Rabbit Adenosine Deaminase Conversion Proteins. Purification and Characterization†

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ABSTRACT: An animal model for the adenosine deaminase (ADase) conversion proteins has been demonstrated in various rabbit tissues. These proteins bind specifically to the type C rabbit or calf ADase (M_r ~35000) to produce a large increase in molecular weight with no significant effect on maximum velocity. Kidney displayed the greatest conversion activity, followed by ileum and lung. The latter tissues were shown to have all of their ADase present as high molecular weight enzyme and were also characterized by low specific ADase activity. The molecular weight of endogenous kidney ADase was estimated as 295000 by gel filtration chromatography. No strict correlation between the level of specific ADase activity and quantity of either ADase or free conversion protein was observed. The kidney conversion protein was purified over 1700-fold to apparent homogeneity. Purification steps included gel filtration chromatography followed either by specific affinity chromatography or, for resolution of electrophoretic variants, by preparative isoelectric focusing. The latter procedure demonstrated four major species of conversion activity with p*I* values of 5.65, 5.05, 4.50, and 4.15, respectively, but only two principal forms of high molecular weight ADase. Various degrees of interaction of the four variants with concanavalin A– and wheat germ lectin–Sepharose resins suggest carbohydrate differences as the basis for the microheterogeneity. The molecular weight of the conversion proteins was estimated by gel filtration chromatography to be ~215000 and to be essentially the same for each of the electrophoretic variants. The subunit molecular weight of 110000 determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis indicates a dimeric structure of apparently identical polypeptide chains. These data should provide a useful model for future studies on the biological function of these proteins.

Adenosine deaminase (EC 3.5.4.4) is a widely distributed aminohydrolase that catalyzes the irreversible hydrolysis of adenosine to inosine and ammonia. This enzyme is of special interest since much evidence supports a role in the regulation of cellular growth and differentiation [e.g., Ishii & Green (1973), Meuwissen et al. (1975), and Trotta & Balis (1978)]. In particular, clinical and in vitro studies strongly suggest a causal relationship between the genetically determined absence of this enzyme activity and the severe combined immunodeficiency disease, which is characterized by severe defects in cellular and humoral immunity (Dissing & Knudsen, 1972; Giblett et al., 1972; Meuwissen et al., 1975). The enzyme is of additional importance because it can also catalyze the deamination and consequent inactivation of several potent antitumor and antiviral nucleosides (Brink & LePage, 1964, 1965; Schabel, 1968; Plunkett & Cohen, 1975).

Several variants of ADase[1] that can be distinguished on the basis of charge and/or molecular weight have been described. The cytosolic vertebrate enzyme can be classified according to three molecular weight ranges: (1) ≥200000 (type A); (2) ~100000 (type B); (3) ~35000 (type C) (Ma & Fisher, 1968, 1969). In man erythrocytes contain exclusively type C enzyme, whereas varying amounts of the type A form are generally found in other tissues (Akedo et al., 1970, 1972; Edwards et al., 1971). Proteins have been described in certain human tissues which can cause a type C to type A conversion (Nishihara et al., 1973; Schrader & Stacy, 1977; Dadonna & Kelley, 1978). These conversion proteins (M_r ~200000) are apparently incorporated into the quaternary structure of the high molecular weight enzyme to form an oligomer containing nonidentical polypeptide chains (Schrader & Stacy, 1977).

The biological function of the ADase conversion proteins remains unknown. Various catalytic and regulatory properties of the high and low molecular weight ADase I are apparently identical (Nishihara et al., 1973; Ma & Magers, 1975). However, data from our laboratory (Trotta & Balis, 1978) suggest that an absent or modified conversion protein may be a fundamental property of the malignant cell. Thus, we have noted that human adenocarcinomas are characterized by a large proportion of the type C form, whereas the corresponding normal tissue has predominantly type A enzyme. Similar data with mitogen stimulation of lymphocytes suggest that a similar molecular change may occur in rapidly proliferating normal cells (Hirschhorn & Levytska, 1974).

Although a high molecular weight ADase has been described and partially characterized in vertebrates other than man [e.g., Ma & Fisher (1968, 1969), Murphy et al. (1969), and Piggott & Brady (1976)], no animal model for the conversion proteins has been previously reported. We present here the tissue distribution of these proteins in the rabbit and the purification and partial characterization of the rabbit kidney proteins.

Experimental Section

Materials. Xanthine oxidase (crystalline suspension in ammonium sulfate; from buttermilk), nucleoside phosphorylase (crystalline suspension in ammonium sulfate; from calf spleen), rabbit muscle pyruvate kinase, bovine serum albumin, yeast alcohol dehydrogenase, ovalbumin, *Escherichia coli* β-galactosidase, rabbit muscle lactic dehydrogenase, rabbit muscle phosphorylase A, bovine liver glutamate dehydrogenase, calf intestinal adenosine deaminase (50% glycerol–phosphate), horse heart cytochrome *c*, and imidazole were obtained from Sigma Chemical Co. (St. Louis, MO). Agar (special grade) and sucrose (enzyme grade) were obtained from Schwarz/Mann (Orangeburg, NY); ampholines were

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[1] Abbreviations used: ADase, adenosine deaminase; $NaDodSO_4$, sodium dodecyl sulfate; Con-A, concanavalin A.

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from LKB Instruments (Hicksville, NY). [8-^{14}C]Adenosine (water–ethanol solution, 1:1; 54.6 mCi/mmol) and iodine-125 (aqueous solution, pH 8–10, 17 Ci/mg) were obtained from New England Nuclear (Boston, MA). Sephadex gel filtration resins, cyanogen bromide activated Sepharose, and molecular weight standards (aldolase, catalase, thyroglobulin, and ferritin) were purchased from Pharmacia Fine Chemicals (Piscataway, NJ). Thin-layer cellulose plates were obtained from Eastman Kodak (Rochester, NY). Spectrin was the generous gift of Dr. Rudy Haschemeyer. Ultrafiltration membranes were purchased from Amicon Corp. (Lexington, MA).

Adenosine Deaminase Activity. ADase activity was most commonly determined spectrophotometrically by a modification of the method of Hopkinson and co-workers (1969) in which the inosine produced is converted to uric acid in the presence of commerical nucleoside phosphorylase (0.1 unit/mL) and xanthine oxidase (0.2 unit/mL). The rate of production of uric acid was monitored by continuous recording in a Beckman Acta III recording spectrophotometer by using a millimolar extinction coefficient of 12.2 at 293 nm (Kalckar, 1947). A reaction volume of 1.0 mL contained 0.2 mM adenosine and 100 mM potassium phosphate, pH 7.0, at 37 °C. An appropriate background blank was subtracted to correct for contaminating ADase activity in commercial preparations of xanthine oxidase and nucleoside phosphorylase.

For the determination of small quantities of activity, as, for example, in heart, liver, and colon homogenates, a radioactivity assay was employed. The sample was incubated for 20 min at 37 °C in a final volume of 0.20 mL containing 0.20 mM [8-^{14}C]adenosine in 100 mM potassium phosphate, pH 7.0. After the reaction was stopped by boiling for 2 min, the radioactive products inosine and hypoxanthine were separated from the substrate on thin-layer cellulose plates by using water-saturated 1-butanol–concentrated ammonium hydroxide (99:1) as the developing solvent. Both substrate and product spots were cut out, and radioactivity was determined by liquid scintillation.

A unit of activity is defined as the amount of enzyme that deaminates 1 μmol of substrate per min under the specified steady-state assay conditions.

Iodination. The general procedure of Hunter (1967) utilizing chloramine-T as the oxidizing agent was employed. Purified calf intestinal enzyme (0.5 mg) was reacted with 2.0 mCi of sodium iodide-125 and 0.01 mg of chloramine-T in 1.0 mL of 250 mM sodium phosphate and 25% glycerol, pH 7.2. After 1 min at room temperature, the reaction was terminated with sodium metabisulfite and sodium iodide at final concentrations of 0.16 and 20 mM, respectively. The labeled calf intestinal enzyme was exhaustively dialyzed against 50 mM imidazole·HCl and 100 mM sodium chloride, pH 7.0, to remove excess reagents. The labeling procedure was accompanied by a 50% loss in enzymatic activity. Approximately 8000–10 000 dpm/mg of protein was typically incorporated.

Conversion Activity. The sample was preincubated for 30 min at room temperature with 0.08 unit of highly purified calf intestinal ADase in 50 mM imidazole·HCl and 100 mM sodium chloride, pH 7.0, containing L-glutamate dehydrogenase (1.0 mg/mL) and ovalbumin (1.0 mg/mL). The latter proteins were added to maximize recovery from the subsequent gel filtration chromatography, which generally ranged from 75 to 80%. After the addition of 8% sucrose, the sample was applied to a Sephadex G-100 column (39 × 1 cm) equilibrated with the same buffer at room temperature (flow rate, 20 mL/h). The amount of high molecular weight ADase formed

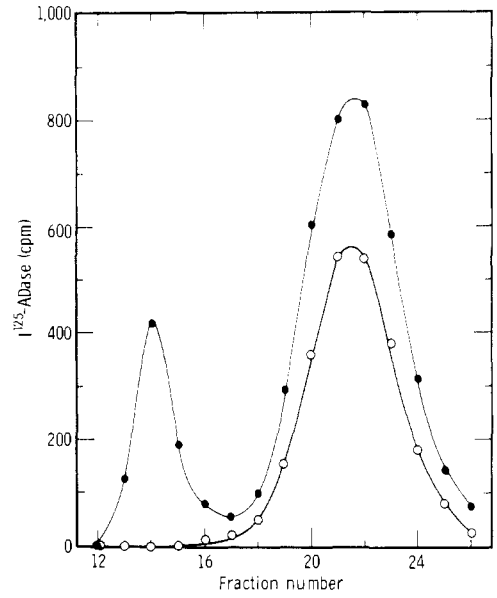

FIGURE 1: Sephadex G-100 chromatography of ^{125}I-labeled calf intestinal adenosine deaminase with and without the addition of rabbit kidney conversion protein: (○) calf intestinal ADase alone; (●) calf intestinal ADase plus purified rabbit kidney conversion protein. The total radioactivity recovered was 75–80% of the amount applied in each case. The amount of high molecular weight ADase produced was approximately proportional to the amount of conversion protein added. The calf enzyme employed in these studies was previously chromatographed on Sephadex G-200 for removal of high molecular weight impurities. Other details of the procedure are given in Experimental Section.

was quantitated by enzymatic activity, or, alternatively, radioactivity was determined in a γ counter when ^{125}I-labeled calf intestinal ADase was employed (Figure 1). The [^{125}I]ADase employed in these studies was chromatographed on Sephadex G-200 to remove contaminating high molecular weight species. Therefore, no background subtraction in the high molecular weight region was required. Parallel experiments employing both ^{125}I-labeled and unlabeled enzyme showed an agreement to within 10–15% of conversion units calculated from each assay. We conclude that iodination does not substantially affect the capacity of the calf intestinal enzyme to interact with the conversion protein.

A unit of conversion activity is defined as the amount of conversion protein which produces 1.0 unit of high molecular weight ADase activity in the excluded volume of a Sephadex G-100 column or the corresponding counts per min of ^{125}I-labeled enzyme, under the specified conditions of preincubation and chromatography.

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis. Electrophoresis was performed in a discontinuous buffer system, essentially as described by Neville (1971). An all-glass vertical slab apparatus was employed with gel dimensions of approximately 8.5 cm × 13.5 cm × 1.0 mm. A gradient in pore size from 6 to 10% polyacrylamide was also included for increased resolution and sensitivity. All samples were boiled in 1% mercaptoethanol–1% sodium dodecyl sulfate, pH 8, for 2 min. The run proceeded until the bromphenol blue marker reached the bottom of the gel. Protein standards included thyroglobulin (M_r 330 000 and 165 000), spectrin (M_r 225 000 and 215 000), β-galactosidase (M_r 130 000), phosphorylase A (M_r 92 000), bovine serum albumin (M_r 67 000), catalase (M_r 60 000), ovalbumin (M_r 45 000), and lactic dehydrogenase (M_r 36 000).

Affinity Chromatography. The general procedure recommended by Pharmacia Fine Chemicals was employed. Calf

intestinal adenosine deaminase (10 mg) was reacted with 1 g of cyanogen bromide activated Sepharose for 2 h at room temperature in 100 mM sodium carbonate and 500 mM sodium chloride, pH 8.3. An assay of the supernatant indicated that over 90% of the enzyme was bound. Unreacted activated residues were blocked by reaction with 1.0 M ethanolamine, pH 9, for 2 h at room temperature. The resin was washed alternatively with 0.1 M acetate and 1.0 M sodium chloride, pH 4, and 0.1 M borate and 1.0 M sodium chloride, pH 8, and the washing was repeated five times. The resin was equilibrated with 50 mM imidazole·HCl and 100 mM sodium chloride, pH 7.0. The sample of conversion factor containing approximately 1.5 units of activity in 0.5 mL was shaken gently at room temperature with 0.5 mL of resin for 30 min. The resin was poured into a column (7 × 0.15 cm) which was washed with 5 volumes of 50 mM imidazole·HCl and 100 mM sodium chloride, pH 7.0, followed by elution of the conversion protein with 50 mM sodium succinate, pH 3.0, containing 0.5 mg/mL cytochrome *c*. Fractions of 0.5 mL were collected, and the pH was immediately adjusted to 7.0–7.5 with 1 M imidazole.

Most of the conversion activity eluted in the first 1.5 mL with a recovery of 85–90% of the bound conversion units. A control resin was also prepared by coupling bovine serum albumin (10 mg) to the cyanogen bromide activated Sepharose. Nonspecific binding to this resin was approximately 5% of the units applied.

Isoelectric Focusing. The run was performed in an LKB Uniphor 7900 apparatus. A 5–50% linear sucrose gradient (160 mL) containing 1% (w/v) ampholines exhibiting p*K* values in the range of 3.5–7.0 was formed with a Pharmacia P-3 peristaltic pump. The sample to be focused was initially present throughout the column. The cathode and anode solutions were composed of 2.9% (v/v) ethanolamine in 64% sucrose and 1% (v/v) H_2SO_4, respectively. Runs were allowed to proceed for 48 h to a final voltage of 1200 V (maximum power output, 3 W). Fractions of ~1.0 mL were collected.

Protein Determination. Protein was determined by the method of Lowry (1951) with bovine serum albumin as standard.

Molecular Weight Estimation. Apparent molecular weight values were estimated by Sephadex G-200 gel filtration in a 76 × 1.6 cm column equilibrated with 50 mM imidazole·HCl and 100 mM sodium chloride, pH 7.0, at 4 °C. Standards included ovalbumin (M_r 45 000), bovine serum albumin (M_r 67 000), aldolase (M_r 160 000), catalase (apparent M_r 195 000; Andrews, 1965), pyruvate kinase (M_r 225 000), and ferritin (M_r 440 000).

Results

Tissue Distribution of Conversion Activity and Adenosine Deaminase Activity. The tissue distribution of specific adenosine deaminase activity in the rabbit (Table I) is similar to that previously described in other species: as, e.g., the mouse (Trotta et al., 1978) or man (Ma & Magers, 1975). Thus, cells of the gastrointestinal tract demonstrate the highest activities, followed by tissues of the lymphoid system, i.e., spleen and thymus. In distinction, the ileum, stomach, and colon represent areas of the gastrointestinal tract with relatively low activities. The specific activities for some of these tissues have been previously reported per gram of fresh tissue (Piggott & Brady, 1976) and are in general agreement in their relative order with the data reported here.

We have characterized the same tissues for the percentage of the high molecular weight type A enzyme by Sephadex G-100 gel filtration (Table I). The separations achieved were similar to those shown in Figure 1, except that enzymatic activities were used to locate the two species. Interestingly, the highest percentage of the type A enzyme is found among tissues with low specific activities, as has been previously noted in man (Ma & Magers, 1975; Van der Weyden & Kelley, 1976). However, no strict correlation between specific activity and type A enzyme was observed. Thus, for example, colon has a low specific activity (0.120 unit/mg of protein), but over 80% of its ADase activity is type C. On the other hand, of the five tissues with the highest specific activity (duodenum, jejunum, spleen, thymus, and stomach), less than 20% of the total activity is a high molecular weight enzyme.

Table I: Tissue Distribution of Rabbit Adenosine Deaminase and Conversion Protein[a]

tissue	specific activity[b] ADase	specific activity[b] conversion activity[d]	% ADase type A[c]
duodenum	20.7	-	1.0
jejunum	18.2	-	9.0
spleen	11.4	-	3.0
thymus	7.5	-	17.0
stomach	3.6	-	3.0
ileum	2.4	0.52	98.0
colon	1.2	-	16.0
lung	1.1	0.32	100.0
kidney	0.86	1.72	100.0
heart	0.62	0	64.0
liver	0.26	0	56.0

[a] A New Zealand white rabbit was sacrificed by gassing with carbon dioxide, and the indicated tissues were excised, cooled immediately on dry ice, and homogenized as 50% (w/v) solutions in a glass homogenizer in 250 mM sucrose–20 mM imidazole·HCl, pH 7.0. Activities were determined as soon as feasible on supernatants obtained after centrifugation at 15000*g* for 30 min. ADase activity was determined spectrophotometrically at 293 nm, except for heart, liver, and colon activities, which were determined by the conversion of [8-^{14}C]adenosine to radioactive products. Conversion units and the percentage of type A enzyme were determined by Sephadex G-100 gel filtration chromatography. Further assay details are described in Experimental Section. [b] Expressed as (units per mg of protein) × 10^2. [c] Obtained by dividing ADase units appearing in the excluded volume of a Sephadex G-100 column by the total units recovered. The latter generally represented 75–80% of the applied units. [d] (-) No determination was performed.

We have examined those tissues with 50% or more of their ADase activity as type A for conversion activity (Table I). As might be anticipated, kidney, ileum, and lung, the only tissues exhibiting 100% of their ADase activity as high molecular weight species, demonstrated the capacity to convert both calf intestinal and rabbit type C jejunal ADase to type A enzyme. No substantial change in the rate of adenosine deamination accompanied this process. Interestingly, kidney and lung are also good sources of human conversion protein (Nishihara et al., 1973; Schrader & Stacy, 1977). The failure to detect conversion activity in tissues exhibiting measurable percentages of low molecular weight enzyme lends further support to an interaction between the two proteins in vivo.

Purification of Kidney Conversion Proteins. The purification procedure summarized in Table II consisted of the following steps.

(*1*) *Homogenization.* A New Zealand white domesticated rabbit was sacrificed by gassing with carbon dioxide. The kidneys were excised and immediately placed on dry ice. For each gram of wet weight 1 mL of 20 mM imidazole·HCl and 250 mM sucrose, pH 7.0, was added. After homogenization with a glass homogenizer, the solution was centrifuged at 15000*g*. The supernatant was concentrated 2–3-fold by

Table II: Purification of Rabbit Kidney Conversion Proteins[a]

step	vol (mL)	protein (mg/mL)	conversion units	conversion units per mg of protein	purification
homogenate	13.0	52.0	11.70	0.0172	1.0
Sephadex G-200	25.0	0.95	2.22	0.089	2.3
isoelectric focusing					
I	1.4	0.30	0.16	0.53	31
II	1.5	0.20	0.14	0.70	40
III	1.2	0.26	0.36	1.38	80
IV	1.2	0.10	0.04	0.40	23
affinity chromatography[b]	3.0	0.11	3.3	30	1740

[a] For determination of conversion activity, sample was incubated with calf intestinal ADase and chromatographed on Sephadex G-100, as described in Experimental Section. For the homogenate and Sephadex G-200 steps, converted enzyme was measured by enzymatic assay. Radioiodinated enzyme conversion was employed for all subsequent steps, as described in Experimental Section. [b] ~8.0 conversion units from the pooled Sephadex G-200 chromatography were applied and eluted as described in Experimental Section.

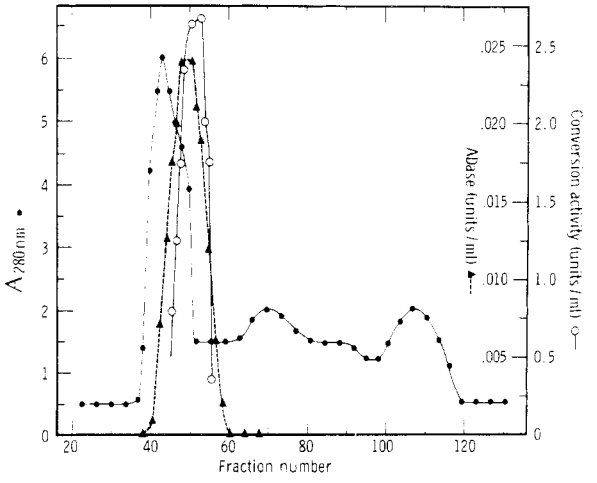

FIGURE 2: Sephadex G-200 chromatography of rabbit conversion proteins. Kidney homogenate concentrated to ~7 mL by ultrafiltration was applied to a Sephadex G-200 column (120 × 3 cm) equilibrated with 50 mM imidazole·HCl and 100 mM sodium chloride, pH 7.0, at 4 °C. Elution was in an upward direction at a flow rate of 20 mL/h. Fractions of 5 mL were collected and assayed as described in Experimental Section.

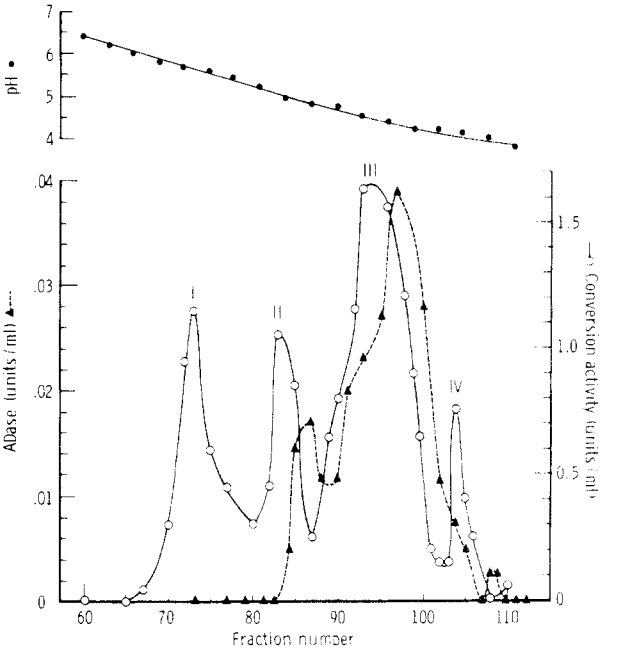

FIGURE 3: Preparative isoelectric focusing of rabbit conversion proteins. Fractions corresponding to the conversion activity peak of the Sephadex G-200 column were pooled, dialyzed overnight against 50 mM imidazole·HCl, pH 7.0, and subjected to isoelectric focusing in a pH 3.5–7.0 gradient, as described in Experimental Section.

Amicon XM-50 ultrafiltration to a final volume of ~7 mL.

(*2*) *Gel Filtration Chromatography.* A Sephadex G-200 column (120 × 3 cm) was equilibrated at 4 °C with 50 mM imidazole·HCl and 100 mM sodium chloride, pH 7, and was chromatographed in an upward direction at 20 mL/h. A single sharp peak of conversion activity was detected just after the appearance of the void volume (Figure 2). A low level of ADase activity was found to peak just before the conversion activity. The descending portion of the conversion activity peak was pooled and concentrated by Amicon PM-30 ultrafiltration.

(*3*) *Isoelectric Focusing.* The Sephadex G-200 concentrate was subjected to preparative isoelectric focusing in a pH gradient from 3.5 to 7.0 (Figure 3). Four peaks of conversion activity were detected corresponding to p*I* values of ca. 5.56 (I), 5.05 (II), 4.50 (III), and 4.15 (IV). Two main peaks of ADase activity were apparent at p*I* values of ca. 4.85 and 4.35. The unsymmetrical nature of both conversion activity and ADase peaks, however, strongly suggested the presence of even greater heterogeneity. For example, distinct shoulders of activity were apparent on the basic side of conversion protein variant III, as well as on the basic side of the p*I* 4.35 ADase variant. Interestingly, no ADase activity was detectable in the pH region in which conversion protein I was located. On the other hand, no fractions with ADase activity free of conversion activity were found.

Significant variation was noted in the specific activities of the conversion protein variants (Table II). This result implied a lack of complete homogeneity at this stage, which was confirmed by $NaDodSO_4$ gel electrophoresis (see below). The relatively low recovery of conversion units, representing ~30% of the applied units, also suggests a partial inactivation produced by instability at the isoelectric point.

(*4*) *Affinity Chromatography.* A specific affinity column was prepared by direct coupling of commercially obtained, purified calf intestinal ADase to cyanogen bromide activated Sepharose, as described in Experimental Section. Fractions from the peak of the Sephadex G-200 eluant were pooled, concentrated by ultrafiltration, and applied directly to this column. Approximately half of the applied units were bound; conversion activity not bound appeared to display a significantly lower affinity for a second ADase–Sepharose column. Eighty to ninety percent of the bound units were readily eluted with 50 mM sodium succinate, pH 3.0. These conditions were chosen since acidic conditions have previously been reported to prevent formation of the conversion protein–ADase complex (Van der Weyden & Kelley, 1976). Maximal yields were obtained by the inclusion of cytochrome *c* (0.5 mg/mL) in the elution buffer to prevent losses due to nonspecific adsorption. Cytochrome *c* was particularly useful for this purpose since

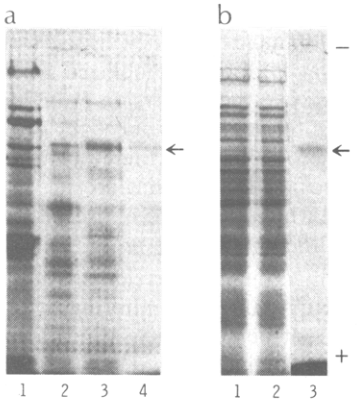

FIGURE 4: $NaDodSO_4$ gel electrophoresis of purified rabbit conversion proteins. (a) Electrophoretic variants. Channels 1, 2, 3, and 4 contain samples of peak fractions corresponding to variants I, II, III, and IV shown in Figure 3. The arrow indicates a species of ~10000 molecular weight. (b) Affinity chromatography eluants: channel 1, sample applied to the resin, which coresponds to the peak of conversion activity from the Sephadex G-200 chromatogram (Figure 2); channel 2, sample which did not bind to the resin; channel 3, sample eluted with 50 mM sodium succinate, pH 3.0. Samples applied to gels in both (a) and (b) were concentrated by ultrafiltration, boiled with 1% $NaDodSO_4$–1% mercaptoethanol for 2 min, and electrophoresed in a discontinuous buffer system with a 6–10% gradient in polyacrylamide, as described in Experimental Section. Samples also contained 30–40 μg of cytochrome *c* as indicated by the dark region at the bottom of the gel. Cytochrome *c* was found to migrate close to the dye front and to have no effect on the relative migration of a mixture of standards of known molecular weight.

it readily passes through the Amicon XM-50 ultrafiltration membrane used for concentration of the eluant while the relatively high molecular weight conversion protein is retained.

The specific conversion activity of the eluted protein indicated a purification of over 1700-fold (Table II). The specific activity exceeded the best value obtained after isoelectric focusing by over 20-fold.

Sodium Dodecyl Sulfate Electrophoresis. The homogeneity of the preparations after both isoelectric focusing and affinity chromatography was monitored by $NaDodSO_4$ gel electrophoresis (Figure 4). The four electrophoretic variants exhibited varying numbers of polypeptide chains. The least homogeneous was variant I, while variants III and IV represented the best preparations at this stage. A major band, indicated by the arrow in Figure 4a and corresponding to a molecular weight of ~110000 ± 5000, was found in variants III and IV. This species represented the only polypeptide chain found in each of the four electrophoretic peaks and therefore can reasonably be assumed to represent a subunit of the protein.

$NaDodSO_4$ gel electrophoresis of the species eluted from the affinity column displayed essentially one polypeptide chain (Figure 4b). This species also yielded a molecular weight of 110000, a value essentially invariant over the gel concentration range of 5–9%. A comparison of the number of distinct polypeptide chains in the sample applied to the affinity column with those which did not bind to the resin supports an extremely high specificity in the conversion factor–ADase interaction (Figure 4b). The 110000 molecular weight form was noted to diminish in intensity but not completely disappear in the mixture of proteins which was not bound to the resin (Figure 4b). This is consistent with the observation that approximately half of the conversion units applied did not bind to the affinity column.

It is notable that the number of contaminants observed in the various preparations did not show a strict correlation with the ratio of specific conversion (Table I). For example, variant I displays a specific activity ~40% of that of variant III, which is not proportional to their relative degrees of homogeneity (Figure 4a). This lack of correlation is most strikingly demonstrated in a comparison of the protein purified by affinity chromatography with, for example, variants III or IV. The latter demonstrate a specific activity far lower than a comparison of their purity with the highest specific activity obtained by the affinity procedure would predict. These data may reflect inherent differences in the binding affinity of each of the variants for ADase, a partial inactivation at the isoelectric point (as previously implied from the low recovery of units), or a combination of both.

Molecular Weight Estimation. The molecular weight value for the predominant form of conversion protein (Figure 2) was estimated on a calibrated Sephadex G-200 column as 215000, as described in Experimental Section. No significant difference in the molecular weight of the four electrophoretic variants could be detected by this technique. ADase activity generally eluted somewhat earlier with an apparent molecular weight of ~295000. Other molecular weight species of conversion protein associated with a low level of ADase activity were noted in some preparations. For example, a shoulder of conversion activity in the excluded volume of the column, probably representing a form of aggregate, was occasionally observed, in addition to a species with a lower molecular weight than the native form. The latter eluted at a position consistent with a dissociation to subunits with one-half the original molecular weight.

In combination with a polypeptide chain molecular weight of 110000 (Figure 4), the most probable subunit structure is a dimer. In addition, since the molecular weight of the rabbit type C ADase is ~–34000 (Murphy et al., 1969; Piggott & Brady, 1976), the relative molecular weight values of conversion protein and high molecular weight ADase suggest that each conversion protein subunit has the capacity to bind 1 mol of type C ADase. To explore this possibility further, we have quantitated the maximal binding of ^{125}I-labeled calf intestinal ADase [M_r ~35000 (Brady & O'Sullivan, 1967)] as ~1.4 ● 0.2 mol/mol of conversion protein utilizing polyacrylamide gel electrophoresis to remove excess calf intestinal enzyme (Trotta & Balis, 1978). A value greater than 1.0 further supports the capacity of at least a portion of the purified conversion protein to bind 2 mol of ADase/215000 daltons. The failure to obtain an integral number of 2.0, as has been reported for the human kidney conversion protein (Dadonna & Kelley, 1978), may reflect a partial dissociation of the ADase–conversion protein complex during the electrophoresis or may represent a partial denaturation of conversion protein when highly purified.

Lectin Affinity. Experiments in our laboratory on the human placental conversion proteins have indicated a differential binding to immobilized lectins which was dependent on the p*I* of the particular species (P. P. Trotta, I. Yamaura, S. Sur, and M. E. Balis, unpublished experiments). We have therefore compared the relative affinities of the rabbit conversion protein electrophoretic variants for both Con-A– and wheat germ lectin–Sepharose resins, as well as for a Sepharose 6B resin in which no lectin was attached (Table III). These resins are useful in detecting different kinds of glycoproteins since wheat germ lectin binds to *N*-acetylglucosamine and sialic acid residues (Nagata & Burger, 1974) while Con-A is specific for α-D-mannopyranosyl and α-D-glucopyranosyl residues (Lloyd et al., 1969). The data in Table III support that each of the conversion protein variants is indeed a glycoprotein which can interact with these two lectins to varying

Table III: Binding of Rabbit Conversion Proteins to Lectin-Sepharose[a]

conversion protein variant	% of conversion activity bound		
	control Sepharose	Con-A	wheat germ lectin
I (p*I* 5.75)	14	94	81
II (p*I* 5.10)	16	92	69
III (p*I* 4.55)	44	88	88
IV (p*I* 4.20)	42	94	91
Sephadex G-200 eluant	17	54	58

[a] Each of the indicated Sepharose resins was incubated with an approximately equal volume (~0.2 mL) of sample containing ~0.1 unit of conversion activity in 50 mM imidazole·HCl and 100 mM sodium chloride, pH 7.0, at room temperature for 30 min with gentle shaking. The resin was removed by centrifugation at 1000*g* for 5 min, and the conversion activity remaining in the supernatant was determined as described in Experimental Section. The percentage of conversion activity bound was calculated from the difference between this value and the original units. Nonspecific binding was determined with a Sepharose 6B resin to which no lectin was bound and is indicated in the column "control Sepharose".

degrees. Consistently high degrees of binding to both resins were observed, but the percentage bound was found to vary significantly with the p*I* of the protein. These differences in apparent affinity were particularly evident with the wheat germ lectin resin. Binding could also be readily demonstrated in the relatively crude mixture of proteins obtained after Sephadex gel filtration.

It is notable that despite the fact that the amount of resin present was several-fold in excess of that required to bind all of the protein present, no electrophoretic variant demonstrated complete binding. Increasing the amount of resin used in each case did not appear to increase the percentage bound significantly. This result suggests the existence within each category of p*I* value of a further microheterogeneity based on carbohydrate content. Such a fact could be the cause of the somewhat asymmetrical character of the isoelectric focusing peaks previously noted. It is of interest that partial adsorption to lectin resins has also been reported for certain variants of the type A human ADase (Swallow et al., 1977). Partial adsorption of each of the variants to the control Sepharose resin was also observed, which could be a consequence of a weak, nonspecific interaction of the carbohydrate moiety with the hydrophilic groups on the agarose matrix. Losses of conversion activity that we have noted on ultrafiltration membranes in the absence of a protecting protein like cytochrome *c* may be another aspect of this interaction.

Discussion

Although several reports exist of a high molecular weight ADase species in vertebrates other than man, there has been no indication of the mechanism of their formation. We report here a very specific ADase conversion protein present only in rabbit tissues characterized by ADase exclusively in the high molecular weight form. This result strongly implies that the high molecular weight enzyme is formed by the interaction of these proteins with the low molecular weight ADase found in tissues without conversion activity. This mechanism is consistent with the relative molecular weight values of the type A ADase (M_r ~295 000) and the conversion proteins (M_r ~215 000). Thus, it is reasonable, although as yet not definitively demonstrated, that a single ADase gene product is the basis for the catalytic activity in both molecular weight ranges. This conclusion is supported in human tissues by the absence of all forms of ADase in the genetically determined severe combined immunodeficiency disease (Hirschhorn et al., 1973). The capacity of the immobilized type C ADase resin to bind exclusively to the conversion protein in the presence of many different proteins is evidence for the high specificity of the interaction. Our data therefore represent the first indication of specific ADase binding proteins from a nonhuman source, a model which should be of great value in studies on the biological function of these proteins.

We have purified these conversion proteins over 1700-fold to a specific activity of ~30 units/mg of protein. A high degree of apparent homogeneity is indicated by the single polypeptide chain obtained on $NaDodSO_4$ gel electrophoresis. The presence of this polypeptide in all electrophoretic variants confirms it as a subunit of the protein. The degree of purification is comparable to the 1690-fold reported for the human kidney preparation (Dadonna & Kelley, 1978). The higher specific conversion activity of the latter (~108 units/mg of protein) most probably reflects, at least in part, the different assay procedures employed. Since neither our assay procedure nor the electrophoretic method employed in studies on the human protein determines the extent of conversion under equilibrium conditions, different degrees of dissociation of the complex in the two assay systems could be expected. In addition, recent studies in our laboratory have indicated that the amount of conversion to high molecular weight ADase can be significantly elevated for both homogenate and purified conversion protein by increasing the amount of ^{125}I-labeled calf ADase in the assay medium. Thus, the absolute number of conversion units is highly dependent on the assay conditions employed.

Similarity exists between the human and rabbit conversion proteins in that each is composed of two polypeptide chains of comparable molecular weight (~110 000). A notable difference is the several electrophoretic forms characteristic of the rabbit proteins, in distinction to the one electrophoretic form reported for the human kidney binding protein. This result may be related to a difference in the carbohydrate moieties since the latter protein is reported not to react with wheat germ agglutinin, in contrast with the data reported here. The human conversion protein does, however, contain carbohydrate residues, which cause it to interact with other lectins. Interestingly, human lung preparations also exhibit one electrophoretic form (Nishihara et al., 1973), whereas the human placental conversion proteins studied in our laboratory exhibit several variants of both conversion protein and ADase over the broad pH range of 4–7 (P. P. Trotta, I. Yamaura, and M. E. Balis, unpublished experiments). Thus, there is a distinct tissue-dependent and species-dependent heterogeneity of these proteins, the functional significance of which remains unknown.

The cause of the microheterogeneity observed on isoelectric focusing has not been established. A reasonable speculation is that the apparent differences in charge are produced by different amounts and types of carbohydrate, as frequently noted in other systems [e.g., Hayes & Wellner (1969)]. Differences in the binding of the variants to the Con-A– and wheat germ lectin–Sepharose support this conclusion. Similar data by Swallow and co-workers (1977) also suggest that the so-called "tissue-specific" type A human ADase enzymes are glycoproteins with heterogeneous carbohydrate content. The data presented here further imply that the multiple electrophoretic forms noted for the tissue-specific ADase are most probably related to differences in the conversion protein portion of the molecule.

An interesting point of comparison between the rabbit conversion proteins and the various forms of ADase derived from them is the marked difference in electrophoretic distribution (Figure 3). Thus, the conversion protein displays four distinct peaks over the pH range of ~4.2–5.8, whereas ADase shows two distinct peaks over the somewhat narrower range of 4.4–5.0. These results may reflect a preferential binding of endogenous low molecular weight ADase to particular conversion protein variants. Alternatively, the isoelectric points of the various ADase–conversion protein complexes may be sufficiently close so as to render these species unresolvable by isoelectric focusing. Regardless of the mechanisms involved, however, the presence of large quantities of conversion protein with electrophoretic properties distinct from any endogenous ADase further indicates the inherent complexity of the system and suggests a unique biological role for these proteins independent of the deamination of adenosine.

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