Biochemistry

 Volume 25, Number 23 November 18, 1986

Accelerated Publications

Isolation of a New Vanadium-Containing Nitrogenase from *Azotobacter vinelandii*†

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Received July 30, 1986; Revised Manuscript Received September 5, 1986

ABSTRACT: A new nitrogenase from *Azotobacter vinelandii* has been isolated and characterized. It consists of two proteins, one of which is almost identical with the Fe protein (component 2) of the conventional enzyme. The second protein (Av1′), however, has now been isolated and shown to differ completely from conventional component 1, i.e., the MoFe protein. This new protein consists of two polypeptides with a total molecular weight of around 200 000. In place of Mo and Fe it contains V and Fe with a V:Fe ratio of 1:13 ± 3. The ESR spectrum of Av1′ also differs from conventional component 1 in that it lacks the $g = 3.6$ resonance that arises from the FeMo cofactor but contains an axial signal with $g_{av} < 2$ as well as inflections in the $g = 4$–6 region possibly arising from an $S = {}^3/_2$ state. This new enzyme can reduce dinitrogen, protons, and acetylene but is only able to utilize 10–15% of its electrons for the reduction of acetylene.

The nitrogen-fixing enzyme nitrogenase has been purified to homogeneity from a large number of different bacteria. This enzyme is highly conserved and, in all the species tested to date, consists of two separable proteins. The smaller protein, called component 2, has a molecular weight of around 60 000 in an α_2 polypeptide pattern and contains a single 4Fe-4S cluster. During enzymatic activity, component 2 binds MgATP and is reduced and, in this state, acts as the sole reductant of the larger protein of nitrogenase, component 1. Component 1 has a molecular weight of about 240 000 in an $\alpha_2\beta_2$ polypeptide pattern and contains 30–33 Fe atoms and 2 Mo atoms in several different clusters of unknown structure. It generally is felt that component 1 contains the site(s) of substrate reduction (at or near the Mo atoms) and is able to reduce not only dinitrogen to ammonia but also acetylene to ethylene and protons to dihydrogen.

In 1980, a second (alternative) nitrogen-fixing enzyme was hypothesized to exist in *Azotobacter vinelandii* (Bishop et al., 1980). It was predicted that this enzyme was expressed during Mo starvation, a condition that would not favor the synthesis of the conventional (Mo-containing) enzyme. Last year, we reported (Hales et al., 1985) on the partial isolation of this second enzyme and indicated that it, like the conventional enzyme, consists of two proteins. Recently we isolated one of these proteins (Av2′)[1] and showed (Hales et al., 1986) that it was structurally similar to conventional component 2 (Av2). In this paper we describe the isolation, purification, and characterization of the larger protein (i.e., alternative component 1) from *A. vinelandii*. As shown in our earlier report (Hales et al., 1985), alternative component 1 (called Av1′) is a smaller protein than conventional component 1 (Av1). We now find that the metal composition of Av1′ is also different from Av1 with the alternative enzyme containing V instead of Mo. Furthermore, Av1′ is paramagnetic but lacks the characteristic spin ${}^3/_2$ ESR spectrum found in Av1, which has been attributed to a Mo-Fe-S cluster in that protein.

Experimental Procedures

Bacterial Strains. Conventional nitrogenase was isolated from wild-type *A. vinelandii* strain UW. In order to obtain

†This work was supported by the National Institutes of Health under Grant GM33965 and by the Research Corp.

[1] Abbreviations: AA, atomic absorption; Av1, conventional component 1; Av2, conventional component 2; Av1′, alternative component 1; Av2′, alternative component 2; ESR, electron spin resonance; ICP, inductively coupled plasma; SDS, sodium dodecyl sulfate; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; mT, millitesla; kb, kilobase(s); Tris, tris(hydroxymethyl)aminomethane; Bicine, *N*,*N*-bis(2-hydroxyethyl)glycine; BSA, bovine serum albumin; DEAE, diethylaminoethyl.

0006-2960/86/0425-7251$01.50/0

the alternative nitrogenase, a strain (LS15) of *A. vinelandii* was constructed and shown to contain a deletion in the genes (*nifHDK*) that encode for the structural proteins of the conventional enzyme (Hales et al., 1986). This construction was achieved by transformation and recombination of plasmid pDB12 with strain UW. Plasmid pDB12 contains a 3.6-kb KpnI deletion in a 12-kb XhoI *nifHDK* fragment of the Av genome (Bishop et al., 1985). LS15, therefore, can only fix nitrogen by an alternative nitrogen-fixing enzyme. Both UW and LS15 were grown on Burk's N-free medium (Hales et al., 1986) with the exception that 10 μM $NaVO_3$ was substituted for 10 μM Na_2MoO_4 in media used for LS15.

Protein Isolation. Av1′ was isolated by using a variation (Hales et al., 1986) of the procedure used to purify the conventional enzyme (Burgess et al., 1980). All procedures were performed anaerobically and all buffers contained 2 mM $Na_2S_2O_4$ (dithionite).

Activity Assays. Acetylene reduction (10% C_2H_2/Ar) was used to monitor enzyme activity during protein isolation (Burgess et al., 1980). Ethylene generated was determined with a gas chromatograph (Varian, Model 3700) containing a Porapac N column.

Absolute quantiation of electron utilization during acetylene reduction was performed as follows. Anaerobic acetylene reduction vials (14.5 mL) contained 1.0 mL of an ATP-regenerating system (Burgess et al., 1980) plus 0.1 mM dithionite. Addition of components 1 and 2 to these vials initiated acetylene reduction, which terminated upon depletion of the dithionite. At this point, 10 μL of a standardized 0.02 M dithionite solution was added to the reaction mixture and the course of acetylene reduction followed to completion. The difference in the amount of ethylene formed before and after the addition of dithionite represents the absolute amount of ethylene formed for a given amount of dithionite. This cycle was repeated 5 times and the average ethylene produced per injection calculated. The dithionite concentration was determined spectrophotometrically (Davis et al., 1975). The amount of component 2 in these experiments was determined by titrating component 1 with component 2 to obtain maximum rate of ethylene production (Shah et al., 1975).

For dinitrogen reduction assays, reaction vials were identical with those used in acetylene reduction except that the gas above the regenerating solution was dinitrogen gas instead of 10% C_2H_2/Ar. Ammonia production was monitored spectrophotometrically (Maryan & Vorley, 1979). To avoid the inhibition of this colorimetric assay normally induced by Tris buffer, all protein and regenerating solutions were made 25 mM Bicine, pH 7.6. All samples were run in triplicate, and the reactions were terminated by injecting 0.1 mL of trichloroacetic acid (30% w/v) at 10-min intervals. Ammonia production was found to be linear for at least 40 min. Saturating concentrations of component 2 were used in all experiments.

Dihydrogen evolution was monitored with a gas chromatograph (Varian, Model 90P3) containing a 5-Å molecular sieve column (40–60 mesh, flow rate 30 mL of Ar min^{-1} at 100 °C). Reaction vials initially contained Ar as the sole gas.

SDS–Polyacrylamide Gels. All gels were 10.5% acrylamide and were prepared according to published procedures (Howard et al., 1986). Quantitation of protein bands was performed with a Bio-Rad video densitometer (Model 620) equipped with a 540-nm filter.

Metal Analysis. Inductively coupled plasma (ICP) emission spectroscopy was used for metal analysis (Kahn et al., 1979). Samples (400–500 μL at 30 mg/mL) were placed in stoppered 15-mL glass tubes containing 0.5 M HNO_3 (Insta Analyzed) and heated in an oil bath at 100 °C for 17.5 h. After digestion, each tube was washed with glass-distilled water to make a total volume of 15 mL. Control samples using BSA in the same buffer were digested according to the above procedure. All glassware was prewashed in hot HNO_3. ICP spectra were recorded on a Perkin-Elmer ICP/6500 spectrometer.

ESR Spectroscopy. ESR spectra were recorded and spin quantitations were performed as previously described (Hales et al., 1986).

RESULTS

A. vinelandii strain LS15 contains a deletion in the genes (*nifHDK*) that encode for the structural proteins of conventional nitrogenase and, therefore, is unable to synthesize this enzyme (Hales et al., 1986). Any nitrogen fixation that is performed by this strain has to arise from a second nitrogenase whose structural proteins are encoded by a different set of genes. We will assume that this second enzyme is the same as the alternative nitrogenase first hypothesized in 1980 (Bishop et al., 1980).

In the preliminary isolation of the alternative enzyme from LS15, we showed (Hales et al., 1985) that it, like the conventional enzyme, is a two-protein system where one of the proteins (Av2′) (Hales et al., 1986) is extremely similar to conventional component 2 (Av2). While Av2′ can be isolated with the same procedure (Burgess et al., 1980) traditionally used in the purification of Av2, difficulty was encountered in the isolation of alternative component 1 (Av1′). This difficulty was due mainly to two factors, the first being the low relative concentration of Av1′ in cell extract. We subsequently have found that the addition of 10 μM sodium metavanadate ($NaVO_3$) to the growth medium increases the specific activity of Av1′ in crude extracts from 0.1 to 3.0 nmol of C_2H_2 reduced min^{-1} (mg of protein)$^{-1}$.

The second difficulty encountered in the isolation of Av1′ was associated with the apparent instability of this protein on gel filtration columns. During the purification of conventional component 1, a 2.5 × 100 cm Sephacryl S-200 column is used as a final purification step. We noted, however, that eluting Av1′ on this long column resulted in the complete loss of its activity. This activity could not be restored by recombining and concentrating all the eluted fractions and, therefore, appears to be irreversible. The source of this loss of activity is unknown. However, we have found that by using shorter, wider columns (5 × 25 cm) the time needed to elute Av1′ is decreased and the total activity retained. Gel filtration is needed in the purification of alternative nitrogenase because, unlike the conventional enzyme where both component proteins are almost completely separable on DEAE (0.12 and 0.22 M NaCl for Av1 and Av2, respectively), Av1′ and Av2′ elute at almost the same salt concentration (0.15 and 0.17 M NaCl, respectively). Gel filtration is, therefore, needed to separate these two proteins after DEAE purification. Finally, since gel filtration separates by size, the apparent molecular weight of a protein often can be estimated from its relative elution volume. By use of *A. vinelandii* flavoprotein (23 000), Av2′ (61 000), and Av1 (240 000) as molecular weight standards, the weight of Av1′ was approximated to be 180 000–200 000.

The specific activity of Av1′ was determined after every step of its isolation. In a typical isolation, the specific activity increased from around 3.0 to 200.0 nmol of C_2H_2 reduced min^{-1} (mg of protein)$^{-1}$ starting at crude extract and ending at the elution from the final gel filtration column, respectively. This activity can be compared to Av1 where the specific activity normally increases from about 100 to 2000 nmol of C_2H_2

Table I: Specific Activity and Electron Utilization Efficiency[a]

	protein	
	Av1	Av1′
H^+ reduction[b]	2200	1400
C_2H_2 reduction[c]	2000 (90 ± 5%)[d]	220 (12 ± 5%)
N_2 reduction[e]	520	330
reduction ratio ($3 \times N_2/C_2H_2$)	0.78	4.2
reduction ratio ($C_2H_2/2 \times H^+$)	0.91	0.16

[a] All activities measured at 30 °C. [b] nmol of H_2 evolved min^{-1} (mg of protein)$^{-1}$. [c] nmol of C_2H_2 reduced min^{-1} (mg of protein)$^{-1}$ from a 10% C_2H_2/Ar mixture. [d] Percentage of electron used for reduction as determined by dithionite titration. [e] nmol of N_2 reduced min^{-1} (mg of protein)$^{-1}$.

reduced min^{-1} (mg of protein)$^{-1}$ during the same steps.

In order to better compare the relative activities of Av1 and Av1′, the total electron flow through each enzyme was determined. Identical acetylene-reduction assays were performed, using either the couple Av1/Av2 or Av1′/Av2′ (see Experimental Procedures). Standardized quantities of $Na_2S_2O_4$ were injected into each vial, and the absolute amount of acetylene reduction was determined. The percentage of electrons used for acetylene reduction were calculated to be 90 ± 5% and 12 ± 5% for Av1/Av2 and Av1′/Av2′, respectively (Table I), showing that acetylene reduction is a poor indicator of absolute enzymatic activity for the alternative system.

In a similar experiment, the rate of nitrogen fixation relative to acetylene reduction was determined for Av1/Av2 and Av1′/Av2′. Identical reaction vials were again used for each nitrogenase system. In one series 10% C_2H_2/Ar was used as the gas mixture while in the second series N_2 was the sole gas. Colorimetric assay was used as the indicator of the ammonia formation (Maryan & Vorley, 1979). The results from these assays (Table I) show that, while Av1′ is much less efficient for acetylene reduction than Av1, it has comparable efficiency in the utilization of electrons for nitrogen fixation.

For the conventional enzyme, it is often felt (Burgess et al., 1980) that H_2 evolution gives the best indication of enzymatic activity. In the absence of other substrates, H_2 evolution for the Av1/Av2 and Av1′/Av2′ pairs was measured (Table I). As expected, the specific activity of H_2 evolution for Av1′/Av2′ is much greater than that of C_2H_2 reduction such that the ratio of the reductions $C_2H_2/2 \times H^+$ is 0.16. This ratio is close to the electron utilization percentage (12 ± 3%) determined by dithionite titration, again showing that acetylene is a poor substrate for the alternative enzyme.

We previously have shown (Hales et al., 1986) that antibody prepared against Av1 does not react with protein in crude extracts of nitrogen-fixing LS15. In the present study, we tested for cross-reactive material to anti-Av1 after each step in the isolation of Av1′. None was ever observed. This result shows not only that Av1′ is antigenically different from Av1 but also that there is no detectable amount of contaminating Av1 present.

SDS–PAGE similarly was used to observe the polypeptide pattern after each purification step. As mentioned earlier, Av1 is an $\alpha_2\beta_2$ polypeptide protein. Figure 1 show a typical gel of Av1′ after the final S-200 purification step. Two major bands are observable at apparent molecular weights of 52 000 and 55 000. Densitometer traces of these bands show them to exist in approximately equal concentration. It should be noted that twice the sum of the two polypeptides (214 000) is less than that of Av1 (240 000), as previously predicted by us (Hales et al., 1985), but is greater than the apparent weight of Av1′ from gel filtration (180 000–200 000).

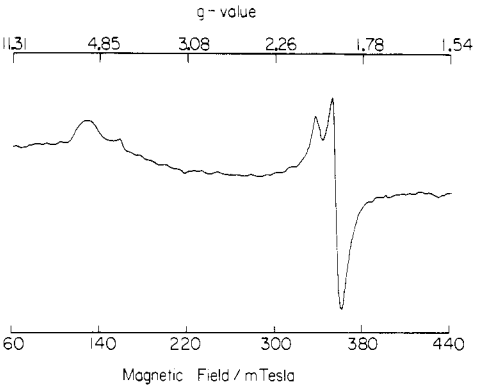

FIGURE 1: ESR spectrum of Av1′ [sp act. 220 nmol of C_2H_2 reduced min^{-1} (mg of protein)$^{-1}$] in 25 mM Tris, pH 7.4, containing 0.25 M NaCl and 2 mM dithionite. The low-field region shows inflection at $g = 5.31$. The signal at $g = 4.34$ probably is due to high-spin rhombic Fe^{3+} adventitious to Av1′. Note the absence of a signal at $g = 3.6$ normally associated with FeMo-co in conventional component 1. The high-field region shows an axial signal with $g = 2.04$ and $g = 1.93$. Experimental conditions: concentration, 15 mg/mL; temperature, 10 K; microwave power, 10 mW; modulation amplitude, 0.50 mT; frequency, 8.994 GHz.

Table II: Metal Analysis[a]

metal[b]	atoms/protein[c]	metal[b]	atoms/protein[c]
V (23)	0.7 ± 0.3	Ni (28)	<0.02
Cr (24)	<0.01	Cu (29)	<0.02
Fe (26)	9.3 ± 2.0	Mo (42)	<0.05
Co (27)	<0.01	W (74)	<0.06

[a] Five separate determinations. [b] Atomic number in parentheses. [c] Assuming a molecular weight of Av1′ of 200 000.

Since Mo is assumed to have an central role in nitrogen fixation by the conventional enzyme but suppresses the synthesis of the alternative enzyme (Bishop et al., 1980), it is important to determine which transition metals are present in Av1′. Traditionally, atomic absorption (AA) spectroscopy has been used to quantitate the concentrations of different metals in proteins. Being an absorption technique, AA is not highly sensitive to refractory metals (e.g., tungsten) and is best utilized in studies where only a small number of metals are being investigated. Not wishing to bias our prediction of which metals were present in Av1′, we decided to use ICP emission spectroscopy. ICP has an advantage over AA in that it is able to detect simultaneously a large number of different elements (up to 60) including refractory metals (Kahn et al., 1979). Table II lists the elemental composition of Av1′ for eight different metals. As expected, Fe is present in high concentrations but Mo is not. Also noteworthy is the significant concentration of V such that the V:Fe ratio is 1:13 ± 3. The high concentration of V is understandable considering the stimulatory effect that it has on the in vivo production of Av1′.

One of the most widely used spectroscopic techniques in the study of Av1 has been ESR spectroscopy. In the presence of 2 mM dithionite, Av1 exhibits a unique rhombic spectrum with apparent g factors of 4.3, 3.6, and 2.0. This spectrum has been theorized (Münck et al., 1975) to arise from the transition of the lowest level of an $S = {}^3/_2$ state. The site of this paramagnetism has been shown to be a Mo-Fe-S cluster called FeMo-co (Shah & Brill, 1977), which contains one Mo atom per cluster. In FeMo-co, the paramagnetism is mainly localized on the Fe atoms with a small amount of interaction with the Mo atom (Venters et al., 1986).

Figure 2 shows the spectrum of Av1′ in Tris-HCl, pH 7.4, at 10 K. This figure shows inflections at $g = 5.31$, 4.34, 2.04, and 1.93 and indicates the complete absence of the FeMo-co signal at $g = 3.6$. The spectrum in Figure 2 was observed in

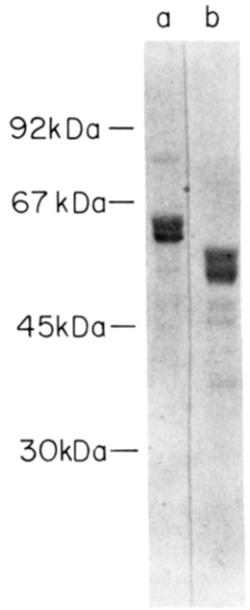

FIGURE 2: SDS gel pattern of polypeptides from Av1 [a; sp act. 2000 nmol of C_2H_2 reduced min^{-1} (mg of protein)$^{-1}$] and Av1′ [b; sp act. 220 nmol of C_2H_2 reduced min^{-1} (mg of protein)$^{-1}$]. Gels were 10.5% polyacrylamide with a 3.5% stacking gel. Integration of the densitometer trace of Av1′ at 540 nm shows both bands in equal concentration (±10%).

all high-activity samples tested. Spin quantitation of just the $g = 2$ region yields approximately 0.4 spin per protein.

Discussion

The possibility of nitrogen fixation occurring in the absence of Mo or in the presence of other transition metals has intrigued scientists for many years. Specifically, several reports (Bortels, 1936; McKenna et al., 1970; Burns et al., 1971; Benemann et al., 1972; Nagatani & Brill, 1974) have appeared in the literature over the last five decades on the stimulatory effect of vanadium (V) on growth of *A. vinelandii* as well as the tentative identification of a V- and Mo-containing conventional component 1. All of these reports show that the inhibition of nitrogen-fixing growth produced by Mo depletion could be reversed by the addition of V to the medium. Recently, it was suggested (Bishop et al., 1982) that this effect was due to the depression of an alternative nitrogen-fixing system. Our present work shows that V actually is incorporated into the alternative enzyme.

The metal analysis of Av1′ shows it to contain both V and Fe in a ratio of approximately 1:13, respectively. It should be remembered, however, that the quantitation of the metal composition depends on the molecular weight of Av1′. Gel filtration implies a weight of 180 000–200 000 while SDS–PAGE suggests a weight closer to 214 000. Since the ability of either technique to estimate molecular weight depends on the shape of Av1′ and its interaction with SDS, we decided to use a molecular weight (200 000) that is based on an average weight from the two techniques.

The absence of a $g = 3.6$ signal in the ESR spectrum of Av1′ implies the lack of a conventional Mo-Fe-type cluster in this protein. The axial signal in the $g = 2$ region of Av1′ (Figure 2) with $g_{av} < 2.0$ has been observed for several other proteins containing Fe-S clusters. In particular, this signal is almost identical in both shape and spin quantitation (i.e., less than 1 spin/molecule) with the signal arising from the 4Fe-4S center of Av2 or Av2′ (Hales et al., 1986) in the presence of MgATP. The inflection at $g = 5.3$ is also reminiscent of those observed for Av2 (Lindahl et al., 1985; Hagen et al., 1985; Watt & McDonald, 1985) whose presence has been associated with an $S = {}^3/_2$ state of the 4Fe-4S cluster. Apparently, the low spin quantitation of the $g = 2$ signal of component 2 is due to distribution of spin density between the $S = {}^1/_2$ and $S = {}^3/_2$ states. A similar situation may prove to be true for the paramagnetism of Av1′, with the spin density being shared among two or more spin states. It is highly unlikely, however, that the signal in Figure 2 arises from an Av2′–MgATP impurity because (1) there is no Av2′ activity in these samples, (2) there is no observable band in SDS gels (Figure 1) that can be associated with the polypeptides (30 000) of Av2′, and (3) there is no MgATP in the sample (nor was it ever used in any step of the isolation). Furthermore, there is no indication that the $g = 2$ signal arises from the vanadium-centered radical. The only stable nuclide of vanadium is ^{51}V with $I = {}^7/_2$. Because the magnetogyric ratio of ^{51}V is large, hyperfine splitting (eight lines) is usually observed. No such splittings are observable in the spectrum in Figure 2.

Both the similarities and differences between conventional and alternative nitrogenase are now apparent. For similarities, both are two protein enzymes with one of the proteins (component 2) essentially unchanged. Both enzymes are dioxygen sensitive and require both MgATP and reducing equivalents (e.g., dithionite) for activity (Hales et al., 1986). Furthermore, these enzymes are each able to reduce not only dinitrogen but also acetylene and protons. Finally, the component 1 protein of each enzyme contains a relatively high concentration of Fe.

As so often happens, it will probably be the differences, rather than the similarities, between these enzymes that will aid us more in our understanding of the mechanism of nitrogen fixation. Aside from the difference in molecular weight, ESR spectrum, and substrate reduction efficiency, the major difference has to be the substitution of V for Mo in component 1. This substitution brings forth questions concerning the role, if any, each metal plays in substrate binding and reduction in their respective enzymes. Recently, an alternative nitrogenase was isolated from *Azotobacter chroococcum* and shown also to contain V (Robson et al., 1986). Similar to Av1′, component 1 of *A. Chroococcum* (called Ac1*) is composed of two polypeptides of weights 50 000 and 55 000. The substrate reduction pattern for the two bacteria, however, differs. For example, the ratios of reduction of N_2 to C_2H_2 (i.e., $3 \times N_2/C_2H_2$) for Ac1 and Ac1* are 1.2 and 1.0, respectively, while the similar ratios for Av1 and Av1′ (Table I) are 0.78 and 4.2, respectively. Looking at this in another way, by using the H_2 evolution activity as a point of reference, the major difference between the alternative enzymes of these two bacteria seems to be that Av1′ is poorer than Ac1* at acetylene reduction but better at nitrogen fixation. It will be interesting to see what other differences exist between these two enzymes.

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Articles

Isolation and Characterization of a Human Colon Carcinoma-Secreted Enzyme with Pancreatic Ribonuclease-like Activity†

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Received June 3, 1986; Revised Manuscript Received July 29, 1986

ABSTRACT: A ribonuclease was isolated from serum-free supernatants of the human colon adenocarcinoma cell line HT-29. It was purified by cation-exchange and C18 reversed-phase high-performance liquid chromatography. The protein is basic, has a molecular weight of ~16000, and has an amino acid composition that is significantly different from that of human pancreatic ribonuclease. The amino terminus is blocked, and the carboxyl-terminal residue is glycine. The catalytic properties of this ribonuclease resemble those of the pancreatic ribonucleases in numerous respects. Thus, it exhibits a pH optimum of ~6 for dinucleotide cleavage and employs a two-step mechanism in which transphosphorylation to a cyclic 2′,3′-phosphate is followed by slower hydrolysis to produce a 3′-phosphate. It does not cleave NpN′ substrates in which adenosine or guanosine is at the N position and prefers purines at the N′ position. Like bovine ribonuclease A, the HT-29-derived ribonuclease is inactivated by reductive methylation or by treatment with iodoacetate at pH 5.5 and is strongly inhibited by the human placental ribonuclease inhibitor. However, in contrast, the tumor enzyme does not cleave CpN bonds at an appreciable rate and prefers poly(uridylic acid) as substrate 1000-fold over poly(cytidylic acid). It also hydrolyzes cytidine cyclic 2′,3′-phosphate at least 100 times more slowly than uridine cyclic 2′,3′-phosphate and is inhibited much less strongly by cytidine 2′-monophosphate than by uridine 2′-monophosphate. Other ribonucleases known to prefer poly(uridylic acid) were isolated both from human serum and from liver and were compared with the tumor enzyme. The physical, functional, and chromatographic properties of the serum ribonuclease are essentially identical with those of the tumor enzyme. The liver enzymes, however, differ markedly from the HT-29 ribonuclease. The potential utility of the tumor ribonuclease in the diagnosis of cancer is considered.

We have recently developed a method for maintaining the human colon adenocarcinoma cell line HT-29 in a serum-free and exogenous protein free medium (Alderman et al., 1985). This advance has made possible the detailed examination of several products secreted by these cells, including the blood vessel forming protein angiogenin (Fett et al., 1985b), lysozyme (Fett et al., 1985a), and a vascular permeability factor (Lobb et al., 1985). Determination of the complete amino acid sequence of angiogenin (Strydom et al., 1985; Kurachi et al., 1985) revealed its extensive homology to human pancreatic ribonuclease (RNase),[1] with 35% identity, including virtually

† This work was supported by funds from the Monsanto Co. under agreements with Harvard University.

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0006-2960/86/0425-7255$01.50/0