. Another set of reactions was performed in the presence of 1 mM $CaCl_2$, which lowers the magnesium ion requirement (C. A. Grosshans and T. R. Cech, unpublished results). In this case, the initial rate of cleavage of the mismatched substrate exceeded that of the matched substrate at 10 and 5 mM $MgCl_2$ and specificity was reversed at 2 and 1 mM $MgCl_2$ (data not shown). Thus, lowering the divalent cation concentration, another method of destabilizing RNA duplexes, also allows the ribozyme to discriminate against mismatched substrates.

Discussion

Previously we have emphasized the importance of base pairing between the ribozyme and its substrate for both substrate specificity and catalysis (Zaug & Cech, 1986a,b; Been & Cech, 1986; Zaug et al., 1986). The current finding that a mismatch in the ribozyme–substrate complex can enhance the cleavage rate by as much as 100-fold seems at first glance to contradict the importance of ribozyme–substrate base pairing. It is therefore useful to review the evidence that initially led us to the base pairing model of Figure 1. First, there is the clear mechanistic similarity between ribozyme-catalyzed cleavage and RNA self-splicing. In the case of self-splicing, base pairing between the CUCUCU sequence at the 3′ end of the 5′ exon and the 5′ exon binding site GGAGGG within the IVS is proven by comparative sequence analysis (Davies et al., 1982; Michel & Dujon, 1983) and by the analysis of single-base mutations and second-site suppressor mutations (Waring et al., 1986; Been & Cech, 1986). Second, ribozymic cleavage of large RNA molecules as well as oligoribonucleotide substrates occurs only at sequences that are closely related to CUCUCU↓N, cleavage occurring at the position of the arrow. For example, a 504-nucleotide mouse β-globin pre-mRNA transcript is cleaved by the L − 19 IVS_β RNA at two major sites, each preceded by CUCU, and an 841-nucleotide pBR322 transcript is cleaved at four major sites, two preceded by CUCU, one by CCUU, and one by UUUU (Zaug et al., 1986). Finally, active-site mutations alter the substrate specificity in a manner predictable by the rules of Watson–Crick base pairing when reactions are carried out in 2.5 M urea (Zaug et al., 1986). We must now modify the base-pairing model for the mechanism of oligonucleotide cleavage in the following manner: formation of a base-paired duplex between the ribozyme and its substrate is necessary for reaction, but a mismatched duplex can be better than a perfect duplex.

To facilitate discussion of the data, it is useful to consider a simple reaction scheme:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E{\cdot}S \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E{\cdot}P \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} E + P$$

where E is the ribozyme, S is the oligonucleotide substrate, and the products P include both the 5′ half of the cleaved substrate and the guanylylated 3′ half. (The guanosine substrate is present in vast excess; its binding is not explicitly shown.) If $K_m \simeq k_{-1}/k_1$, the dissociation constant for E·S, then changes in the substrate at position −3 relative to the cleavage site which result in a mismatched ribozyme–substrate complex should increase K_m. The expected trend is seen in rows 5–8 of Table I. The substrate predicted to form the most stable ribozyme–substrate complex ($\Delta G° = -7.8$ kcal/mol) has the lowest K_m, and the three substrates with weaker binding (−3.6 kcal/mol) have considerably higher K_m's. Quantitatively, however, the variation in K_m is much less than expected from the differences in $\Delta G°$; the effect of a mismatch seems to be buffered by other interactions between ribozyme and substrate [cf. Sugimoto et al. (1988)]. Futhermore, for the substrates in rows 1–4 of Table I, K_m is not significantly affected by mismatches.

Models for the Effect of Mismatches on the Cleavage Rate. During the course of the experiments described here, three models were considered to explain the increased k_{cat} of mismatched substrates. Some tests of the models were performed.

(*1*) *Nonproductive Binding.* The CUCU-containing substrates would be predicted to have more nonproductive modes of binding to the G-rich active site than CGCU- or CACU-containing substrates; this might explain the reduced k_{cat} of the former. This model was tested by synthesis of the two CCCU-containing substrates, which were expected to have at least as many nonproductive binding modes as CUCU-containing substrates. The high k_{cat} of the CCCU-containing substrates (Table I) was opposite to the expectation for nonproductive binding. Furthermore, nonproductive binding can reduce k_{cat} and K_m but not k_{cat}/K_m (Fersht, 1985). If the k_{cat} values of the CUCU-containing substrates are 10–100-fold low due to nonproductive binding, then their K_m's in the absence of nonproductive binding would have to be 10–100-fold larger than the measured K_m's. This seems most unlikely, because the CUCU-containing substrates are the ones that match the active site the best. It remains quite possible that some of the K_m values are reduced because of nonproductive binding. For example, the C_5 portion of the $GGCCCCCUA_5$ substrate could have multiple nonproductive modes of binding to the G-rich active site of the ribozyme.

(*2*) *Rate-Limiting Product Release.* If product release were rate limiting, k_{cat} could be the rate constant for dissociation of the ribozyme–product complex (k_3) instead of representing the rate constant for the chemical step (k_2). Mismatched substrates would also form mismatched ribozyme–product complexes, thereby increasing k_{cat}. This model was tested by performing cleavage with excess ribozyme; with each ribozyme participating in a single turnover, product release is not expected to contribute to k_{cat}. The continued faster cleavage of the mismatched substrate observed in ribozyme excess does not support this model.

(*3*) *Mismatches Destabilize E·S.* In terms of transition-state theory, mismatches could increase k_{cat} by destabilizing the ground state of E·S without destabilizing the transition state ($E{\cdot}S^‡$) for the rate-limiting step (Fersht, 1985). Such destabilization would leave k_{cat}/K_m unchanged, in accordance with the data in rows 4–8 of Table I. If a mismatch also stabilized $E{\cdot}S^‡$, k_{cat}/K_m would increase as observed in rows 1–3 of Table I. The enhanced cleavage of matched substrates afforded by denaturants such as urea and formamide could

be explained if they also destabilized E·S. Inhibition of cleavage of mismatched substrates by the denaturants might be explained if destabilization of the transition-state E·S‡ became dominant.

How could mismatches destabilize E·S and have a different effect on E·S‡? The nucleotides preceding the reactive phosphate could be bound to the ribozyme in E·S‡ in a manner different from that in the E·S complex. As an extreme possibility, the substrate might become unpaired from the internal template in E·S‡. Note the E·S‡ is the transition state for the rate-limiting step; the data do not distinguish between a rate-limiting chemical step and rate-limiting conformational change in E·S preceding the chemical step.

We propose that optimal cleavage requires a finely tuned degree of stability of the ribozyme–substrate complex. This idea is attractive because it explains the cleavage data in the absence and presence of urea. According to the model, substrates that bind the tightest ($GGC^{C}_{U}CUCUA_5$, $\Delta G° \simeq -10.9$ and -7.8 kcal/mol) have the lowest reaction rates in the absence of urea; addition of urea destabilizes their binding and increases their reaction rate (Figure 6 and Table I). The next class of substrates ($GGCCC^{C}_{G}CUA_5$, $\Delta G° \simeq -6.7$ kcal/mol) binds with slightly more than optimal stability; addition of 1 M urea gives the maximum reaction rate, while further addition of urea decreases the reaction rate because E·S‡ is destabilized. The final class of substrates ($GGCUC^{C}_{G}CUA_5$, $\Delta G° \simeq -3.6$ kcal/mol) binds too poorly to give an optimal reaction rate, so further destabilization caused by addition of urea further lowers the reaction rate. It will be informative to see if mismatches at position −4 affect cleavage rates as predicted by the model.

Implications for Self-Splicing. In the self-splicing reaction, guanosine addition occurs in the sequence $CUCUCU^{\downarrow}A_3$ at the position of the arrow. Single-base changes at positions −1, −2, −3, and −4 relative to the cleavage site all decrease the rate of splicing (Been et al., 1987). In particular, pre-rRNA containing CUCUCU is much more reactive in self-splicing than pre-rRNA containing CUCGCU (Been & Cech, 1986). In the catalytic cleavage of oligonucleotides by L − 21 *Sca*I RNA, the specificity is reversed, a substrate containing CUCGCU being much more reactive than one containing CUCUCU (except at ≥1 M urea). The enzymatic system is more promiscuous than the self-splicing system, the former tolerating and even preferring a limited amount of mismatch in the substrate–active-site complex. The implication is that some of the exon or intron sequences removed when the self-splicing pre-rRNA is converted into the L − 21 *Sca*I RNA enhance the specificity of splice site selection.

Use of Ribozymes for Sequence-Specific Cleavage of RNA. The present work has implications for the use of ribozymes as tools for the sequence-specific cleavage of RNA. First, from our previous work (Zaug et al., 1986), we could make no conclusion about the importance of nucleotides at positions −5 and −6 from the cleavage site. It is now clear that the substitution of a C for a U at position −5 has a minimal effect on k_{cat} but can have a very large effect on the K_m of the cleavage reaction (Table I). The effect of purines at position −5 or of mismatched nucleotides at position −6 has not been studied. Second, and much more fundamentally, certain mismatched sequences are efficiently cleaved even in the presence of urea. Most dramatic is $GGCCCCCU^{\downarrow}A_5$; although its cleavage is strongly inhibited by urea, it is such a good substrate that it is still cleaved more rapidly than the cognate sequences $GGC^{C}_{U}CUCU^{\downarrow}A_5$ in 2–2.5 M urea. A similar mismatched sequence ($CCCU^{\downarrow}$) has been found to be a major site of cleavage of a 602-nucleotide SV40 transcript by the L − 21 *Sca*I RNA (A. J. Zaug and T. R. Cech, unpublished results). It seems likely that discrimination against mismatched substrates can be further improved by increasing the urea concentration to 3 M or by decreasing the magnesium ion concentration to 2 mM. Unless reaction conditions are carefully titrated, however, ribozyme cleavage of RNA molecules will generally occur at a family of sites that includes the sequence that matches the ribozyme active site as well as related sequences that contain one or two mismatched nucleotides.

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Properties of Purified Actin Depolymerizing Factor from Chick Brain†

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ABSTRACT: Actin depolymerizing factor (ADF) from 19-day embryonic chick brains has been purified to >98% homogeneity with a yield of 7.2 mg/100 g of brain. Quantitative immunoblotting with a monospecific antibody to ADF indicated that ADF comprises 0.3% of the total brain protein, resulting in an actual purification yield of about 20%. Brain ADF migrates as a single polypeptide of 19000 kDa on SDS-containing polyacrylamide gels. The molecular weight of the native protein determined from sedimentation equilibrium in buffers containing from 50 to 200 mM KCl is 20000. The secondary structure of ADF calculated from the circular dichroic spectrum consists of about 22% α-helix, 24% β-sheet, and 18% β-turn. ADF contains a blocked N-terminus, a single tryptophan residue located about one-third of the way from one end of the protein, and six cysteine residues (all in reduced form in the native protein). All six cysteine residues could be chemically modified with eosinylmaleimide under nondenaturing conditions; however, ADF activity was lost when more than one cysteine residue was modified. ADF microheterogeneity has been observed upon nonequilibrium pH gradient electrophoresis in polyacrylamide gels containing 9 M urea, the major isoform having a p*I* of $\simeq$7.9–8.0. ADF can interact with either monomeric or filamentous actin to give a complex which can be isolated by gel filtration chromatography. Both major and minor isoforms of the ADF are found in the complex. Assembly-competent actin and active ADF can both be recovered from the complex by chromatography on ATP-saturated DEAE-cellulose. A 1:1 stoichiometry of ADF to actin in the complex has been determined from the elution position of the complex on gel filtration and by SDS–PAGE of the complex, chemically cross-linked through sulfhydryls with *N,N'-p*-phenylenedimaleimide.

Alterations in cellular morphology are often accompanied by shifts in the distribution of actin between the globular and filamentous forms (G- and F-actin, respectively) (Markey et al., 1981; Fox et al., 1981; Heacock & Bamburg, 1983; Heacock et al., 1984; Clark et al., 1983). The regulation of this assembly process has been attributed to a variety of proteins which have been found associated with both F-actin and the actin in the monomer pool (Stossel et al., 1985; Pollard & Cooper, 1986). In 1980, we reported the presence of protein factors in embryonic chick brain and porcine brain which had the ability to depolymerize rapidly F-actin (Bamburg et al., 1980). The chick brain protein, called brain actin depolymerizing factor (ADF),[1] was partially purified and was shown to be directed in its activity toward actin filaments lacking tropomyosin (Bernstein & Bamburg, 1982). More recently, several other groups have reported the presence of actin fragmenting, capping, and/or depolymerizing proteins in extracts of brain or spinal cord from different animals (Kilimann & Isenberg, 1982; Isenberg et al., 1983; Petrucci et al., 1983; Berl et al., 1983; Maekawa et al., 1984; Nishida et al., 1984a,b; Wanger & Wegner, 1985). Two of these proteins, the one from bovine brain (Berl et al., 1983) and one from porcine brain (Nishida et al., 1984a), have properties which are similar to those reported previously for the partially purified chick brain protein. Other proteins with similar actin depolymerizing properties have been found in starfish oocytes (Mabuchi, 1983), porcine kidney (Nishida et al., 1985), and Acanthamoeba (Cooper et al., 1986).

Recent studies which used an antibody to chick brain ADF have shown that this protein is found in substantial amounts in a variety of chick tissues and that immunologically cross-reactive proteins occur in birds and mammals (Bamburg & Bray, 1987). The major calcium-independent regulator of actin assembly in a cultured fibroblast cell line (BHK/C21 cells) is ADF (Koffer et al., 1988). Thus, proteins of this type appear to be ubiquitous in eukaryotes and probably play a general role in the regulation of actin polymerization.

Although there have been several studies on the interactions of a number of these ADFs with actin, most purification

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[1] Abbreviations: ADF, actin depolymerizing factor; SDS, sodium dodecyl sulfate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; DTE, dithioerythritol; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid).

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