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On the Molecular Weight and Subunit Composition of Calf Thymus Ribonuclease H1†

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ABSTRACT: We have reinvestigated the molecular weight and subunit composition of calf thymus ribonuclease H1. Earlier studies suggested a variety of molecular weights for the enzyme in the range of 64K–84K and reported that the enzyme either was a single polypeptide of 74 kDa or consisted of from two to four subunits in the range of 21–34 kDa. Although we too find bands in this lower molecular weight range in our highly purified preparations following SDS–PAGE, our data suggest that the native structure of RNase H1 is a dimer of 68-kDa subunits. The evidence includes the following: (1) Western blot analysis of fractions taken at various stages of the purification indicates that the predominant antigenic form of the enzyme in crude extracts has a molecular weight of 68K but that during purification in the absence of sufficient protease inhibitors a variety of lower molecular weight forms appear concomitant with the disappearance of the 68-kDa band. (2) Activity gel analysis of the highly purified enzyme prepared in the presence of a battery of protease inhibitors reveals that the 68-kDa band (as well as several bands of lower molecular weight) possesses RNase H activity. (3) The 68-kDa band recognized by Western blotting with anti-RNase H immune sera is not detected by using preimmune sera. Furthermore, when immune sera are used, a trace of a 140–150-kDa antigenic form can sometimes be detected, consistent with the existence of a dimeric form of the enzyme. (4) Gel filtration analysis of highly purified RNase H1 reveals that nearly all the observed RNase H activity is found at an elution volume corresponding to a molecular weight in the range of 140K–150K, although under the conditions of sucrose gradient centrifugation, the majority of the enzyme appears to sediment as a 68-kDa monomer. We therefore suggest that the proteolytic lability of RNase H1 may account for earlier reports that the enzyme contains low molecular weight subunits and that the enzyme is composed of a 68-kDa polypeptide which can dimerize and remain active.

Ribonucleases H (RNases H)[1] are enzymes that specifically degrade the RNA strand of RNA/DNA hybrids. They are not active against the DNA of the hybrid or against single- or double-stranded RNAs (Crouch & Dirksen, 1982). In bacteria, RNase H plays an important role in determining whether DNA replication proceeds either from the normal origin or from cryptic origins that are activated in the absence of RNase H (Kogoma, 1986), and also aids in removing the RNA primers from Okazaki pieces formed during lagging-strand DNA synthesis (Ogawa & Okazaki, 1984). The enzyme also plays an important role in the control of col E1 plasmid replication (Polisky, 1989). The role(s) of RNases

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[1] Abbreviations: EDTA, ethylenediaminetetraacetic acid; kDa, kilodalton(s); GdnHCl, guanidine hydrochloride; NP-40, Nonidet P40; PMSF, phenylmethanesulfonyl fluoride; RNase H, ribonuclease H; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; SRB, standard renaturation buffer; TCA, trichloroacetic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; 2-MCE, 2-mercaptoethanol.

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H in eukaryotic cells is (are) largely unknown, although several studies of RNases H have been reported in organisms suitable for genetic analysis such as yeast (Wyers et al., 1973, 1976; Karwan et al., 1983) and *Drosophila* (DiFrancesco & Lehman, 1985). The studies on yeast RNases H are particularly relevant to the present paper since Karwan et al. reported that their attempts to duplicate the earlier studies of Weyers et al. describing the purification of multiple low molecular weight forms of RNase H were unsuccessful, probably due to the proteolytic instability of RNase H. The yeast RNase H purified by Karwan et al. was a single polypeptide of molecular weight 68K. More recently, Karwan and Kindas-Mugge (1989) reported still other RNase H activities in yeast.

RNase H activity was first detected in calf thymus by Stein and Hausen (Stein & Hausen, 1969; Hausen & Stein, 1970). Busen and Hausen (1975) reported the existence of distinct forms of the enzyme from calf thymus that differed in molecular weight, ionic requirements for maximum activity, and sensitivity to inhibitors. The principal RNase H activity of calf thymus (RNase H1) has been variously reported to be a 76-kDa monomer (Haberkern & Cantoni, 1973), a 64-kDa dimer (Stavrianopolis & Chargaff, 1973, 1978), and an 80-kDa trimer or tetramer (Busen, 1980, 1982).

We are interested in understanding the in vivo role of RNase H1. During our studies, it became clear that the "subunits" described by earlier authors were probably proteolytic degradation products. We present here evidence that RNase H1 activity is associated with a dimer of a 68-kDa polypeptide that is readily subject to proteolytic degradation.

Materials and Methods

Materials

RNase H1 was purified from commercial calf thymus (Pel Freez) by a slight modification of the procedure of Stavrianopolis and Chargaff (1978). Buffers were as indicated in the original paper except that all buffers were supplemented with 1 mM PMSF, 1 μM pepstatin and leupeptin, 0.1 mM sodium tetrathionate, and 1 mM sodium hydrogen sulfite. Furthermore, the CM-Sephadex step used by Stavrianopolis and Chargaff to separate what they believed to be two closely related forms of RNase H was omitted, since the amount of activity attributable to RNase H2 was negligible in our preparations. We also omitted the final affinity chromatography step on oligo(dT)–cellulose since it produces only a very slight change in specific activity and the extra processing involved often led to low yields and degradation of the enzyme. Our final enzyme preparation had a specific activity of 2.2 $\times$ 10^6 units/mg. Some earlier preparations of the enzyme followed the cited procedure in omitting protease inhibitors but added to the steps utilized by Stavrianopolis and Chargaff several additional chromatography steps as noted in Figure 1. Poly(dT) was from Pharmacia and *Escherichia coli* RNA polymerase from New England Biolabs. ^{125}I Protein A was obtained from Amersham, protein molecular weight markers were from Bio-Rad, and Immobilon filters were from Millipore. SRB buffer for activity gel renaturation contains 50 mM Tris-HCl, pH 8.0, 0.1 mM EDTA, 1 mM 2-MCE, 10 mM $MgCl_2$, 100 mM NaCl, 10% glycerol, and 1 mM DTT.

Methods

Assay for RNase H. RNase H activity was assayed by the conversion of the radioactivity of [^{3}H]poly(rA)$_m$·poly(dT)$_n$ into acid-soluble form (Carl et al., 1980). One unit of RNase H activity corresponds to the release of 1 nmol of [^{3}H]AMP to an acid-soluble form in 15 min at 37 °C. The preparation of the substrate was described (Arendes et al., 1982). Protein was measured by the Bradford method (Bradford, 1976) using the reagent from Bio-Rad.

SDS–PAGE and Activity Gel Measurements. Slab gel SDS–PAGE was carried out as described (Laemmli, 1970). Following electrophoresis, gels were stained with Coomassie Brilliant Blue R-250 (Stephano et al., 1986). To identify polypeptides having RNase H activity, activity gels were prepared by incorporating [^{32}P]poly(rA)$_m$·poly(dT)$_n$ (300 000 cpm per 13 $\times$ 15 cm gel) into a 10% running gel. Samples were boiled in sample loading buffer for 2 min prior to loading. Following electrophoresis, the bulk of the SDS was removed from the samples by washing the gel with three changes of 25% 2-propanol in 50 mM Tris-HCl, pH 8.0, 1 mM 2-MCE, and 0.1 mM EDTA for 20 min each. Following this, the 2-propanol was removed by washing twice for 15 min each with 0.01 M Tris-HCl, pH 7.5, and 5 mM 2-MCE. At this point, gels containing proteins to be renatured with GdnHCl were washed with 6 M GdnHCl in SRB for 2 h followed by washing with SRB alone for 20 h with three changes of buffer. Gels with proteins to be renatured with NP-40 were treated for 2 h with SRB alone followed by washing with SRB plus 2.5% NP-40 for 20 h with three changes of buffer. Finally, gels were fixed, and the last traces of acid-soluble radioactivity were removed by washing 4 times with 5% TCA/1% sodium pyrophosphate for 15 min each. After the last wash, the gels were left for 21 h in the TCA solution. All washes were at room temperature using about 200 mL of buffer per 13 $\times$ 15 cm gel with gentle agitation. Finally, the gels were dried and autoradiographed at −70 °C. Marker proteins were run on the same gel and stained after transfer with India ink.

Preparation of Antisera. Protein (0.5 mg) of a highly purified RNAse H1 preparation that contained no detectable 68-kDa band was emulsified with Freund's complete adjuvant and injected subcutaneously at multiple sites into a New Zealand White rabbit. The rabbit was given two booster injections of 0.2 mg of protein emulsified with Freund's incomplete adjuvant at approximately 3-week intervals. The animal was bled periodically thereafter and the collected serum stored at −70 °C.

Western Blot Analysis. Crude thymus extracts were prepared by extraction in a blender at medium speed [1 part thymus to 2 parts extraction buffer (50 mM Tris-HCl, pH 7.8, 0.1 M LiCl, 20 mM $MnCl_2$, 0.06% α-thioglycerol, 0.2 mM PMSF, 1 μM pepstatin and leupeptin, and 1 mM Na_2EDTA)]. After 30 s, the extracts were centrifuged for 30 min at 20000g. The supernatant fluid was freed of fat by filtration through glass wool, and the extracts were stored at −20 °C. Typically, 200 μg of crude protein in sample buffer was boiled for 2 min and loaded on one lane of a 0.75 mm $\times$ 13 cm $\times$ 15 cm gel. Following SDS–PAGE, gels were blotted to Immobilon filters using a semi-dry apparatus and three-buffer system (Kyhse-Andersen, 1984). The filters were blocked for 1 h at room temperature with blocking buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% NP-40, 0.05% NaN_3, and 5% dry milk) and then incubated with anti-RNase H1 rabbit serum or preimmune serum from the same rabbit diluted in blocking buffer. Following three washes with TBST buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 0.05% Tween 20), the filters, were incubated with ^{125}I protein A (0.5 μCi/mL) for 2 h, again washed 3 times with TBST, dried, and autoradiographed. In the experiment shown in Figure 1, transfer to nitrocellulose filters was by diffusion blotting for 3 days. After similar blocking and washing procedures, the filters were incubated with primary antibody diluted in blocking buffer, washed, incubated with biotinylated protein A (30 μg/mL)

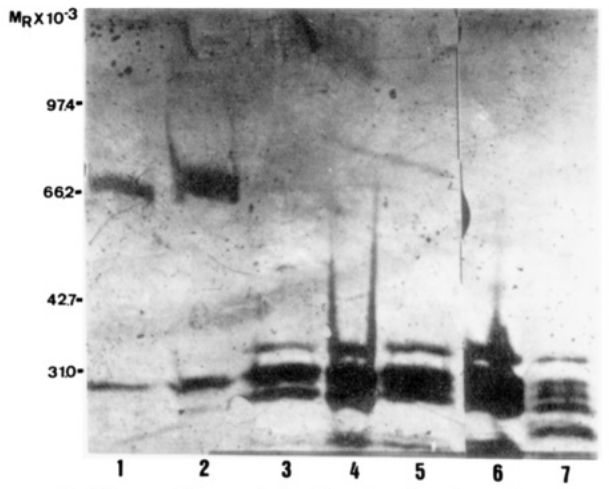

FIGURE 1: Western blot analysis of various fractions of an RNase H1 purification carried out in the absence of protease inhibitors. The serum was used at a 1:10 dilution, and detection was via biotinylated protein A and avidin–horseradish peroxidase as noted under Materials and Methods. The protein concentration in each lane was as follows: lanes 1 and 2, 98 and 292 μg of crude extract; lanes 3 and 4, 62 and 155 μg of CM-Sephadex fraction; lane 5, 3 μg of hydroxyapatite fraction; lanes 6 and 7, 50 and 20 μg of Affigel Blue fraction.

(30 min/37 °C), washed, incubated with glutaraldehyde-cross-linked avidin/horseradish peroxidase (10 μg mL^{-1} for 1 h at 37 °C), and developed with 3-amino-9-ethylcarbazole and 0.05% hydrogen peroxide.

Determination of Molecular Weight by Gel Filtration. A 0.9 × 90 cm column of Sephacryl S-200 was equilibrated with 50 mM Tris-HCl, pH 7.5, 100 mM KCl, 1 mM 2-MCE, 1 mM PMSF, 0.1 mM sodium tetrathionate, and 1 mM sodium hydrogen sulfite. Samples (2 mL) of marker proteins (2–10 mg/mL) or the most purified preparation of RNase H1 (8 μg) were added in 50 mM Tris-HCl, 100 mM KCl, 0.1% 2-MCE, and 5% glycerol. The column was eluted with the equilibration buffer at a flow rate of 20 mL/h. Fractions of 2 mL were collected and analyzed for protein by A_{280} or for RNAse H activity.

Determination of $s_{20,w}$ of RNase H1 by Sucrose Gradient Centrifugation. Samples (0.1 mL) were centrifuged at 45 000 rpm on 15.5–33% linear sucrose gradients (total volume 4.5 mL) at 4 °C for 15.5 h in a Beckman SW 50.1 rotor. The buffer used was the same as that used for the Sepharose column determination of molecular weight. Fractions of 0.1 mL were collected and assayed for either RNase H activity or A_{280} to determine the position of marker proteins.

RESULTS

Western Blotting of Calf Thymus Extracts with Anti-RNase H1 Antibody. Preliminary attempts to utilize the procedure of Stavrianopolis and Chargaff (1978) to purify RNase H1 gave low yields and low specific activity of our final product. SDS–PAGE analysis of the most highly purified fractions, however, revealed a banding pattern quite similar to what these authors had published, i.e., approximately four bands in the range of 21–34 kDa. We prepared a polyclonal rabbit antibody to our final preparation and used it for Western blotting of the various fractions obtained during the purification. The results are shown in Figure 1. Whereas the antibody detected 68- and 30-kDa bands in the crude extract and early steps of the purification, the 68-kDa band was not detectable in more highly purified fractions. Instead, these fractions contained an increasing number of bands in the range 21–34 kDa. In order to determine if the 68-kDa band was

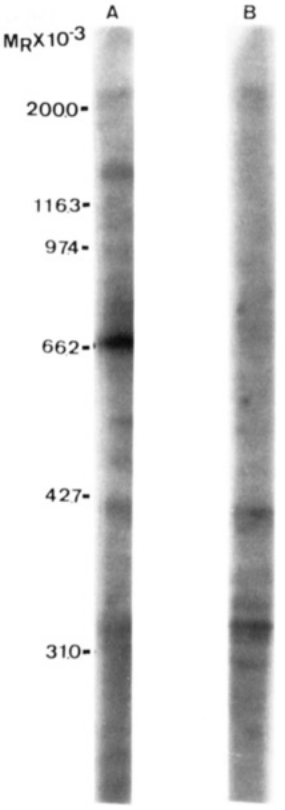

FIGURE 2: Western blot analysis of crude calf thymus extracts. SDS–PAGE, transfer to Immobilon filters, and probing were carried out with ^{125}I protein A as described under Materials and Methods using a 25-fold dilution of sera. (A) Postimmune serum. (B) Preimmune serum.

a possible artifact, we prepared a fresh extract of thymus in the presence of a battery of protease inhibitors and again carried out Western blotting, this time with both preimmune and postimmune sera. The results, shown in Figure 2, show that even though in this experiment we used a different method of detecting the blotted antigens compared to that utilized in Figure 1, we again could detect a 68-kDa band and furthermore this band was detected only by postimmune serum. There was no detectable band at 30 kDa in this experiment, but there is a faint band of immunoreactive material at approximately 140–150 kDa. (There is a faint band of immunoreactive material above 200 kDa, but this band is also detected by the preimmune serum.) These results suggested to us that the 68-kDa band might well be authentic RNase H1, but that it is proteolytically nicked during purification of the enzyme and gives rise following SDS–PAGE to the multiple lower molecular weight bands we detected in our purified enzyme. These results suggested further that even higher molecular weight forms of the enzyme might exist.

SDS–PAGE Analysis and Activity Gel Analysis of Purified RNase H1. To further explore the possibility that the native molecular weight of RNase H1 might reside in a polypeptide of 68 kDa or even more, we repeated the enzyme purification, but this time included in all buffers a battery of protease inhibitors. This time both the yield and specific activity of the final preparation were much improved. The progress of the purification is outlined in Table I. When we examined our most highly purified fraction by SDS–PAGE, we found the results shown in Figure 3A. The 68-kDa band is the predominant band in this preparation, but a second major band of about 34 kDa is also quite prominent. In addition, there are approximately six other very faint bands ranging from 68 to 27 kDa.

Table I: Purification of Ribonuclease H from Bovine Thymus[a]

fraction	vol (mL)	total protein (mg)	total act. (units × 10^{-6})	sp act. (units/mg)
(I) crude extract	1160	13340	5.4	402
(II) poly(ethylene glycol)	428	7148	5.0	703
(III) $(NH_4)_2SO_4$	60	1800	3.2	1760
(IV) poly(ethylene glycol)	40	293	1.95	6692
(V) CM-Sephadex	17	10.5	2.5	235000
(VI) DEAE-cellulose	0.5	0.42	0.92	2200000

[a] The fractionation steps were carried out as described (Stavrianopolis & Chargaff, 1978) using the minor modifications described under Materials and Methods.

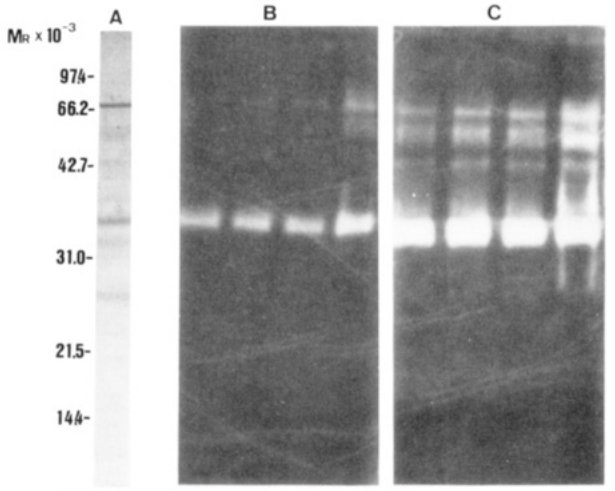

FIGURE 3: (A) SDS–PAGE analysis of polypeptides present in the purified RNase H1 (4 μg) following SDS–PAGE on a 10% gel. (B and C) Activity gel analysis of RNAse H activity present in the preparation shown in (A). An SDS–PAGE gel containing [^{32}P]-poly(rA)·poly(dT) was run and renatured as described under Materials and Methods. (B) Renaturation with guanidine hydrochloride. (C) Renaturation with NP-40. In (B) and (C), the lanes contain the following amounts (micrograms) of purified enzyme: (1) 1.3; (2) 2.5; (3) 5.0; (4) 8.4.

In order to determine if the higher molecular weight polypeptides in our preparation were contaminants or higher molecular weight forms of RNase H, we carried out activity gel measurements after renaturation (Dottin et al., 1987; Bertazzoni et al., 1986). Briefly, following SDS–PAGE on a gel containing a [^{32}P]poly(rA)·poly(dT) hybrid, purified RNase H1 was renatured by washing the gel with 2-propanol to remove the bulk of the SDS followed by further washing with either a buffered GdnHCl or NP-40 solution. Following further washing steps and drying, the gel was autoradiographed. Regions of RNase H activity reveal themselves as clear bands in an otherwise dark background. The results demonstrate that the NP-40 procedure (Figure 3C) is far more efficient than the GdnHCl (Figure 3B) procedure in promoting renaturation, and that although the most active bands displaying RNase H activity are those at approximately 34 kDa higher molecular weight bands including the 68K band also demonstrate activity. There is even a very slight trace of activity at still higher molecular weights observable only in the NP-40 renatured gel that may represent an active dimer of the 68-kDa band (see below).

Estimation of the Molecular Weight of Native RNase H1. In order to estimate the molecular weight of native RNase H1, we chromatographed our most highly purified fraction on a Sephacryl 200 column that had been calibrated with a group

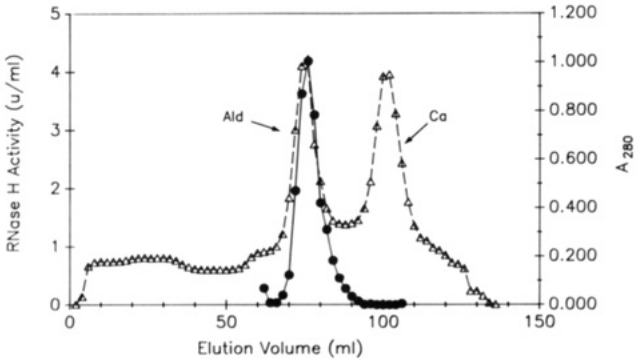

FIGURE 4: Elution of RNase H1 and marker proteins during gel filtration chromatography. Standard proteins and purified RNase H1 (1760 units, 0.8 μg) were chromatographed separately on a Sephacryl S-200 column. The elution volume of marker proteins was determined from A_{280} measurements. Ald, alcohol dehydrogenase; Ca, carbonic anhydrase. RNase H activity was determined as described under Materials and Methods. Solid line, RNase H1 activity; dashed line, A_{280}.

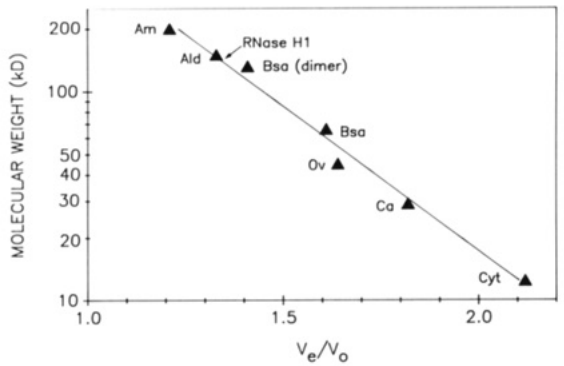

FIGURE 5: Determination of the molecular weight of RNase H1 by gel filtration chromatography. Partition coefficients (V_e/V_0) determined as indicated in Figure 4 are plotted against the molecular weight of the proteins. Am, potato β-amylase; Ald, yeast alcohol dehydrogenase; Bsa, bovine serum albumin; Ov, chicken ovalbumin; Ca, bovine erythrocyte carbonic anhydrase; Cyt, horse heart cytochrome *c*.

of standard proteins. In order to reduce the possibility of aggregation of the protein, very low amounts of protein were used in the absence of any carrier. Only a single peak of enzymatic activity was seen at a partition coefficient (V_e/V_0) of 1.33 (Figure 4). When the logarithm of the molecular weights was plotted against the partition coefficients of the standard proteins, the results were as shown in Figure 5. From this plot, we estimated that the molecular weight of RNase H1 is 150K, assuming that the protein is a typical globular protein. This result suggests that the active form of the enzyme is a dimer of the 68-kDa monomer. The absence of significant activity in the range of 34 kDa (Figure 4), where the activity gel (Figure 3) reveals significant activity, indicates that despite the proteolytically induced nicks in the polypeptide backbone, the enzyme remains intact during gel filtration.

We also attempted to determine the molecular weight of the enzyme by sucrose gradient centrifugation (Martin & Ames, 1961). Purified RNase H1 was sedimented in the presence of 1 mg/mL BSA with a variety of marker proteins in separate tubes. The $s_{20,w}$ of the marker proteins was plotted against the distance sedimented and the $s_{20,w}$ of RNase H estimated by linear regression (Figure 6). The $s_{20,w}$ determined by this method was 4.5 S. Using this value together with the empirical equation of Martin and Ames (1961) relating the $s_{20,w}$ of proteins to their molecular weight, we estimated the molecular weight of RNase H1 as 70.9K. This

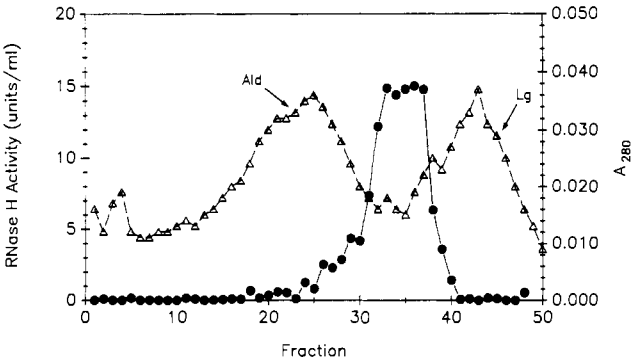

FIGURE 6: Determination of the sedimentation of RNase H1 and marker proteins on sucrose gradients. RNase H1 (930 units, 0.4 μg) was sedimented in the presence of BSA (1 mg/mL) as described under Materials and Methods. The marker proteins Ald (yeast alcohol dehydrogenase) and Lg (bovine lactoglobulin) were sedimented in a separate tube. Protein was determined by A_{280} and RNase H1 activity as described under Materials and Methods. Solid line, RNase H activity; dashed line, A_{280}.

result suggests that under the conditions of sucrose gradient centrifugation RNase H1 sediments as a monomer.

Discussion

The results presented here lead us to the conclusion that calf thymus RNase H1 probably consists of a dimer of 68-kDa subunits contrary to several earlier claims that the enzyme is composed of a variety of low molecular weight "subunits" in the range of 21K–34K (Stavrianopolis & Chargaff, 1978; Busen, 1980, 1982). We believe that the 68-kDa band represents undegraded RNase H1 and that the lower molecular weight bands observed following SDS–PAGE of highly purified preparations represent degraded forms of the enzyme for the following reasons. First, Western blot analysis using a polyclonal rabbit antiserum prepared against highly purified RNase H1 containing "subunits" recognizes a 68-kDa polypeptide in crude thymus extracts. This polypeptide, which is not recognized by preimmune serum, is lost during purification of the enzyme in the absence of protease inhibitors, and lower molecular weight polypeptides recognized by the antiserum appear (Figures 1 and 2). This results suggests that epitopes present on the low molecular weight protein bands used for immunization are also present on the 68-kDa species. While we have not explicitly shown by a kinetic analysis that the 68-kDa band is a precursor of the lower molecular weight bands, this seems to be the most reasonable explanation for the disappearance of the former and appearance of the latter during the purification. Second, the results of our activity gel measurements (Figure 3B,C) clearly demonstrate that the 68-kDa band visible in preparations of RNase H1 prepared in the presence of protease inhibitors (Figure 3A), as well as the other lower molecular weight bands in our preparation, possess ribonuclease H activity and therefore are unlikely to represent contaminants. Although it is true that the majority of the activity seen on the activity gels (Figures 3B,C) is accounted for by a band of approximately 34K molecular weight, despite the fact that this band is only a minor component of the preparation as indicated by CBB staining (Figure 3A), it is important to note that such gels cannot be used to deduce the specific activity of a given band, since one is measuring not merely enzymatic activity but also the ability of a given band to renature to an active form. It seems likely that relatively large molecules are more difficult to renature than are small ones, and indeed Cathala et al. (1979), who were successful in renaturing a low molecular weight RNase H from Krebs ascites cells, failed in their attempt to renature a high molecular weight form of the enzyme. Third, there is precedent for the view that RNase H1 does not consist of multiple low molecular weight subunits in that Haberkern and Cantoni (1973) also concluded that calf thymus RNase H could be purified as a single polypeptide chain whose molecular weight they estimated by sedimentation equilibrium to be 75 K. Furthermore, Busen, who initially claimed the 80–84-kDa enzyme consisted of four polypeptide chains (Busen, 1980), later concluded that two of the peptides were in fact produced by proteolytic nicking during purification (Busen, 1982). Finally, we have observed that during purification of the enzyme and upon storage −20 °C, high molecular weight bands disappear from the preparation and are replaced by lower molecular weight ones (data not shown).

Although our data demonstrate that the RNase H1 molecule contains a 68-kDa polypeptide, measurements of the molecular weight of the protein by gel filtration suggest a native molecular weight of 135K–150K, indicating that the active form of the enzyme is probably a dimer (Figures 4 and 5). One caveat should be noted, however, about the evidence suggesting a dimeric structure for the enzyme. We used our most purified fraction of RNAse H for the gel filtration studies in an effort to minimize the possibility of possible protein complexes that could produce an artificially high value for the molecular weight. As a result, the amount of protein loaded on the column and gradients was very low which resulted in a very low yield (about 3–10%) of the applied activity. The instability of RNases H during gel filtration has previously been noted (Wyers et al., 1976; Cathala et al., 1979). Although it seems unlikely, it is possible that the dimeric form of the enzyme that we recover following this procedure is not the predominant form of the enzyme and that an active monomeric form is selectively lost.

When we attempted to improve the yield of enzyme by incorporating 0.5% BSA into the elution buffer, the yields were improved, but still only about 10%. Under these conditions, however, we were able to detect approximately 2% of the activity eluting nearly coincident with the BSA marker, consistent with the activity being a monomer of the 68-kDa polypeptide, while 68% of the activity still eluted between the β-amylase and aldolase markers, consistent with this activity being a multimer of the 68-kDa polypeptide with perhaps some aggregated material (Wyers et al., 1976). Of course, it is difficult to rule out some slight amount of nonspecific binding of the enzyme to BSA under these conditions.

Additional evidence for the existence of a dimeric structure of native RNase H1 is provided by the high molecular weight band of immunoreactive material seen in Figure 2 and a slight suggestion of a higher molecular weight RNase H activity seen in the activity gel (Figure 3C). We cannot rule out the possibility that the native form of RNase H1 is a monomer of 140–150 kDa and that the 68-kDa form is derived from the larger form by proteolytic nicking. However, the fact that the 140–150-kDa band is seen only occasionally and even then only in low yield, despite the presence of numerous protease inhibitors in our extracts, argues that the 68-kDa form is probably not a proteolytic fragment of a still higher molecular weight precursor. This view is supported by our failure to observe any putative precursor of the 68-kDa polypeptide in our preliminary immunoprecipitation experiments carried out with human cell lines, in which cell lysis is achieved by the rapid lysis of the cells in the presence of boiling SDS (data not shown).

The results of our gel filtration measurements for the molecular weight of native RNase H1 do not agree with the

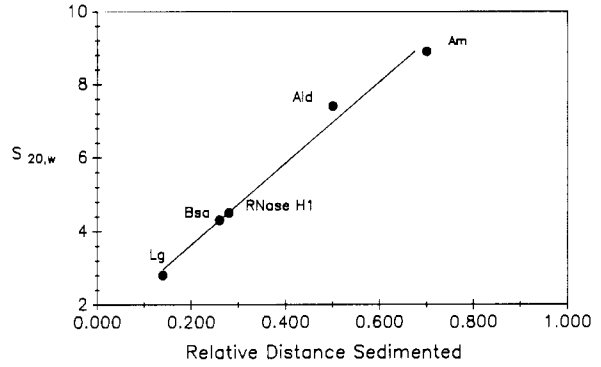

FIGURE 7: Determination of $s_{20,w}$ of RNase H1. The distances sedimented as determined in Figure 6 are plotted against the $s_{20,w}$ of the proteins.

earlier studies of Stavrianopolis and Chargaff (1973), who utilized gel filtration to arive at a molecular weight of 64K for the native enzyme. Our results are in closer agreement with the molecular weight of 105K–110K that Cathala et al. (1979) estimated for Krebs/Ascites RNase H1 which these authors suggest is a dimer of a 60-kDa subunit. Furthermore, *Drosophila* RNase H is also a dimer (DiFrancesco & Lehman, 1985), and even the *E. coli* enzyme may be active as a dimer (Crouch & Dirksen, 1982). In view of these findings and the results reported here suggesting that the enzyme preparation studied by Stavrianopolis and Chargaff was nicked by proteases during purification, it seems likely that the formation of the dimeric forms of RNAse H1 may depend on an interaction of portions of the molecule easily subject to proteolytic nicking.

Although as indicated above the preponderance of evidence suggests a dimeric structure for the enzyme, the results of our determination of the $s_{20,w}$ of the enzyme demonstrate that the enzyme sediments as a 68-kDa monomer (Figures 6 and 7). The results of our sucrose gradient sedimentation experiments ($s_{20,w}$ = 4.5S) agree fairly closely with those of Busen (1980), who estimated an $s_{20,w}$ of 5.0–5.1 S for his preparation of RNase H1. Although the experiment in Figure 6 was carried out in the presence of a low concentration of BSA in an attempt to stabilize the enzyme, the results were similar when the enzyme was sedimented in the absence of BSA (data not shown) although the yield was lower. This result, and the observation noted above that even in the presence of far higher amounts of BSA that 98% of the activity eluting from a gel filtration column appears as a dimer, makes it unlikely that the appearance of the 68-kDa monomer during sucrose gradient centrifugation is simply due to binding of the enzyme to BSA. Instead, it seems likely that the dimer dissociates during sucrose gradient centrifugation probably due to pressure effects (Paladini & Weber, 1981).

The large size and proteolytic lability of yeast RNase H and calf thymus RNase H1 raise several questions about the in vivo role of this enzyme. *E. coli* RNase H is a relatively small protein, and although it demonstrates molecular heterogeneity on activity gels (Carl et al., 1980) and upon isoelectric focusing (Arendes et al., 1982) due to some undetermined posttranslational modification, it does not appear to be especially proteolytically labile. Since the job of hydrolyzing RNA strands in duplexes can clearly be carried out by a relatively small enzyme, why are some yeast RNases H and mammalian RNase H1 so large? It is tempting to speculate that these enzymes carry out some additional functions in vivo that do not depend on their ability to hydrolyze RNA, a point already suggested by Kane (1988).

The proteolytic lability of the enzymes also suggests a mechanism by which the activity of RNases H might be controlled. Indeed, Wada et al. (1980) observed some time ago that proteolysis could inactivate RNase H activity in rat intestine, although the physiological significance, if any, of this observation is unclear.

Knowledge of the molecular structure of RNase H1 should prove helpful in identifying RNase H activities in other systems. In this regard, the observation of Bialek et al. (1988) that an immunoaffinity-purified DNA polymerase α–primase complex from human lymphoblasts contained a polypeptide of 68 kDa and demonstrated RNase H activity is of interest. It seems likely this enzyme plays important and perhaps presently unsuspected roles in cell function. Further understanding of these roles will only come once the gene coding for RNase H1 has been cloned. Work underway in our laboratory is directed to this end.

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Crystal Structures of Phosphonoacetamide Ligated T and Phosphonoacetamide and Malonate Ligated R States of Aspartate Carbamoyltransferase at 2.8-Å Resolution and Neutral pH[†,‡]

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ABSTRACT: The T → R transition of the cooperative enzyme aspartate carbamoyltransferase occurs at pH 7 in single crystals without visibly cracking many of the crystals and leaving those uncracked suitable for single-crystal X-ray analysis. To promote the T → R transition, we employ the competitive inhibitors of carbamoyl phosphate and aspartate, which are phosphonoacetamide (PAM) and malonate, respectively. In response to PAM binding to the T-state crystals, residues Thr 53–Thr 55 and Pro 266–Pro 268 move to their R-state positions to bind to the phosphonate and amino group of PAM. These changes induce a conformation that can bind tightly the aspartate analogue malonate, which thereby effects the allosteric transition. We prove this by showing that PAM-ligated T-state crystals (T_{pam}), space group *P*321 (a = 122.2 Å, c = 142.2 Å), when transferred to a solution containing 20 mM PAM and 8 mM malonate at pH 7, isomerize to R-state crystals ($R_{pam,mal,soak}$), space group also *P*321 (a = 122.2 Å, c = 156.4 Å). The R-state structure in which the T → R transition occurs within the crystal at pH 7 compares very well (rms = 0.19 Å for all atoms) with an R-state structure determined at pH 7 in which the crystals were initially grown in a solution of PAM and malonate at pH 5.9 and subsequently transferred to a buffer containing the ligands at pH 7 ($R_{pam,mal,crys}$). In fact, both of the PAM and malonate ligated R-state structures are very similar to both the carbamoyl phosphate and succinate or the *N*-(phosphonoacetyl)-L-aspartate ligated structures, even though the R-state structures reported here were determined at pH 7. Crystallographic residuals refined to 0.16–0.18 at 2.8-Å resolution for the three structures.

Aspartate carbamoyltransferase [from *Escherichia coli*, EC 2.1.3.2; for a recent review, see Kantrowitz and Lipscomb (1988)] catalyzes the formation of phosphate and *N*-carbamoyl-L-aspartate from carbamoyl phosphate and L-aspartate, initiating the first step in the synthesis of pyrimidines (Jones et al., 1955; Reichard & Hanshoff, 1956). In response to saturating concentrations of substrates, the enzyme undergoes large conformational rearrangements in the T → R[1] transition as illustrated and described in Figure 1. The changes in the quaternary structure of the molecule as a result of the allosteric transition are evident in the difference of the c axis unit cell dimensions of *P*321 crystals. For the T state the c axis length is 142 Å, and for the R state the length is 156 Å; the length of the a axis of 122 Å changes less than 0.5 Å as a result of the transition. The increase in the c-axis dimension of the R-state crystals, consistent with a molecular expansion along c by 8 Å, was noted by Monaco (Monaco, 1978; Monaco et al., 1978). Ultracentrifugation experiments that demonstrated an increase in the frictional coefficient of the enzyme in the presence of *N*-(phosphonoacetyl)-L-aspartate (PALA) were interpreted in terms of a 3.5% expansion of a spherical model or an expansion (or contraction) of an ellipsoid model (Gerhart & Schachman, 1968). Moody et al. (1979) predicted the

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[‡] The coordinates of all structures have been submitted to the Brookhaven Protein Data Bank. The entry names for the coordinates are as follows: 1AT1 ATC (PAM and malonate ligated R state, crystal soak); 2AT1 ATC (PAM and malonate ligated R state, cocrystal); 3AT1 ATC (PAM ligated T state).

[1] T is an abbreviation for tense and is used here to indicate the conformational state of the enzyme that has unit cell dimensions of a = 122 Å and c = 142 Å in the space group *P*321; R, an abbreviation for relaxed, indicates the conformational state of the enzyme that has unit cell dimensions of a = 122 Å and c = 156 Å also in the space group *P*321. To signify that a structure contains a ligand bound at the active site, we will add an abbreviation of the ligand name as a subscript to either T or R. Alternatively, if the ligand is bound to the regulatory site, then an abbreviation for that ligand will be added as a superscript. Functionally, the T form shows low activity and low aspartate affinity, while the R form has a high activity and a high affinity for aspartate. See Monod et al. (1965) for a discussion on a theory of allosteric transitions in proteins and for an explanation of the nomenclature.

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