Purification of the Xenopus laevis Double-stranded RNA Adenosine Deaminase* 

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A double-stranded RNA adenosine deaminase that catalyzes the conversion of adenosines to inosines in duplex RNA substrates was purified to near homogeneity from Xenopus laevis eggs. The final specific activity was ~2.9 mmoles of inosine min⁻¹ mg⁻¹ at 25 °C and pH 7.9 with a 704-base pair RNA substrate. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis revealed a single major ~120-kDa protein band by silver staining. The purified enzyme migrated with an apparent molecular mass of 90 × 10⁶ kDa during high performance liquid chromatography. Gel filtration of the partially purified enzyme gave an apparent molecular mass of 210 ± 20 kDa, suggesting that the enzyme may dimerize or associate with other cellular components. Substrate modification was inhibited by excess substrate, thiol reagents, heparin, and moderate concentrations of monovalent cations.

A daRNA unwinding/modifying activity, first identified in Xenopus laevis embryos, was originally thought to unwind duplex RNA formed after microinjection of antisense RNA (1, 2). The apparent unwinding was later demonstrated to be a hallmark modification of adenosines to inosines (3, 4) by hydrolytic deamination of purine C-6 (5). In vitro studies show that >100 bp of dsRNA are preferred substrates, yet smaller intramolecular (23 bp) or intramolecular (15 bp) dsRNAs are also modified (6).

The activity is released from Xenopus oocyte nuclei into the cytoplasm at germinal vesicle breakdown and is stable in whole egg homogenates (1, 3). It is also present in HeLa cells (7) and in the nuclear fractions of a broad spectrum of mammalian cell and tissue extracts (8). A recent report describes cytoplasmic activity in follicular cells during choriongenesis in silk moths (9), leaving open the possibility of an extranuclear role.

Several possible functions of A to I conversion have been suggested, including commitment of the substrate RNA to deg

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3 The abbreviations used are: daRNA, double-stranded RNA; bp, base pairs; daRAD, double-stranded RNA adenosine deaminase; BSA, bovine serum albumin; PFLC, fast protein liquid chromatography; ssRNA, single-stranded RNA; HPLC, high performance liquid chromatography; TBS, Tris-buffered saline; CAT, chloramphenicol acetyltransferase; PAGE, polyacrylamide gel electrophoresis; PKR, RNA-dependent protein kinase.

4 L. Baccamanno and B. L. Bass, manuscript submitted for publication.

5 Radiation, an antiviral role, modulation of translational efficiency, and RNA editing (for review, see Ref. 10). As a first step toward exploring potential biological functions and developing a minimal in vitro system, we have undertaken bulk purification of the enzyme.

This report describes the isolation of an ~120-kDa polypeptide from Xenopus eggs, hereafter referred to as the dsRNA adenosine deaminase (dsRAD). The enzyme is a minor cellular protein that was substantially purified by anion-exchange and RNA affinity chromatography of the 100,000 × g supernatant. Subsequent affinity chromatography produced a nearly homogeneous protein preparation that remained essentially unmodified after an additional purification step by hydrophobic interaction chromatography.

EXPERIMENTAL PROCEDURES

Materials—Trizma (Tris base), acrylamide, bisacrylamide, dithiothreitol, EDTA, protease inhibitors, proteinase K, nucleoside triphosphates, and PI nucleases were purchased from Boehringer Mannheim. Pregnant mare gonadotropin was from Biosynth B. V. Acetylated BSA (ribonuclease-free) was from Life Technologies, Inc. Radiomutases were from DuPont NEN. Single-stranded V-linked agarose, poly(1-C) (type 6), and FPLC accessories were from Pharmacia LKB Biotechnologie Inc. RNAase was from Promega, Human chorionic gonadotropin, CNBr-activated Sepharose 4B, poly(G), poly(C), dsDNA-cellulose, dsDNA-cellulose, DNA (salmon sperm), and RNA (tobacco yeast) were obtained from Sigma. Toyopearl TSK resins and a G4000PW HPLC column were purchased from Toyo Asahi. X. laevis was from Xenopus 1 (Ann Arbor, MI).

Buffers—Tris-buffered saline (TBS), which was used for all purification steps, contained 10 µM Tris-HCl, pH 7.9, 20% (v/v) glycerol, 25 mM KCl, 1.0 mM MgCl₂, 1.0 mM dithiothreitol, and 0.1 mM EDTA. Standard assay buffer contained 40 mM Tris-HCl, pH 7.0, 5% glycerol, 25 mM KCl, 10.0 mM NaCl, 1.3 mM MgCl₂, 1.0 mM dithiothreitol, 5.0 mM EDTA, 0.2 mM ATP, 800 units/ml RNAsin, 25 µg/ml acetylated BSA, 1.0 µg/ml DNA, and 1.0 µg/ml RNA. Activity Assays—The substrate for all assays was a 794-base dsDNA formed between the sense and antisense RNAs of chloramphenicol acetyltransferase (CAT). The dsRNA was internally labeled with α-32P-ATP (2000 Ci/m mole) and prepared as described, except the RNAs were not capped (1). Activity assay conditions for fractions after TSK-DEAE-650M chromatography were as follows: 5 µl of the appropriate column fraction and 10 fmol of α-32P-labeled CAT duplex RNA in 100 µl of standard assay buffer incubated for 1–3 h at 25 °C. The TSK-DEAE-650M column fractions were assayed at 1:20 dilution with 5 fmol of CAT dsRNA for 2 h at 25 °C in standard assay buffer (final volume of 20 µl) with additional carrier protein, DNA, and RNA 125 µg/ml BSA, 5 µg/ml DNA, and 5 µg/ml RNA. In addition, for each purification step, various amounts (0.1–5 µl) of the active pools were mixed with 20,000 cpm CAT dsDNA (2–50 fmol) in 20 µl of reaction buffer to estimate maximum activity, calculated from 100% to 1 conversion. All reactions were quenched by addition of 0.33 volume of 3 × proteinase K/SDS buffer stock (1 × stock = 2 mg/ml proteinase K, 0.25 µg/ml Triton X-100, 25 mM EDTA, and 2.5% SDS) and incubation for 1 h at 37 °C, followed by extraction with phenol and chloroform; RNA was precipitated with ethanol for subsequent analysis. Reaction products were assayed by electrophoresis on 4% polyacrylamide gels or by TLC in cellulose plates as described (1, 3). Quantitative image analyses were done using a Molecular Dynamics Model 4000 PhosphorImager with Image Quanti software.
Xenopus dsRNA Adenosine Deaminase

RNA Affinity Column Preparation—Poly-L-lysine acid and poly(γ-glutamic acid were coupled to cyanogen bromide-activated Sepharose 4B according to standard procedures (11) in 0.1 M NaHCO3 and 0.5 M NaCl, pH 8.0, to 0.94 mg of RNA/ml of resin determined by A260 difference measurements. Unreacted sites were blocked with 1 M ethanolamine, pH 9.0. Equal volumes of poly(G)-Sepharose and poly(C)-Sepharose were washed with 50 mL Tris-HCl, 100 mM NaCl, and 20 mM EDTA, pH 8.0, combined, and mixed overnight at 4°C. A DE-2 protein solution, from which the deaminase was removed by prior poly(G + C) affinity chromatography, was mixed with the matrix (3 mg of resin) to block nonspecific binding sites, followed by washes in TBS and in TBS plus 0.5 M NaCl and then re-equilibration in TBS – 0.2 X NaCl until the eluent was free of A260 absorbing material. This matrix was stable for repeated use for at least 1 year. The column was stored at 4°C in TBS supplemented with 1.0 M NaCl and 0.02 mM EDTA.

Purification of the dsRNA Adenosine Deaminase—Frogs were injected (11) by eggs by giving a sperm injection (premature egg gonadotropin, 50 units), followed by a booster injection (human chorionic gonadotropin, 50 units) 4 days later. Eggs were collected during the ensuing 20-24-h period in 20 mL NaCl. Two separate batches of eggs (combined total of 4.7 liters), each from 72 frogs, were collected 10 weeks apart. The S100 supernatant prepared as described (1) from each batch of de-jellied eggs (12) was clarified again at 100,000 g for 90 min after extended dialysis for 3 days at 4°C against three changes of TBS buffer (13). The clarified supernatant was dialyzed against 0.1 M potassium phosphate (pH 6.8) and 0.9% NaCl in 0.001 M EDTA. The clarified supernatant was loaded on a 1.6 × 60-cm column of Sephacryl S100 superfine and 100 μL containing the AF-Blue-650 M active pool in 2% (NH4)2SO4 was loaded onto a 1.6-mL Blue-650 M column (HR 5/20) equilibrated in TBS at 0.1 M/m in and then developed with a 40-mL 0.0–1.0 M NaCl gradient at 0.5 mL/min into 1.0-mL fractions. The active fractions obtained between 0.28 and 0.43 M NaCl were pooled (15 mL) and made 25% (v/v) (NH4)2SO4 by the addition of saturated ammonium sulfate.

Phosphorylation—Approximately 20 mL containing the AF-Blue-650 M active pool in 2% (NH4)2SO4 was loaded onto a 1.6-mL Blue-650 M column (HR 5/20) equilibrated in TBS plus 25% (NH4)2SO4 at 1.0 mL/min and then developed with a 1.25-mL 25–45% (NH4)2SO4 gradient, at 0.5 mL/min. The 4–mL active pool (12%–ammonium sulfate) was dialyzed extensively against TBS. The final preparations from the DE-1 and DE-2 parallel purifications were designated phosphoryl-1 and phosphoryl-2, respectively.

Analytical HPLC—200 μL of partially purified dsRAD (see below) and the final purified dsRAD preparation (pH 6.8) were subjected to HPLC. A GPC 6000PW column (7.8 × 30 cm) equilibrated in TBS was pumped at a flow rate of 0.5 mL/min using a Beckman System Gold 126 HPLC apparatus in line with a Model 168 diode array detector monitoring 260 and 230 nm. Fractions (250 μL) were assayed for dsRNA deaminase activity, and the positions of the active peaks were compared to a standard curve generated with the following proteins: thyroglobulin (670 kDa), IgG (160 kDa), acetylated BSA dimer (120 kDa), hemoglobin (64.5 kDa), and cytochrome c (12,500 kDa).

Partial Purification of dsRAD—Partial purification of dsRAD was conducted for different purposes as follows. (a) To characterize inhibitors of the deaminase reaction (see Table II), we used a DE-2 dsRAD pool that was salt-fractionated and concentrated by precipitation in ammonium sulfate solution (ammonium sulphate, fractionation), followed by gel-f density gradient centrifugation (10–40%) as described (13) for 28 h in an SW 28 rotor at 25,000 rpm (9–18 S activity pool). (b) To ascertain dsRAD binding preferences for various RNA-Sepharose mixtures (see Table III), and to determine the size of partially purified dsRAD by analytical HPLC on GPC 6000PW columns(Fig. 4), we used ammonium sulfate-concentrated dsRAD (from DE-2) subsequently purified by gel filtration on TSK-HW 55 (2.5 × 90 cm). (c) To determine the apparent sizes of dsRAD obtained from the DE-1 and DE-2 pools after RNA affinity chromatography, sedimentation studies were done as described above, except that the ammonium sulphate-concentrated pools were first step-fractionated (total bound material, eluted in 3.5 mL NaCl) on poly(G + C). (d) To study further the activity of smaller dsRAD forms identified in c above, the DE-2 pool was bulk-purified after RNA affinity chromatography by gel-gel density gradient centrifugation for a longer period (40 h), followed by RNA affinity chromatography of the active pool (4–5.6 S). (e) To study the effect of smaller dsRAD forms identified in c above, the DE-2 pool was bulk-purified after RNA affinity chromatography by gel-gel density gradient centrifugation for a longer period (40 h), followed by RNA affinity chromatography of the active pool (4–5.6 S) obtained from 10–40% gel fractions (see Fig. 5).

Miscellaneous Protein Methods—Discontinuous SDS-PAGE (33.6% C) was performed by the method of Laemmli (14), and SDS gels were silver-stained as described (15). Gel images were captured with a 35-mm f 1.7 monochrome video camera coupled to a Macintosh Ii using NIH Image 1.49 for densitometric analysis. Protein concentration in crude solutions was estimated by the method of Bradford (16) using Coomassie (see Table IV). For the very dilute fractions obtained after poly(G + C) affinity chromatography, protein concentration was estimated by absorbance (A260) using an average A260 = 10 for an ~1 mg/mL solution. This value was determined by assuming that an ~1 mg/mL crude protein solution gives an absorbance of 1 A260 unit and averaging the 220/280 nm extinction coefficient ratios of five standard proteins (range of 0.6–1.79). Estimates of relative protein concentration were further refined by trypsinogen fluorescence measurements (excitation at 280 and emission at 340 nm) of the dilute fractions and denative-
RESULTS

Purification of the dsRNA Adenovirus Deaminase—The double-stranded RNA adenovirus deaminase, derived from 4.7 liters of Xenopus eggs, was purified ~8000-fold with respect to the S100 supernatant (Table 1). A 784-base pair dsRNA internally labeled with [α-32P]ATP was the substrate to monitor deaminase activity during purification. Two assays were used to identify activity profiles and to estimate total yields. Quantitative data (e.g., Table 1) were obtained by PI nucleic acid digestion of the dsRNA reaction products, followed by TLC to determine the proportion of 5'-[α-32P]AMP converted to 5' α-32P]AMP. A gel mobility assay provided an alternative and more rapid method for the identification of active column fractions. The latter assay relies on the fact that the modified dsRNA substrate is retarded on polyacrylamide gels approximately in proportion to the number of mismatched I U base pairs (3).

The purification consisted of five steps, which are outlined in Table 1. A profile of the first chromatographic step, TSK-DEAE-650M, using the gel mobility assay is shown in Fig. 1. Two peaks of activity were recovered by TSK-DEAE-650M chromatography of S100 extracts prepared from Xenopus eggs (Fig. 1) or embryo nuclei (data not shown). Similar results were also obtained with nuclear fractions from human placenta and bovine liver (data not shown). Although the amount of deaminase activity recovered in the first peak (DE-1) was variable, the second peak (DE-2), whether obtained from amphibian or mammalian sources, consistently eluted in TBS plus 0.17-0.23 mM KCl and contained most of the total activity. We do not know if the two active peaks obtained by ion-exchange chromatography on TSK-DEAE-650M are true isomers. Isolation of two regions of activity was not merely the result of overloading the column, however, as activity was recovered at the same respective positions after rechromatography of the two peaks. In the purification from Xenopus eggs described here, both DEAE pools were carried independently through succeeding purification steps and displayed identical elution characteristics (Table 1: poly(G + C)-Sepharose (0.8-1.2 mM NaCl), AF-Blue-650M (0.28-0.43 mM NaCl), and phenyl-650M (12-25% (NH4)2SO4).

Aliquots from each step of the purification were analyzed on an 8% SDS-polyacrylamide gel and visualized by silver staining (Fig. 2). The final preparations, phenyl-650M-1 and phenyl-650M-2 (derived from DE-1 and DE-2, respectively), contained a single major polypeptide (~120 kDa) and minor bands ranging in size from 96 to 112 kDa. The ~120-kDa protein first appeared as a prominent band after RNA affinity chromatography on poly(G + C), a 1:1 mixture of single-stranded poly(G)-Sepharose and poly(C)-Sepharose beads. Since deaminase activity eluted from poly(G + C) at the trailing edge of a broad protein peak, the active region was divided approximately in half into two pools (GC-A and GC-B) to assure purity and specific activity across the peak. It can be seen in Fig. 2 that similar amounts of an ~120 kDa polypeptide were present in both poly(G + C) pools. Densitometric measurements of silver-stained 8% (Fig. 2) and 11% (not shown) SDS-polyacrylamide gels confirmed that the relative amount of the ~120-kDa band at each step in the purification directly correlated with relative activity measurements. Overall, the protein complement changed very little in the last two steps of the purification, AF-Blue-650M and phenyl-650M chromatography. The specific activity also remained nearly constant in these final purification steps.

The amount of the ~120-kDa protein correlated with measured dsRAD activity and argued against consideration of the minor SDS-PAGE bands (96-112 kDa) in the purified preparations as dsRAD candidates. For example, comparison of the relative amount of the ~120-kDa band in the phenyl-650M lanes by densitometry gave a phenyl-2:phenyl-1 ratio of ~4, whereas a similar comparison between the 96-112-kDa group gave a ratio of ~0.7. Only the ratio for the ~120-kDa bands agrees with the measured activity difference (Fig. 3). The kinetic data in Fig. 3 also show that both preparations had similar thermal optima (~30 °C). The activity of both dsRAD preparations was stimulated by brief incubation at 37 °C (~20 min), but decayed significantly after 1 h (data not shown). Experiments using partially purified deaminase also showed slightly more deamination at 37 °C relative to 25 °C during a 1-h incubation, but showed nearly complete loss of activity by prior incubation for 1 h at 37 °C in the absence of substrate (data not shown).

The phenyl-2 pool also contained a minor ~30-kDa band (dye front in Fig. 2) as revealed by 11% SDS-PAGE (data not shown), which can be excluded as a deaminase candidate since it is not present in the final phenyl-1 preparation. Although various bands in the range of ~30-40 kDa were frequently, but inconsistently, found to co-migrate with dsRAD in the DE-1 and DE-2 pools, they typically eluted slightly after the activity peak from poly(G + C). Furthermore, a highly purified preparation obtained by an independent protocol, culminating in affinity chromatography on poly(A + G + U), contained two major polypeptides (~145 and ~60 kDa) and two less abundant polypeptides (~120 and ~100 kDa) in addition to several minor bands (~40-60 and ~18 kDa) as shown by silver staining after 6-12% SDS-PAGE (data not shown). The specific enrichment of the ~120-kDa band in the present purification is again consistent with its identification as dsRAD.

Analytical HPLC of the Phenyl-650M-purified Deaminase—A partially purified dsRAD preparation (see "Experimental Procedures") as well as the highly purified phenyl-1 and phenyl-2 enzyme preparations were subjected to HPLC on G4000PWXL to ascertain the size of the deaminases and to purify further the active species in the phenyl-650M preparations. The elution position of the purified enzymes obtained from phenyl-1 and phenyl-2 corresponded to a mean size of ~90 ± 10 kDa (Fig. 4). In contrast, when partially purified dsRAD was similarly fractionated, the activity profile depicted a much larger size range of 160-270 kDa (mean of ~215 kDa).
**Fig. 1. TSK-DEAE-650M chromatography of S100 extract.** Proteins bound to TSK-DEAE-650M (2.6 x 83 cm) were resolved with a 0.0-0.3 M KCl gradient and fractions were assayed as described under "Experimental Procedures." Reaction products of assays performed with 1 µl of the even-numbered column fractions were electrophoresed on 4% acrylamide/Tris borate/EDTA gels for 60 V-h. The autoradiograph of the dried gel shows two activity peaks that eluted at positions separated by a factor of 2 in overall lane strength: DE-1 eluted in TBS + 85-86 mM KCl, and DE-2 eluted in TBS + 170-230 mM KCl. Shown are control reactions, from left to right: no enzyme (0), full modification with a 1:2 dilution (10 µl) of S100 (5), and no apparent activity in the DEAE breakthrough volume (B) at 1:2 dilution (10 µl). A complete reaction corresponded to ~50% A to 1 modification shown by the slowest migrating radiolabeled dsRNA.

**Fig. 2. SDS-PAGE of purified dsRNA adenosine deaminase fractions.** Various amounts of pools obtained at each step in the purification (Table I) were resolved by 8% SDS-PAGE, fixed, and stained with silver. **First lane, S100, 1 µg; second and third lanes, equal volumes of the 200-ml starting DEAE pools DE-1 (~2.7 µg) and DE-2 (~1 µg), respectively; fifth and sixth lanes, leading one-half of polyG + C peak (GC-A (60 µl)) and trailing one-half of polyG + C peak (GC-B (60 µl)) derived from DE-2, respectively; seventh lane, AP-Blue-650M pool (10 µl) from DE-2; ninth and tenth lanes, 125 µl of the final phenyl-650M-1 and phenyl-650M-2 derived from DE-1 and DE-2, respectively.** Lanes marked Buffer contained 120 µl of TBS + 40 µl of SDS sample buffer as controls for artifact bands (+, arising from epidermal keratins (38) and aggravated by large sample volumes. The poly(G + C) and AP-Blue-650M pools from the DE-1 parallel purification are not shown since 11% SDS-PAGE revealed very little protein present in aliquots of these pools maintained on ice during isolation of the phenyl-1 deaminase. Re-examination of the DE-1 intermediate pools (10 times concentrated by trichloroacetic acid precipitation with cytochrome c as carrier protein) revealed only smaller polyepptides (<56 kDa) after silver staining, suggesting that substantial proteolysis had occurred (see "Experimental Procedures"). The estimated molecular masses (~2 kDa) of bands in the AP-Blue-650M and phenyl-650M fractions relative to markers (β-galactosidase (116 kDa), phosphorylase b (97.4 kDa), BSA (66 kDa), ovalbumin (45 kDa), and glyceraldehyde-3-phosphate dehydrogenase (36 kDa)) were 122, 112, 107, 105, and 96 kDa, respectively.

The HPLC results are consistent with other observations made during these studies that suggest a change in molecular size after RNA affinity chromatography. For example, prior to RNA affinity chromatography, sedimentation in 10-40% glycerol gradients produced a dsRAD activity profile with an apparent sedimentation coefficient of 9-10 S, and gel filtration on TSK-HW55(F) gave an apparent molecular mass of 210 ± 20 kDa (data not shown). However, sedimentation of poly(G + C)-purified dsRAD (from either DE-1 and DE-2; data not shown) revealed an activity peak about midway (~6 S) between

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[Institution’s City, State, Zip]

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two marker proteins (IgG, 6.9 S; and hemoglobin, 4.5 S), consistent with the estimated molecular mass of 90 ± 10 kDa obtained by HPLC of the final preparations (Fig. 4). Therefore, size analyses done prior to RNA affinity chromatography repeatedly gave higher estimates, suggesting either dimerization of the enzyme or association with other, unknown cellular components, while even under the low ionic strength conditions used for gel filtration on G4000PWG3 (Fig. 4), purified dsRAD did not self-associate. Thus, it appears that RNA affinity chromatography produced monomeric forms of the deaminase that remained active.

Of importance, for both phenyl-1 and phenyl-2 preparations, HPLC partially resolved the contaminating ~96-kDa band from the ~120-kDa band, and the peak fractions of dsRAD activity precisely corresponded with the distribution of the ~120-kDa band (data not shown). The apparent specific activities of the HPLC active peaks from phenyl-1 and phenyl-2 preparations were estimated to be ~1.6 and ~1.8 nmol of inosine min⁻¹ mg⁻¹ at 25 °C and pH 7.9, respectively.

**Characterization of Partially Purified dsRAD—Prior to the full purification, dsRAD was characterized using partially purified enzyme (see “Experimental Procedures”). These studies were helpful in the development of the final purification scheme, providing general information on the properties of dsRAD. Table II shows that modification of duplex RNA was completely inhibited by thiol reagents, the basic histone proteins and lysosome, spermidine, heparin, and moderate concentrations of monovalent cations (Na⁺, K⁺, NH₄⁺). In contrast, the reaction was unaffected by the acidic proteins BSA and casein, polyglutamic acid, DNA and RNA, the divalent cations Mg²⁺ and Ca²⁺, EDTA, EGTA, ATP, GTP, 5′-AMP, adenosine, and hypoxanthine. A broad pH optimum (~6.0–8.5) was observed in either Tris or phosphate buffers (data not shown).

A number of RNA affinity matrices were also tested prior to the full purification. As shown in Table III, heparin, poly(I), poly(G + C), and bona fide dsRNA (poly(I-C)) all bound dsRAD in partially purified preparations. The most significant improvement in protein purification occurred with poly(G + C). Therefore, this matrix was used during bulk purification. In separate experiments not shown, all mixtures containing poly(I), or bases with the potential to form base pairs (A + U, A + G + U, and G + C), gave good yields. Interestingly, passage of partially purified deaminase over any mixture containing poly(U) activated a single-stranded ribonuclease evident by degradation of modified, but not unmodified, dsRNA in reaction mixtures incubated without RNAsin (data not shown).

The mechanism of binding of the deaminase to a mixture of poly(G + C) is not known. Presumably, it is located to limited regions of dsRNA (or other complex RNA structures) formed upon mixing of the two affinity resins because partially purified dsRAD does not bind poly(C) alone and only weakly interacts with poly(G) (Table III). It is noteworthy that partially purified preparations of dsRAD contained dsRNA-binding proteins in addition to dsRAD (identified by gel-shift and Northwestern analyses) that also bound the poly(G + C) matrix (data not shown). Since activated Sepharose 4B contains potential ssRNA linkage sites distributed throughout the matrix, regions available for hybridization between covalently linked complementary polyribonucleotides are limited to a small portion of the total surface area where the packed beads adjoin one another. This limitation may be one reason for the selectivity of the poly(G + C) matrix under our chromatographic conditions.
Table II
Inhibitors of dsRNA adenosine deaminase

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Conc</th>
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<tbody>
<tr>
<td>L-koic acid</td>
<td>10 μM</td>
</tr>
<tr>
<td>NEM</td>
<td>10 μM</td>
</tr>
<tr>
<td>p-HMPS</td>
<td>1.0 μM</td>
</tr>
<tr>
<td>ZnCl₂</td>
<td>1.0 μM</td>
</tr>
<tr>
<td>TLCX</td>
<td>1.0 μM</td>
</tr>
<tr>
<td>Heparin</td>
<td>0.5 mg/ml</td>
</tr>
<tr>
<td>Spermide</td>
<td>0.5 mg/ml</td>
</tr>
<tr>
<td>Basic proteins (histones and lysozyme)</td>
<td>0.5 mg/ml</td>
</tr>
<tr>
<td>KCl and NaCl</td>
<td>0.2 μM</td>
</tr>
<tr>
<td>Potassium and sodium phosphate</td>
<td>0.2 μM</td>
</tr>
<tr>
<td>Ammonium acetate</td>
<td>0.4 μM</td>
</tr>
</tbody>
</table>

No inhibition

DNA (salmon sperm) | 0.3 mg/ml
RNA (torula yeast) | 0.3 mg/ml
Acidine proteins (BSA and casein) | 2.0 mg/ml
Polyglutamic acid | 2.0 mg/ml
Glutamic acid | 2.0 mg/ml
Glutamine | 2.0 mg/ml
Glyceraldehyde 6-phosphate | 2.0 mg/ml
CaCl₂ and MgCl₂ | 5.0 mg/ml
EDTA and EGTA | 10 μM
ATP and GTP | 10 μM
5′-AMP adenine, hypoxanthine | 10 μM

*NEM, N-ethylmaleimide; p-HMPS, p-hydroxymercuriipropionio sulfonic acid; TLCX, N′-p-tosyl-L-lysine chloromethyl ketone.

Table III
Affinity chromatography of partially purified dsDNA adenosine deaminase

Aliquots (~0.9 ml, 2.8 mg) of partially purified dsRAD (see "Experimental Procedures") were loaded onto 1.6-ml columns containing heparin-Sepharose or various RNA homopolymer-linked agarose columns in TBS. After elution, the breakthrough and bound (eluted with TBS + 3.5 mM NaCl) fractions were analyzed under standard conditions for 2 h at 25°C (Experiment 1). Active breakthrough fractions from Experiment 1 were pooled, and aliquots (~1.8 ml, 1.4 mg) were chromatographed and assayed similarly (Experiment 2).

<table>
<thead>
<tr>
<th>Column</th>
<th>dsRAD activity</th>
<th>Specific activity change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exp. 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heparin-650 M</td>
<td>-</td>
<td>+ +</td>
</tr>
<tr>
<td>Poly(A)</td>
<td>+++</td>
<td>-</td>
</tr>
<tr>
<td>Poly(C)</td>
<td>+++</td>
<td>-</td>
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<tr>
<td>Poly(U)</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td>Poly(Y)</td>
<td>Trace</td>
<td>+</td>
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<td>Exp. 2</td>
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<tr>
<td>Poly(G)</td>
<td>++</td>
<td>Trace</td>
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<td>Poly(C)</td>
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<tr>
<td>Poly(U)</td>
<td>Trace</td>
<td>+</td>
</tr>
<tr>
<td>Poly(LC)</td>
<td>+++</td>
<td>-</td>
</tr>
<tr>
<td>Poly(G + C)</td>
<td>-</td>
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Substrate Inhibition—An early observation that significantly affected our ability to monitor deaminase activity in column fractions was the apparent substrate inhibition of A to I conversion. It can be seen in Fig. 5 that excess substrate inhibited production of inosine. This property was observed in pools obtained at each stage of the purification. In no case, however, have we observed significant skewing of activity peaks as a function of substrate concentration. Focusing the peak active fractions by substrate inhibition (Fig. 5) proved to be a useful technique for the identification of the most likely dsRAD candidates by subsequent SDS-PAGE.

Discussion
We have shown that the dsDNA adenosine deaminase purified from Xenopus egg extracts is an ~120-kDa protein by SDS-PAGE, with a native size of 90 ± 10 kDa by gel filtration. The purified monomeric form of dsRAD is active, but it is not known if the enzyme functions strictly as a monomer. It may dimerize in the presence of substrate or associate in crude mixtures with other cellular constituents as suggested by gel filtration and sedimentation studies with partially purified preparations (Fig. 4). Under our assay conditions, deamination of adenosines in CAT dsDNA using purified dsRAD proceeded with an apparent kcat of ~0.2 min⁻¹. No obvious differences have yet been detected between partially purified dsRAD and purified dsRAD, except for apparent size.

The dsDNA adenosine deaminase is the ~120-kDa protein band as shown by SDS-PAGE (Fig. 2) is further corroborated by the identification of an ~115-kDa protein with dsDNA.
adenosine deaminase activity from calf thymus. Moreover, the two additional enzyme preparations reported here were isolated from distinctly different starting protein populations (DE-1 and DE-2), yet they appear nearly identical. This conclusion is strengthened by experiments showing identical A to T conversion patterns in a 36-bp dsDNA by the purified deaminase and partially purified Xenopus preparations.

The apparent larger size of dsDNA determined by SDS-PAGE compared to the size determined by gel filtration is not an uncommon observation. Post-translationally modified proteins (e.g., glycopeptides, lipoproteins, and phosphorylated proteins) and proteins that are unusual, such as very basic or acidic proteins, migrate anomalously during SDS-PAGE (17).

For example, the acidic protein U, an abundant heterogeneous nuclear ribonucleoprotein, contains long tracks rich in glutamic acid residues (18). Protein U is an 860-amino acid polypeptide of 88,939 Da that migrates during SDS-PAGE as a 120-kDa band. Small fragments of protein U (<60 to 120 kDa) have been revealed by immunoblots with two different monoclonal antibodies (18–20). By analogy, it is quite possible the smaller polypeptides in our preparations are dsDNA fragments. In many respects, dsDNA appears similar to protein U. The hallmark of protein U, however, are binding to dsDNA, celluose and enhanced ability to bind poly(G) in the presence of 0.5 mM NaCl (20), properties not shared by the dsDNA adenosine deaminase.

The substrate inhibition we have observed in these studies may not be physiologically relevant, but nonphysiological substrate inhibition is frequently observed in vivo with purified enzymes and can be useful for mechanistic studies (21). Our results cannot be explained by product inhibition as production of inosine-containing duplex RNA was inhibited by excess unmodified duplex RNA (Fig. 5). Rather, the substrate inhibition results are reminiscent of the inhibition of dsDNA-activated protein kinase (PKA, DAI, Dsl, 68k, or PI kinase) by excess dsDNA (22–26). Like dsDNA, PKR is a dsDNA-binding protein, and recent work demonstrates that both dsDNA-binding motifs are responsible for the ability of PKR to bind dsDNA (26). Given the similarity between dsDNA and PKR with respect to dsDNA binding and substrate inhibition, it will be of interest to see if dsDNA also contains dsDNA-binding motifs (27). Our preliminary protein sequencing results suggest that the NH2 terminus of dsDNA is blocked, so we are simultaneously proceeding to obtain internal sequence and mouse antibodies for cloning and comparison with other dsDNA-binding proteins.

Other studies suggest a further relationship between PKR and dsDNA. For example, treatment of HeLa cells with poly(I:C), which activates PKR, results in the apparent inactivation of dsDNA (28). Similarly, pluripotent P19 embryonal carcinoma cells induced to differentiate accumulate endogenous dsDNA and show a reduction of dsDNA activity coincident with PKR activation (29). At present, the meaning of the observations suggesting that PKR activation correlates with dsDNA inactivation is unclear. The purification described here will make it possible to approach some of the questions related to dsDNA inducible phenomena with direct biochemical experiments using purified components (see Ref. 30 for dsDNA review).

Some studies suggest that additional factors in vivo may alter Xenopus dsDNA specificity or assist in some way to promote site selection for specific adenosine deamination (31). Many mechanisms can be envisioned, which need not be mutually exclusive, invoking multimeric complexes. In this regard, studies of editing of apolipoprotein B messenger RNA, which is also thought to occur by deamination (C to U), may be relevant. While some studies suggest that this editing event requires more than one protein (32–34), it remains possible that the sequence recognition and catalytic domains reside in a single protein, with other proteins modulating specificity and editing activity. In vivo (35). Although the dsDNA adenosine deaminase appears to consist of a single protein, in vivo modification of natural substrates may, in some cases, require other factors and may differ from the promiscuous modification of CAP duplex RNA we have exploited to purify the enzyme.

Finally, experiments described in a recent report make it highly likely that dsDNA is the enzyme responsible for editing glutamate receptor mRNAs in mammalian cells (36). The observed RNA editing is dependent on a dsDNA structure formed between exon 1 and in-frame sequences and changes a genomically encoded A to a nucleotide that appears as a G in a CDNA. Since G and I both prefer to pair with C, the sequence change is consistent with an inosine in the edited RNA (see Ref. 37). Although we suspect that dsDNA may have multiple biological functions, these recent experiments suggest the exciting possibility that the isolation of dsDNA represents the purification of an RNA editing enzyme and further sets the stage for studying glutamate receptor RNA editing in vitro.

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