Identification of a Membrane Adenosine Deaminase Binding Protein from Human Placenta†

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ABSTRACT: A class of proteins has been described that binds specifically to type C adenosine deaminase (ADase; M_r 35000) to produce a high molecular weight, tissue-specific form. In the present study an ADase binding protein has been identified in membrane fractions and, in particular, syncytiotrophoblastic plasma membrane in human placenta and purified to a high degree of homogeneity. Extraction of both 12000*g* and 105000*g* pelleted fractions with deoxycholate–Triton X-100 yielded high specific ADase binding activity and resulted in a large stimulation of ADase activity. When purified trophoblastic plasma membrane was extracted with the same detergent mixture, similarly high levels of ADase binding activity and 6-fold increase in ADase activity were observed. Both membrane-derived and soluble ADase were characterized by gel filtration as type A enzymes (M_r ≥200000). With an ADase affinity resin ADase binding protein was purified over 480-fold to greater than 95% homogeneity as judged by sodium dodecyl sulfate electrophoresis; the subunit molecular weight was estimated as 110000. Binding to concanavalin A–Sepharose and wheat germ lectin–Sepharose resins and elution with specific sugars supported the presence of carbohydrate. The ADase binding activity of the purified protein was characterized by the following: (1) a concave-downward Scatchard plot with an apparent limiting value for the association constant of 3.5×10^{11} M^{-1}; (2) a stoichiometry of 0.95 mol of ADase bound/M_r 110000; (3) a strict linear relationship between the concentration of binding protein and the conversion of ADase to high molecular weight species; (4) a rapid time course of binding. These data lead to the hypothesis that a form of ADase anchored to the plasma membrane through a specific binding protein functions to protect the mammalian cell from the toxic effects of adenosine and 2′-deoxyadenosine entering from the extracellular space.

The proteins that control the metabolism of adenosine and 2′-deoxyadenosine are of special interest because of a substantial body of in vitro and clinical data that supports an important role for these nucleosides in regulating the growth and differentiation of mammalian cells. Both cytotoxic and cytostatic effects of these naturally occurring nucleosides have been reported in mammalian cells [e.g., Green & Chan (1973) and Ishii & Green (1973)] in addition to bacteriostatic action [e.g., Shobe & Campbell (1973)]. The fact that this toxicity is generally potentiated by inhibitors of ADase[1] (adenosine aminohydrolase, EC 3.5.4.4) suggests an important role for the deamination reaction in detoxification. It is consistent with this function that an inherited deficiency of ADase in man is the apparent cause of a severe combined immunodeficiency disease, in which both B-cell development and T-cell development are severely impaired (Dissing & Knudsen, 1972; Giblett et al., 1972). Similarly, administration of an ADase inhibitor to mice is highly lymphocytotoxic (Trotta et al., 1981).

ADase, which catalyzes the hydrolytic deamination of adenosine, 2′-deoxyadenosine, and a number of synthetic adenosine analogues to the corresponding 6-oxy derivatives, is a ubiquitous enzyme found in virtually all mammalian tissues and in a variety of organisms [e.g., Brady & O'Donovan (1965) and Ma & Fisher (1968, 1969)]. It is polymorphic in both size and charge. The normal human enzyme is characterized by two cytoplasmic forms that differ in molecular weight: type A (M_r ≥200000) and type C (M_r ca. 35000) (Ma & Fisher, 1968, 1969). The intermediate type B enzyme (M_r ca. 100000) is found mainly in tissues of lower vertebrates (Ma & Fisher, 1972). Much evidence strongly implies that the relative amounts of the type A and type C enzymes, which are tissue dependent, are determined by the presence of an ADase binding protein, also referred to as a conversion or complexing protein (Akedo et al., 1970, 1972). This high molecular weight glycoprotein, which previous studies have implied to be cytoplasmic in origin (Van der Weyden & Kelley, 1976), specifically and stoichiometrically combines with type C enzyme and thus becomes incorporated into the quaternary structure of the type A ADase (Schrader & Stacey, 1977; Dadonna & Kelley, 1978, 1979). Free binding protein in excess of enzyme is found in selected tissues as, for example, human kidney and lung (Nishihara et al., 1973; Van der Weyden & Kelley, 1976). Similar proteins also occur in certain tissues of the rabbit (Trotta et al., 1979).

The precise biological significance of the ADase binding protein or of two molecular weight forms of the enzyme remains elusive. It is notable that both types of ADase are similar in substrate specificity and various catalytic properties (Nishihara et al., 1973; Ma & Magers, 1975) and that addition of ADase binding protein to purified type C enzyme does not produce a significant alteration in the maximum velocity (Dadonna & Kelley, 1978) or the Michaelis–Menten constant for hydrolysis of adenosine (Schrader & Pollara, 1978). Previous studies (Trotta & Balis, 1978) have suggested that changes in the properties of these proteins or in their rate of biosynthesis may be a fundamental property of the malignant cell.

An indication of a possible function for these proteins is provided by the observation that although ADase is generally

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[1] Abbreviations: ADase, adenosine deaminase; M_r, molecular weight; AMP, adenosine 5′-monophosphate; $NaDodSO_4$, sodium dodecyl sulfate; PBS, Dulbecco's phosphate-buffered saline; NADH, reduced nicotinamide adenine dinucleotide; Con A, concanavalin A; Tris, tris(hydroxymethyl)aminomethane.

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found in the cytoplasmic fraction, in some mammalian cells, as, for example, human erythrocyte (Agarwal & Parks, 1975), it may be closely associated with the plasma membrane. The data presented here establish for the first time that a binding protein can be extracted and purified from the particulate fractions, and, in particular, the plasma membrane, of human placental syncyticial trophoblast. It is consistent with these findings that the majority of the ADase in this tissue was found to be membrane bound and was released from its latent state by extraction with detergent. These results lead to the hypothesis that in human placenta, and perhaps in other tissues as well, these binding proteins function to juxtapose at least a part of the cellular ADase to the plasma membrane.

Materials and Methods

Calf intestinal ADase (50% glycerol–0.01 M potassium phosphate, pH 6.0), bovine liver glutamate dehydrogenase, horse heart cytochrome *c*, β-glycerophosphate (disodium salt), *N*-acetyl-D-glucosamine, imidazole, Triton X-100, 2,6-dichlorophenolindophenol, *Escherichia coli* β-galactosidase, and rabbit muscle phosphorylase A were purchased from Sigma Chemical Co. (St. Louis, MO). Sodium deoxycholate and Coomassie brilliant blue were obtained from Fisher Scientific Co. (Fair Lawn, NJ), and methyl β-D-mannopyranoside was from Calbiochem-Behring Corp. (La Jolla, CA). Ampholines (pH 5–7) were obtained from LKB Instruments (Hicksville, NY). Dulbecco's phosphate-buffered saline (2.7 mM KCl–1.5 mM KH_2PO_4–8.1 mM NaH_2PO_4–136.9 mM NaCl, pH 7.2) and Hank's balanced salt solution were purchased from GIBCO (Grand Island, NY). The source of [^{14}C]AMP (diammonium salt; water–ethanol solution, 1:1; 54.6 mCi/mmol; uniformly labeled) was New England Nuclear (Boston, MA). Molecular weight standards (thyroglobulin, bovine serum albumin, and ovalbumin), cyanogen bromide activated Sepharose, Sephadex resins, and blue dextran 2000 were from Pharmacia Fine Chemicals (Piscataway, NJ). Thin-layer cellulose plates were purchased from Eastman Kodak (Rochester, NY). Acrylamide and bis(acrylamide) were purchased from Bio-Rad Laboratories (Richmond, CA).

ADase Binding Activity. The amount of ADase binding protein was quantitated by incubating sample containing binding activity with a saturating amount of ^{125}I-labeled calf intestinal ADase and measuring the number of picomoles of ^{125}I-labeled calf intestinal ADase converted to radiolabeled high molecular weight enzyme. The radiolabeled high molecular weight enzyme was separated from excess low molecular weight [^{125}I]ADase by chromatography on Sephadex G-150. In brief, the sample to be assayed for binding activity was incubated for 20 min in a final volume of 0.3 mL containing ^{125}I-labeled calf intestinal ADase (6.6 μg; 50000 dpm), L-glutamate dehydrogenase (300 μg), and 13% sucrose in 50 mM imidazole hydrochloride–100 mM sodium chloride, pH 7.0. This solution was applied to a Sephadex G-150 column (39 × 1 cm) equilibrated with 50 mM imidazole hydrochloride–100 mM sodium chloride, pH 7.0. Elution was performed with the same buffer at a flow rate of 8–10 mL/h at room temperature; 1.0-mL fractions were collected. Radioactivity appearing in the excluded volume, which was determined with dextran 2000, was fully resolved from low molecular weight ADase and was quantified in a Beckman Radioimmuno-Analyzer. So that the background of contaminating high molecular weight enzyme present in the ^{125}I-labeled calf intestinal ADase preparation could be determined, a control solution consisting of ^{125}I-labeled calf intestinal ADase (6.6 μg; 50000 dpm), L-glutamate dehydrogenase (300 μg), 13% sucrose, and 50 mM imidazole hydrochloride–100 mM sodium chloride, pH 7, in a 0.3-mL final volume was also chromatographed under identical conditions. This background value, which represented ca. 2% of the total radioactivity present, was subtracted from the dpm appearing in the excluded volume to yield the net radioactivity produced by ADase binding protein. The mean recovery of radioactivity after chromatography was 79 ± 3% (±SD) (mean of 10 determinations) of the total radioactivity applied to the column. This recovery was constant over the entire range of radiolabeled ADase employed in the Scatchard analysis (Figure 3).

A unit of ADase binding activity[2] is defined as the amount of protein that produces 1 pmol of radiolabeled high molecular weight enzyme under the specified conditions of preincubation and chromatography. On the basis of the above considerations, the units of ADase binding activity are calculated from the ratio (net dpm appearing in excluded column volume)/[specific radioactivity of calf intestinal ADase × 0.79 (dpm/pmol of enzyme)].

Iodination. Commercially obtained purified calf intestinal ADase (0.5 mg) was iodinated with Na^{125}I (5 μCi) by employing Chloramine T as the oxidizing agent, essentially as described by Hunter (1973) and previously performed in this laboratory (Trotta et al., 1979).

Adenosine Deaminase. ADase was determined by a sensitive radioactive assay in which the conversion of [8-^{14}C]-adenosine to radioactive products was quantified under the reaction conditions previously employed (Trotta et al., 1979). Thin-layer cellulose chromatography in 1-butanol–concentrated ammonium hydroxide (99:1) was employed for resolution of product and substrate, followed by quantitation by liquid scintillation spectrometry. The micromoles of adenosine deaminated is calculated from the percentage of the total radioactivity appearing as the products inosine and hypoxanthine. Incubation times and sample volumes were chosen to produce a maximum of 20% conversion to product. A unit of activity is defined as the amount of enzyme that deaminates 1 μmol of substrate per minute under the specified steady-state conditions.

Polyacrylamide Gel Electrophoresis. Samples were boiled for 2 min in a final volume of 0.1 mL containing 1% Na-DodSO_4, 1% mercaptoethanol, and 0.1 M Tris, pH 9–10. After addition of sucrose (final concentration 10%) and bromphenol blue marker, electrophoresis was performed in a discontinuous buffer system (Neville, 1971) with 6%–15% gradient in polyacrylamide gel concentration in an all-glass vertical apparatus (85 mm × 135 mm × 1.0 mm). All samples including standard proteins were boiled for 2 min in 1% mercaptoethanol–1% NaDodSO_4, pH 8. Gels were stained with 0.25% Coomassie brilliant blue in 50% methanol–7.5% acetic acid–3% glycerol.

Affinity Chromatography. Purified calf intestinal ADase (10 mg) was reacted with cyanogen bromide activated Sepharose, as previously performed in this laboratory (Trotta et al., 1979). A control resin in which bovine serum albumin (10 mg) replaced ADase was prepared similarly. A 10-mL sample of each resin, previously equilibrated with 50 mM imidazole hydrochloride–100 mM sodium chloride, pH 7.0, was reacted with 25 mL of a deoxycholate–Triton X-100 extract of the 12000*g* pellet (prepared as described below) containing 5000 units of ADase binding activity. Reaction

[2] ADase binding activity is distinct from ADase enzymic activity. The former, sometimes referred to simply as "binding activity", refers to the capacity of the specific protein studied here to bind to low molecular weight type C ADase, while the latter refers to the capacity of ADase to hydrolyze adenosine to inosine and ammonia.

proceeded with end-to-end turning for 60 min at room temperature. The resin was collected by filtration on scintered glass and washed with a minimum of 10 volumes of 50 mM imidazole hydrochloride–100 mM sodium chloride, pH 7.0, containing 0.5 mg/mL cytochrome *c* and 0.1% ampholines (pH 5–7). The resin was poured into a column (14 × 1 cm) and eluted at 10 mL/h with 50 mM sodium succinate, pH 3.0, containing cytochrome *c* and ampholines as described above. Fractions of 1.0 mL were collected and immediately neutralized to pH 7–8 with 1 N Tris. For removal of cytochrome *c* and ampholines, the purified binding protein was concentrated by Amicon XM-50 ultrafiltration to 1.0 mL and applied to a Sephadex G-100 column (50 × 0.9 cm) equilibrated with 50 mM imidazole hydrochloride–100 mM sodium chloride, pH 7.0, and eluted with the same buffer. Fractions of 1.0 mL containing ADase binding protein were pooled and concentrated by Amicon XM-50 ultrafiltration.

Subcellular Fractionation. All solutions contained penicillin (100 units/mL) and streptomycin (0.1 mg/mL) and were maintained at 4 °C through all procedures. A full-term, normal human placenta obtained within 20 min of delivery was immediately placed on ice and trimmed of connective tissue. After blood was removed by extensive flushing with 0.9% sodium chloride, the placenta was homogenized at low speed in a Waring Blendor with 10 volumes of 0.25 M sucrose, 1 mM magensium chloride, 1 mM ATP, and 10 mM Tris-HCl, pH 7.4, conditions described by Fox & Marchant (1976). The homogenate was centrifuged at 12000*g* for 30 min in a Sorvall 5B centrifuge, and the pellet was washed 3 times with PBS. The postmitochondrial, 12000*g* supernatant was centrifuged for 65 min at 105000*g* in an OTD-65 Sorvall ultracentrifuge to obtain the microsomal pellet, which was washed as described above.

For extraction of membrane components, the washed 12000*g* pellet was stirred for 30 min successively with 200, 100, and 100 mL, respectively, of 2% deoxycholate–1% Triton X-100–0.1% ampholines (pH 5–7) in 10 mM Tris-HCl, pH 7.4. Ampholines were included in the extraction medium for stabilization of 5′-nucleotidase, as described by Fox & Marchant (1976). After each treatment the suspension was centrifuged at 20000*g* for 20 min. The washed 105000*g* pellet was extracted by homogenization in a motor-driven glass homogenizer successively with 60, 50, and 40 mL of the deoxycholate–Triton X-100 solution described above. After each extraction the suspension was centrifuged at 20000*g* for 20 min.

Plasma Membrane Isolation. Syncyticial trophoblastic plasma membrane was isolated by a minor modification of the procedure of Smith et al. (1977). Procedures were performed at 4 °C, and all solutions contained penicillin (100 units/mL) and streptomycin (0.1 mg/mL). A fresh, normal human full-term placenta was trimmed of connective tissue and flushed extensively with 0.9% sodium chloride. Trophoblast weighing ca. 150 g was cut into 1–2-cm strips and placed immediately in 200 mL of Hank's balanced salt solution. The tissue was collected on gauze in a funnel and washed with 3 volumes of 100 mM calcium chloride, followed by 3 volumes of PBS. The villous tissue was transferred to a beaker containing 200 mL of PBS and stirred gently for 40 min to remove the microvilli. The suspension was centrifuged at 800*g* for 10 min; the pellet consisting of erythrocytes and cellular debris was discarded. The supernatant was centrifuged at 10000*g* for 20 min, and the pellet was discarded. The latter supernatant was centrifuged at 105000*g* for 35 min. The pellet was washed 2 times with PBS and resuspended in 5 mL of PBS. The preparation is highly enriched in plasma membrane, as judged by the enzyme composition in Table II and the electron microscope studies of Smith et al. (1977).

Enzyme Marker Assays. For establishment of the purity of the plasma membrane preparation, several enzymes that are readily assayed and have previously been demonstrated to monitor the presence of specific organelles in either placental trophoblast (Carlson et al., 1976; Smith et al., 1977) or various other tissues [e.g., Leighton et al. (1968) and Steck & Wallach (1970)] were employed as markers. Briefly, these assays were as follows. (1) Plasma membrane markers: 5′-Nucleotidase was determined by a radioactive assay in which product and substrate were resolved by thin-layer chromatography. Sample was incubated for 20–40 min at 37 °C in 0.1 mL containing 0.3 mM [^{14}C]AMP, 50 mM magnesium chloride, and 100 mM β-glycerophosphate in 100 mM Tris-HCl, pH 8.2. After the reaction was terminated by boiling for 2 min, 0.01 mL of 4 mM adenosine–4 mM inosine was added as a marker for product. A 0.02-mL sample was chromatographed on thin-layer cellulose in a solvent composed of 71 mL of 95% ethanol, 30 mL of 1 M ammonium acetate, and 4 mL of H_2O, pH 7.5. Spots corresponding to substrate and product were cut out and counted by liquid scintillation spectrometry. Alkaline phosphatase was determined by monitoring the hydrolysis of *p*-nitrophenyl phosphate at 493 nm (Sussman et al., 1968). (2) Mitochondrial marker: Glutamate dehydrogenase was determined by the decrease in absorbance at 340 nm (Ellis & Goldberg, 1972). (3) Microsomal marker: NADH diaphorase was determined by reduction of 2,6-dichlorophenolindophenol (Mahler et al., 1952). (4) Peroxisomal marker: Catalase was quantitated by following the decomposition of hydrogen peroxide at 240 nm (Luck, 1965). (5) Cytoplasmic markers: Lactic dehydrogenase was monitored by the oxidation of NADH at 340 nm (Kornberg, 1955), and pyruvic kinase was determined in the presence of excess lactic dehydrogenase by a similar assay at 340 nm (Bücher & Pfleiderer, 1955).

Protein Determination. Protein was quantified by the method of Lowry et al. (1951) with bovine serum albumin as the standard. Samples that contained interfering substances (e.g., ampholines) were dialyzed against 50 mM imidazole hydrochloride–100 mM sodium chloride, pH 7.0, prior to protein determination.

Results

An analysis of a typical distribution of ADase binding protein, ADase, and the plasma membrane marker 5′-nucleotidase in supernatants and particulate fractions obtained from a fresh, full-term human placenta is summarized in Table I.

A significant level of ADase binding protein in the placental homogenate indicates that this tissue belongs to the class in which ADase binding protein is present in excess of enzyme. The most notable feature of this distribution, however, is the extraction of high levels of ADase binding activity from membrane-derived fractions. Thus, detergent extractions of 12000*g* and 105000*g* pellets revealed a total number of ADase binding units of 32 880 and 9704, respectively. The specific ADase binding activity in these extracts exceeded by 1 order of magnitude the corresponding value in the 105000*g* supernatant. In comparison, the addition of a final concentration of 2% deoxycholate–1% Triton X-100 to this soluble fraction was observed to have no effect on the level of ADase binding activity.

The general distribution of ADase enzymic activity paralleled that observed for the binding protein. The specific activity of ADase in the placental homogenate of 1.7 units/mg

Table I: Subcellular Distribution of Human Placental Adenosine Deaminase Binding Protein, Adenosine Deaminase, and 5′-Nucleotidase[a]

fraction	volume (mL)	protein (mg/mL)	ADase binding units/mg	ADase binding total units	ADase units/mg[b]	ADase total units[b]	5′-nucleotidase units/mg[b]	5′-nucleotidase total units[b]
homogenate	3350	5.0	1.1	18425	1.7	28.5	19.4	325.0
12000*g* supernatant	2650	3.4	1.6	14416	2.3	20.7	23.6	212.6
12000*g* pellet[c]	2650	1.6[d]	ND[e]	ND[e]	0.16	0.7	16.8	71.2
12000*g* pellet extract[f]	420	4.5	17.3	32880	17.6	33.3	6.1	11.5
105000*g* supernatant	2540	3.1	1.4	11024	1.1	12.3	2.6	20.5
105000*g* pellet[c]	2540	0.3[d]	ND[e]	ND[e]	6.6	5.0	304.4	232.0
105000*g* pellet extract[f]	150	1.6	40.6	9704	51.9	12.4	172.8	41.3

[a] A fresh human full-term placenta was homogenized and fractionated into supernatants and pellets by differential centrifugation as described under Materials and Methods. Assays were performed in duplicate by procedures described under Materials and Methods and generally agreed to within 5%. The 12000*g* and 105000*g* pellets were extracted 3 successive times with 2% deoxycholate-1% Triton X-100, 0.1% ampholines, and 10 mM Tris-HCl, pH 7. Pellets were obtained after each extraction by centrifugation at 15000*g* for 30 min. [b] These values have been multiplied by 10^{-3}. [c] Pellets were resuspended in PBS, and an aliquot of the suspension was assayed. [d] These values (i.e., 1.6 and 0.3 mg/mL) were obtained from the difference between the protein concentrations before and after centrifugation, i.e., between the protein concentrations of the 12000*g* supernatant and homogenate and between the 105000*g* supernatant and 12000*g* supernatant, respectively. [e] Not determined. [f] These data are the composite of three extractions with 2% deoxycholate–1% Triton X-100.

of protein, which is comparable in magnitude to the value reported by Sim & Maguire (1970), is over 10-fold higher than values reported for other human tissues (Van der Weyden & Kelley, 1976). Of the total units of ADase detectable in the homogenate, 74% and 44% were recoverable in the 12000*g* and 105000*g* supernatants, respectively. A significant level of ADase activity was also present in particulate suspensions of each of the pellets, representing together ca. 20% of the total units of ADase in the homogenate. The specific ADase activity in the resuspended 105000*g* pellet exceeded specific activities in the homogenate and both supernatant fractions. An analysis of the activity of the cytoplasmic marker lactic dehydrogenase indicated that less than 1% of the activity of this enzyme in the homogenate could be detected in either of the resuspended particulate fractions, supporting that the measured ADase activity was not attributable to cytoplasmic contamination.

The highest specific ADase activity was found as a latent form in each of the particulate fractions. The specific ADase in the detergent-extracted pellets was 1 order of magnitude greater than the corresponding value in the homogenate. These values represent apparent stimulations of 133-fold and 10-fold of the ADase activity in the 12000*g* and 105000*g* resuspended pellets, respectively. In distinction, the addition of a final concentration of 2% deoxycholate–1% Triton X-100 to the 105000*g* supernatant produced no significant stimulation of activity. Thus, the majority of the membrane ADase appears to be sequestered and is detectable only after distruption of the membrane integrity with detergent. It is notable that a greater portion of the ADase activity is latent in the 12000*g* pellet compared to that in the 105000*g* pellet. Negligible amounts of the cytoplasmic markers lactic dehydrogenase and pyruvic kinase were detectable in any of the detergent extracts.

Both the 105000*g* supernatant and the 12000*g* deoxycholate–Triton X-100 extract were chromatographed on Sephadex G-150 under the conditions described for the ADase binding assay under Materials and Methods. Assay of ADase in the eluted fractions indicated that the activity in both samples appeared exclusively in the excluded volume and is therefore classified as type A enzyme ($M_r \geq 200000$). No evidence for the presence of low molecular weight (type C) enzyme was obtained.

A bimodal distribution of an enzyme activity in the 12000*g* and 105000*g* pellets can be considered to represent preliminary evidence for a plasma membrane origin since plasma membrane and its fragments are generally detectable in both fractions. So that the distribution of plasma membrane in the fractionation of human placenta by differential centrifugation could be established, the plasma membrane marker 5′-nucleotidase was assayed in each of the subcellular fractions (Table I). High specific activities of 5′-nucleotidase in both pelleted fractions strongly supported the presence of plasma membrane or plasma membrane fragments. It is notable that extraction of the pellets with deoxycholate–Triton X-100 depressed the specific activity of 5′-nucleotidase, in distinction to the striking stimulation of the ADase activity that was observed. The 5′-nucleotidase activity in the 105000*g* supernatant suggests a distinct, low-activity enzyme located in the cytoplasm, as previously observed in other mammalian cells (Drummond & Yamamoto, 1971).

Identification of ADase and ADase Binding Activity in Purified Plasma Membrane. In light of the potential plasma membrane origin for ADase and its binding protein indicated by the subcellular distribution, syncytiotrophoblastic plasma membrane was highly purified from fresh full-term placenta by gentle stirring in PBS (Smith et al., 1977). The purified plasma membrane preparation was examined for ADase and ADase binding activity as well as for selected enzyme markers. These assays were performed both on particulate suspensions and on detergent extracts (Table II).

The plasma membrane preparation exhibited a high specific ADase activity that was substantially greater than that observed in the homogenate or in any of the particulate suspensions (Table I). The activity was more than 5-fold stimulated by detergent, providing further support for the existence of a latent form of the enzyme. The degree of stimulation, however, was significantly less than that observed in either of the pelleted fractions, suggesting a greater accessibility of the enzyme to exogenous substrate in the purified plasma membrane. Sephadex G-150 chromatography performed as described under Materials and Methods classified this enzyme as completely in the type A form. The detergent extract was also characterized by a high specific ADase binding activity that was more than 6-fold higher than the binding activity in the placental homogenate and was comparable in magnitude to the highest value observed in the 105000*g* pellet extract (Table I).

Plasma membrane markers 5′-nucleotidase and alkaline phosphatase assayed in the absence of deoxycholate–Triton X-100 demonstrated enrichments of 27.5 and 33.9-fold, respectively, in the purified plasma membrane preparation compared to those of the homogenate. The specific activity of these two markers in the final preparation is comparable to values previously reported (Carlson et al., 1976; Smith et al., 1977). In distinction, glutamate dehydrogenase (mitochondrial marker), catalase (peroxisomal marker), NADH diaphorase (endoplasmic reticulum marker), and lactic de-

Table II: Enzymic Composition of Purified Human Placental Syncytiotrophoblastic Plasma Membrane[a]

	sp act. (units/mg of protein)[b]			
		plasma membrane		
enzyme marker	homogenate	−detergent[c]	+detergent[d]	ratio[e]
ADase binding protein	77.6[f]	ND[g]	124.2	1.6
ADase (×10³)	12.7	21.5	113.1	1.7
5′-nucleotidase (×10³)	40.8	1120.0	82.4	27.5
alkaline phosphatase (×10)	4.7	159.4	78.5	33.9
glutamate dehydrogenase	99.0	18.8	15.4	0.19
catalase (×10³)	82.5	3.8	7.9	0.05
NADH diaphorase (×10)	20.0	4.5	20.8	0.23
lactic dehydrogenase (×10)	11.0	0.3	0.9	0.03

[a] Human placental syncytiotrophoblastic plasma membrane was purified as described under Materials and Methods. The pelleted membrane was extracted with 1.0 mL of 2% deoxycholate–1% Triton X-100, 0.1% ampholines (pH 5–7), and 10 mM Tris-HCl, pH 7.4, containing 0.1 mg/mL streptomycin and 100 IU/mL penicillin, followed by centrifugation at 12000*g* for 30 min. The protein concentration of the extract was 1.54 mg/mL. [b] A unit of enzyme activity is defined as 1 μmol of product formed per minute under the specified assay conditions described under Materials and Methods. [c] Assays were performed on purified plasma membrane suspended in PBS at a concentration of 2.0 mg/mL. [d] Assays were performed on supernatants obtained from deoxycholate–Triton X-100 extracts, prepared as described above. [e] Enzyme activity of purified plasma membrane in the absence of detergent divided by enzyme activity in the homogenate. The ratio for the ADase binding protein was obtained from the binding activity in the 2% deoxycholate–1% Triton X-100 extracts of the purified plasma membrane and homogenate. [f] This value was obtained after extraction of the homogenate with 2% deoxycholate–1% Triton X-100, as described above. [g] Not determined.

hydrogenase (cytoplasmic marker) were reduced to ca. 20% or less of the original value of the homogenate.

Although the preparation contains minor contamination with other subcellular organelles, the enhanced activity of both ADase and its binding protein in the purified preparation (1.7- and 1.6-fold, respectively) supports a plasma membrane origin for a least a portion of these activities. It is notable that these enrichments are significantly less than those observed for 5′-nucleotidase and alkaline phosphatase. This result may reflect a partial loss of these proteins during the preparation of the plasma membrane or perhaps a distribution into other subcellular fractions. In addition, a comparison of the data in Tables I and II indicates somewhat higher specific activities for both ADase and its binding protein in the homogenate employed for plasma membrane preparation compared to those in the homogenate utilized for subcellular fractionation. Although not completely understood, these results may well reflect the fact that different placentas obtained from unrelated donors were employed for each of these experiments.

Purification of Membrane ADase Binding Protein. The fraction employed for the purification of the membrane-derived ADase binding protein was the first deoxycholate–Triton X-100 extract of the 12000*g* pellet. Following incubation of this extract with an ADase–Sepharose resin, the immobilized enzyme–binding protein complex was poured into a column and eluted at acidic pH, as described under Materials and Methods. For assessment of nonspecific binding effects, a parallel binding and elution were performed with a Sepharose 4B column to which bovine serum albumin was covalently attached in place of ADase.

A summary of the purification of the ADase binding protein and the ADase activity associated with it is summarized in Table III. Only ca. 20% of the ADase binding activity in the initial extract could be recovered in the combination of unbound material and imidazole–NaCl wash. In distinction, in the control resin these fractions together accounted within experimental error for all of the units applied to the column. The low level of ADase associated with this preparation demonstrated a similar recovery pattern. Only ca. 10% of the ADase activity initially present was recoverable in the unbound and wash fractions, compared to over 80% in the control resin. These data strongly imply a specific interaction of the binding activity with the ADase affinity resin.

On the basis of the previous observation that an acidic pH weakens the ADase–binding protein interaction (Van der Weyden & Kelley, 1976), a pH 3.0 buffer was employed for elution of the ADase binding protein (Figure 1). Binding activity was typically eluted as a single, somewhat asymmetrical peak with a sharp maximum. The first fraction with detectable binding activity (fraction 6 in Figure 1) corresponds to a decrease in elution pH to 3.0. Immediately after elution the pH of each fraction was adjusted to 7–8 to prevent potential loss of biological activity. A substantial quantitiy of

Table III: Purification of Human Placental Adenosine Deaminase Binding Proteins by Affinity Chromatography[a]

			ADase binding act.				ADase			
fraction	volume (mL)	protein (mg/mL)	units/mL	units/mg	total units	% recovered[b]	units/mL[c]	units/mg[c]	total units	% recovered[b]
12000*g* extract	25	11.3	195.7	17.4	4893	100.0	306.0	27.1	7.65	100
ADase affinity resin										
unbound	25	7.5	23.0	3.1	575	11.8	25.7	3.4	0.64	7.0
wash	50	ND[d]	8.9	ND[d]	445	9.1	4.2	ND[d]	0.21	2.8
eluted	10	ND[d]	127.9	8378[e]	1279	26.1	259.0	5450[e]	2.59	33.9
control affinity resin										
unbound	25	7.5	175.0	23.3	4375	89.4	196.0	26.0	4.90	77.7
wash	50	ND[d]	17.1	ND[d]	855	17.5	6.0	ND[d]	0.30	3.9
eluted	10	ND[d]	0	0	0	0	15.0	ND[d]	0.15	2.0

[a] The affinity resin was prepared by immobilizing commercially obtained calf intestinal ADase to a cyanogen bromide activated Sepharose 4B column. Binding and elution were performed at room temperature in a 14 × 1.0 cm column as described under Materials and Methods. A control resin was prepared by coupling bovine serum albumin in place of ADase. "Unbound" refers to the fraction of the original sample that passes through the column unretarded: "wash" refers to the fraction obtained by washing with 50 mL of 50 mM imidazole hydrochloride–100 mM NaCl, 0.5 mg/mL cytochrome *c*, and 0.1% ampholines, pH 7.0; and "eluted" refers to the pooled fractions obtained by elution with 50 mM sodium succinate, pH 3.0, containing 0.5 mg/mL cytochrome *c* and 0.1% ampholines. [b] These values represent the percentages of units in the 12000*g* extract that were recovered in the unbound, wash, and eluted fractions. [c] ×10³. [d] Not determined. [e] Specific activity was calculated on purified binding protein from which cytochrome *c* and ampholines were removed by chromatography on Sephadex G-100 as described under Materials and Methods. ADase, ADase binding activity, and protein concentration were determined on this chromatographed material and are the basis for the calculated values of 8378 and 5450 units/mg of protein.

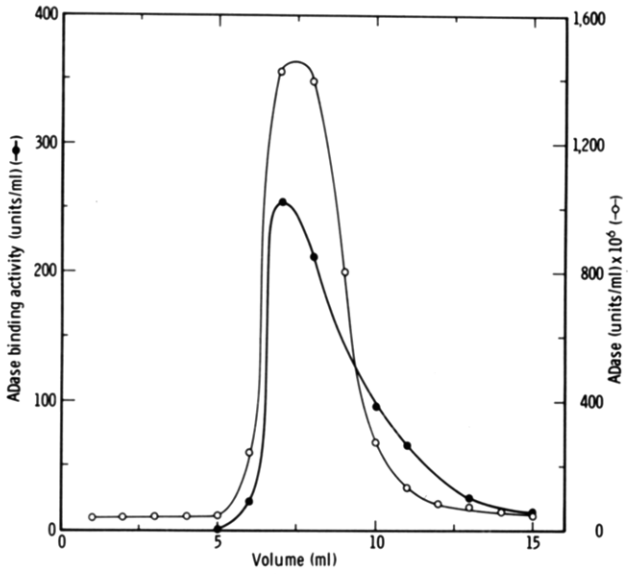

FIGURE 1: Affinity chromatography of human placental ADase binding protein. The 12000*g* pellet was extracted with 2% deoxycholate–1% Triton X-100 as described under Materials and Methods, and the extract was centrifuged at 15000*g* for 30 min. 11 mL of the supernatant containing 19.4 units of ADase and 1200 units of binding activity was incubated with 5 mL of an ADase–Sepharose resin for 1 h at room temperature with end-to-end turning. The resin was poured into a 13 × 7 mm column at room temperature and washed with 50 mL of 50 mM imidazole hydrochloride–100 mM NaCl, pH 7, containing 0.5 mg/mL cytochrome *c* and 0.1% ampholines (pH 5–7), followed by elution with 50 mM sodium succinate, pH 3, containing the same concentrations of cytochrome *c* and ampholines. 1.0-mL fractions of the pH 3 eluant were collected at a flow rate of 6–8 mL/h and were assayed for binding activity and ADase. Experimental details are further described under Materials and Methods.

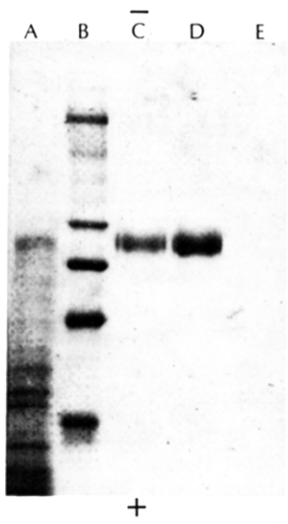

FIGURE 2: Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of purified human placental ADase binding protein. Samples were boiled for 2 min in 1% $NaDodSO_4$–1% mercaptoethanol, followed by gel electrophoresis in a 5–15% polyacrylamide gradient employing a discontinuous buffer system as described under Materials and Methods. (A) 2% deoxycholate–1% Triton X-100 extract of a 12000*g* pellet (100 μg); (B) mixture of standard protein markers, which are [from top (−) to bottom (+)] thyroglobulin (M_r 330 000), β-galactosidase (M_r 130 000), phosphorylase A (M_r 92 000), bovine serum albumin (M_r 67 000), and ovalbumin (M_r 45 000) (3–5 μg of each standard); (C and D) ADase binding protein purified by affinity chromatography (Figure 1) and concentrated by ultrafiltration on an Amicon XM-50 membrane (2 and 4 μg, respectively); (E) pH 3 eluant from a control bovine serum albumin–Sepharose resin (Table III). The column fractions that produced the sample in channel E were pooled and concentrated exactly as performed in the ADase affinity chromatography, and the volume of sample treated with $NaDodSO_4$ was identical with that employed in channel D.

ADase binding activity was eluted that represented ca. 26% of the initial number of units (Table III). In distinction, no binding activity was detectable in the pH 3 eluant from the bovine serum albumin–Sepharose resin, consistent with the total recovery of the original units in the "unbound" and "wash" fractions.

Of special interest was the observation that ADase activity was associated with the eluted binding protein (Figure 1 and Table III). The ADase reached a maximum value in the same fraction as binding activity and generally demonstrated a more symmetrical elution profile. The ratio of binding activity to ADase was approximately constant in the central portion of the peaks but increased in the latter half of the elution. The recovery of ADase in the pH 3 eluant was comparable to that observed for binding activity.

Fractions containing ADase binding activity were pooled, concentrated by ultrafiltration, and subjected to $NaDodSO_4$–polyacrylamide gel electrophoresis (Figure 2). Channel A contains a profile of proteins present in the crude 12000*g* deoxycholate–Triton X-100 extract. At least 10 distinct polypeptide chains were observed in the molecular weight range 30 000–150 000. In distinction, the eluted protein from the ADase affinity resin demonstrated a single, somewhat broadened band both at normal and overloaded protein concentrations (channels C and D, respectively). The shape of the band suggested a microheterogeneity, perhaps related to the presence of variable amounts of carbohydrate, as described below. In comparison to a mixture of standard proteins (channel B), the purified binding protein migrated between phosphorylase A and β-galactosidase with an apparent average molecular weight of 110 000. Varying the percentage of polyacrylamide over the range of 4–8% did not appear to change significantly this estimate for the subunit molecular weight.

In spite of the fact that ADase activity is associated with the purified protein (Table III), no polypeptide chain was detectable in the molecular weight range characteristic of the human enzyme [M_r ca. 36 000 (Schrader et al., 1976)] on $NaDodSO_4$–polyacrylamide gel electrophoresis even at the higher amount of binding protein applied (channel D). This observation is consistent with theoretical calculations, indicating that the ADase present must represent a very low percentage of the total protein. Thus, on the basis of a specific activity for human ADase of ca. 500 units/mg of protein (Schrader et al., 1976; Dadonna & Kelley, 1977), the amount of ADase present per milligram of protein is 10.9 μg, or approximately 1% of the total. However, increasing the amount of protein applied 5-fold to ca. 20 μg did reveal several minor additional polypeptide chains, including a band at M_r ca. 36 000 molecular weight. These minor components were estimated as representing together less than 2% of the total protein.

$NaDodSO_4$–polyacrylamide gel electrophoresis was performed on the pH 3 eluant obtained from a control of bovine serum albumin–Sepharose resin. This column was washed and eluted identically with the ADase affinity chromatography. Fractions corresponding to those in which ADase binding activity appeared in the affinity resin (Figure 1) were pooled and concentrated by ultrafiltration. As shown in Figure 2, channel E, no protein bands at any molecular weight range could be detected in this effluent. This result is consistent with the absence of ADase binding activity and the presence of only traces of ADase in these control fractions and further supports a high specificity in the interaction of the binding protein with the ADase affinity resin.

For quantitation of the specific ADase binding activity of the purified preparation per milligram of protein, cytochrome *c* and ampholines, both of which produce a positive Lowry reaction, were removed by Sephadex G-100 gel filtration chromatography, as described under Materials and Methods. Fractions containing ADase binding activity were pooled and concentrated by ultrafiltration. The specific binding activity of this concentrated fraction represented a purification of 482-fold from the initial 12000*g* detergent extract (Table III). The specific ADase activity associated with this preparation represented a 202-fold purification. The latter specific activity, however, is less than 10% of the value reported for the human type A ADase (Schrader & Stacy, 1977), further supporting that ADase constitutes only a minor fraction of this preparation.

In addition, a low level of 5′-nucleotidase was consistently observed to be associated with the purified binding protein. The specific activity of this enzyme, however, was lower than the specific activity in the initial detergent extract. Similar levels of 5′-nucleotidase were also observed in the pH 3 eluant from the control bovine serum albumin–Sepharose resin, suggesting that the presence of 5′-nucleotidase is a consequence of nonspecific interaction with the Sepharose matrix.

Purified syncytiotrophoblastic plasma membrane (Table II) was also subjected to ADase affinity chromatography for purification of ADase binding protein. $NaDodSO_4$–polyacrylamide gel electrophoresis of the pH 3 eluant indicated the presence of a single species that was identical in migration with the ADase binding protein obtained from the 12000*g* detergent extract (Figure 2C). This result further supports a plasma membrane origin for at least a portion of the ADase binding protein.

Characterization of Purified ADase Binding Protein. (*1*) *Concentration and Time Dependence.* Varying amounts of purified binding protein ranging from 9 to 90 μg were incubated with a molar excess of [^{125}I]ADase, and the amount of binding was quantified. The relationship between ADase binding activity and the amount of binding protein added was observed to be strictly linear over the entire range. A linear relationship was also characteristic of unpurified binding protein present in the 12000*g* deoxycholate–Triton X-100 extracts. The time course for the interaction of binding protein and ADase was sufficiently rapid that no significant time dependence could be measured with the technique employed. Virtually complete binding was obtained by mixing binding protein and enzyme, followed by immediate application to a column of Sephadex G-150 for quantitation.

(*2*) *Scatchard Analysis.* The extent of binding was studied as a function of free ADase concentration, and the data were analyzed according to Scatchard (1949) (Figure 3). A markedly concave downward shape of the plot was observed, indicative of a strong apparent positive cooperativity in binding. The most straightforward interpretation of this result is that the affinity of binding shows an apparent increase in the presence of increasing amounts of ADase. The alternative explanation of the dissociation from the calf intestinal ADase of an inhibitor of the ADase–binding protein interaction appears less likely as an explanation in light of the high degree of homogeneity of the commercially obtained calf intestinal ADase. The shape of the Scatchard plot was dependent on the age of the [^{125}I]ADase. In comparison to ADase stored at 4 °C for 3 months, ADase employed within 7 days of iodination produced a Scatchard plot characterized by the following: (1) a higher maximum value of $\bar{\nu}/[\text{ADase}]_f$ (moles of ADase bound per mole of binding protein divided by the concentration of free ADase); (2) a steeper decline in $\bar{\nu}/[\text{ADase}]_f$ as a function of $\bar{\nu}$ (Figure 3). These data suggest that the average association constant is substantially lower when aged enzyme is employed as the ligand.

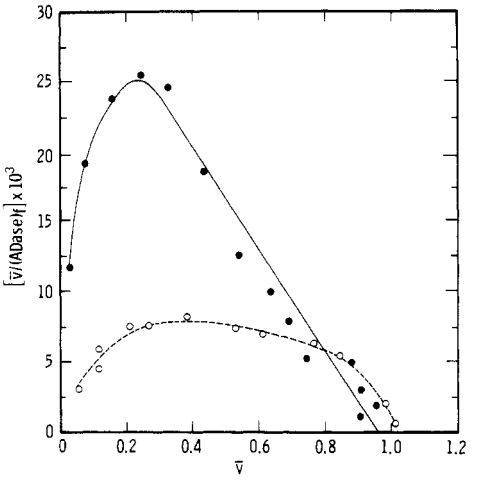

FIGURE 3: Scatchard analysis of [^{125}I]ADase binding to purified ADase binding protein. Varying volumes of [^{125}I]ADase (0.66 mg/mL) containing between 600 and 10000 dpm were incubated with purified ADase binding protein (45 μg) for 20 min at room temperature in a final volume of 0.3 mL containing [^{125}I]ADase (6.6 μg, 49315 dpm), L-glutamate dehydrogenase (300 μg), and 10% sucrose in 50 mM imidazole–100 mM NaCl, pH 7. The amount of high molecular weight [^{125}I]ADase produced was quantified by gel filtration chromatography on Sephadex G-100, followed by determination of the total radioactivity in the void volume. The binding protein employed was purified by ADase affinity chromatography (Figure 1) and separated from cytochrome *c* and ampholines by Sephadex G-100 chromatography, as described under Materials and Methods. $\bar{\nu}$ represents the moles of [^{125}I]ADase (M_r 35000) bound per mole of ADase binding protein (subunit M_r 110000). $[\text{ADase}]_f$ is the concentration of unbound [^{125}I]ADase expressed in pmol/mL. Each data point represents the mean of two or three determinations, which generally agreed to within 5%. ADase binding data were obtained by employing ^{125}I-labeled calf intestinal ADase within 7 days after iodination (closed circles) or after storage at 4 °C for 3 months (open circles).

The maximum binding obtained at high concentrations of ADase was similar for both aged and fresh preparations of enzyme, as indicated by the similarity of the *x* intercepts. Linear least-squares analysis of the approximately linear portion of the curve for the freshly prepared [^{125}I]ADase, which extends from a $\bar{\nu}$ value of ca. 0.3 to maximal binding, yields an apparent limiting maximum value for the association constant of 3.5×10^{11} M^{-1}. Extrapolation to the *x* intercept by linear least-squares analysis indicates a maximum binding of 0.95 mol of ADase/mol of binding protein (subunit M_r 110000).

Lectin Affinity. So that evidence for the presence of carbohydrate could be obtained, the affinity of the binding protein for wheat germ lectin and Con A immobilized to Sepharose 6B was examined. The former binds to *N*-acetylglucosamine and sialic acid residues (Nagata & Burger, 1974), while the latter interacts with α-D-glucopyranosyl and α-D-mannopyranosyl moieties (Lloyd et al., 1969). Sepharose 4B to which no lectin was attached was employed as a control. Binding and specific elution with selected sugars were evaluated (Table IV). Specific interaction with the lectin resins was supported by the fact that only 5.7 and 7.3% of the initial units applied to the Con A– and wheat germ lectin–Sepharose resins, respectively, passed through unretarded, compared to 100% for the control Sepharose 4B resin. These values did not substantially change by increasing the column volume by 50%. Thus, a small fraction of the binding protein appears to be

Table IV: Affinity of ADase Binding Protein for Immobilized Lectins[a]

	% unretarded[b]	% bound[c]	% eluted[d]
control Sepharose 4B	100	0	0
Con A–Sepharose	7.3	92.7	92.5
wheat germ lectin–Sepharose	5.7	94.3	69.0

[a] Columns (2.5 × 0.7 cm) of Con A-Sepharose 6B, wheat germ lectin–Sepharose 4B, and Sepharose 4B with no lectin attached were equilibrated at room temperature with 50 mM imidazole hydrochloride–100 mM NaCl containing cytochrome *c* (0.1 mg/mL) and ampholines (0.1%, pH 5–7). ADase binding protein (55 units) purified by affinity chromatography was incubated with the resin for 5 min, followed by washing with 1.5 mL of the imidazole–NaCl solution with which the column was equilibrated. Elution was performed with 1.5 mL of 10% solutions of methyl α-D-mannopyranoside and *N*-acetyl-D-glucosamine (dissolved in the same imidazole–NaCl solution) for the Con A–Sepharose and wheat germ lectin–Sepharose resins, respectively. [b] [(ADase binding units in imidazole–NaCl wash)/55.0] × 100. [c] 100% − % unretarded. [d] [(ADase binding units eluted with specific sugars)/(ADase binding units attached to the resin)] × 100.

noninteracting with each of the lectin resins. The sugars methyl α-D-mannopyranoside and *N*-acetyl-D-glucosamine were effective in eluting substantial proportions of the ADase binding units.

Discussion

A significant aspect of the data presented here is that the major fraction of ADase in human placenta is masked in a membrane-associated form and is revealed maximally only after disruption of the membrane integrity with detergent. The ADase–membrane interaction is apparently mediated through a specific ADase binding protein. It is consistent with an in situ interaction between the enzyme and binding protein in that (1) specific activities of ADase and ADase binding protein display a similar distribution, i.e., both being significantly higher in detergent extracts than in either homogenate or soluble fractions, and (2) the detergent-extracted ADase is completely high molecular weight ADase (type A) enzyme rather than the low molecular weight (type C) form characteristic of ADase that is not complexed with binding protein (Schrader et al., 1976).

The data also indicate that at least a portion of the ADase binding protein originates from the trophoblastic plasma membrane. This conclusion is supported by the following: (1) the bimodal distribution of ADase and ADase binding protein at high specific activities in the 12000*g* and 105000*g* pellet extracts, which are also characterized by high levels of the plasma membrane marker 5′-nucleotidase; (2) high specific activities of ADase and ADase binding protein in purified plasma membrane at values significantly greater than observed in the homogenate; (3) isolation of an ADase binding protein from purified plasma membrane by affinity chromatography. It is not yet established whether the plasma membrane ADase is cytoplasmic or extracellular in orientation. These results, however, do not exclude the presence of these proteins in other subcellular fractions or in plasma membrane derived from endothelial, cytotrophoblastic, or connective tissue cells. The recovery of moderate levels of ADase and ADase binding protein in the 105000*g* supernatant does suggest that a portion of these proteins may be cytoplasmic in origin.

Although other mammalian cell types have been reported in exhibit ADase in particulate fractions, the level of activity was generally low or represented a minor fraction of the total tissue enzyme. The human placenta appears to be unique in that the major portion of the enzyme is sequestered in a membrane. For example, low levels of particulate enzyme have been observed in cultured glial cells although most of the activity in central nervous system cells is soluble (Trams & Lauter, 1975). In these studies the association of ADase with fractions from discontinuous sucrose centrifugation that were also enriched with 5′-nucleotidase suggested a plasma membrane origin. Similarly, plasma membrane prepared from cultured mouse fibroblasts (Li & Hochstadt, 1976) exhibited ADase activity although the degree of stimulation by detergent was 1.4-fold compared to the greater than 5-fold that we have observed for ADase from purified human placental plasma membrane (Table II). Van der Weyden & Kelley (1976) have observed that up to 3% of human leucocyte ADase is particulate. However, no data on subcellular distribution were reported. Although it was originally reported that Triton X-100 revealed a latent ADase in mitochondria from mouse cerebral cortex (Mustafa & Tewari, 1970), subsequent studies indicted that the apparent increase in ADase was due to lysis of contaminating synaptosomes (Pull & McIlwain, 1974).

Several previous reports have indicated the existence of a glycoprotein with properties similar to the ADase binding protein described here in the soluble fraction of selected animal and human tissues. The latter include human kidney and lung (Nishihara et al., 1973; Van der Weyden & Kelley, 1976), rabbit kidney, ileum, and lung (Trotta et al., 1979), and human plasma (Schrader et al. 1979). The existence of multiple molecular weight variants of calf intestinal ADase also suggests the presence of an ADase binding protein in this species (Constine et al., 1978). The specific activity that we have noted for the binding protein in the 105000*g* supernatant from human placenta (i.e., 1.4 units/mg of protein) is comparable in magnitude to the values reported for human kidney and lung (Van der Weyden & Kelley, 1976). These tissues, as well as human placenta, contain exclusively high molecular weight type A ADase, consistent with the theory that this enzyme arises by the stoichiometric combination of ADase and binding protein. However, although most human tissues exhibit varying levels of type A ADase, relatively few have an excess of soluble binding protein. In distinction to these data the soluble ADase isolated in homogeneous form from the maternal component of bovine placenta of early gestation has been demonstrated to be exclusively type C enzyme (Sim & Maguire, 1971). This result, which implies the complete absence of ADase binding protein, may reflect the difference in species or gestational age.

The relationship between the soluble ADase binding protein and the membrane-derived form reported here, as well as the physiological role of each, remains unknown. Certain properties of the human placental membrane ADase binding protein and the soluble kidney binding protein (Schrader & Stacy, 1977; Dadonna & Kelley, 1978) are similar, including the following: (1) subunit molecular weight (110000 for the membrane form and 85000–97000 for the soluble kidney protein); (2) stoichiometry of binding [one ADase monomer (M_r ca. 35000) per polypeptide chain]; (3) weakening of the ADase–binding protein interaction at pH 3; (4) ability of the binding protein in a crude homogenate to interact specifically with an affinity resin of purified ADase; (5) the presence of carbohydrate. It is yet to be established whether the plasma membrane of human kidney and lung, or of various rabbit tissues, will yield an ADase binding activity upon extraction with detergent and whether its physicochemical properties are distinct from those of the soluble form.

It is notable that the ADase activity associated with the purified binding protein itself binds to an affinity resin of

purified enzyme (Figure 1). A straightforward interpretation of these data is that the type C ADase is bound to a multisubunited protein that contains one or more unoccupied sites that are free to interact with the immobilized enzyme. The failure to detect free low molecular weight enzyme in any of the membrane detergent extracts or in the preparation of purified binding protein is consistent with this interpretation. The concave-downward appearance of the Scatchard plot further suggests the possibility of multiple sites interacting with positive cooperativity.

Of additional interest is the fact that the binding protein is present in large molar excess over the amount of ADase in each of the membrane extracts. For example, on the basis of a maximum specific activity for the human type C enzyme of ca. 500 units/mg of protein (Schrader et al., 1976; Dadonna & Kelley, 1977) and a molecular weight of 35 000 (Andrews, 1964), the quantity of bound enzyme in the 12 000*g* detergent extract is 1903 pmol, a value less than 6% of the capacity of the ADase binding protein to bind ADase (i.e., 32 880 pmol). Indeed, in all extracts examined, including purified plasma membrane, as well as in supernatant fractions, the amount of ADase binding protein is in large molar excess over the amount of bound enzyme. This conclusion is further strengthened by the fact that Scatchard analysis indicates that a maximum of ca. 1 mol of [^{125}I]ADase binds per polypeptide chain of purified binding protein in spite of the presence of endogenous ADase activity in the preparation (Figure 3). Thus, the molar levels of enzyme that are present are sufficiently low that the stoichiometry of the interaction is not significantly perturbed. Although the significance of the excessive quantities of ADase binding protein is not at present understood, these data imply a potential physiological role for these proteins in addition to their interaction with ADase.

The cytotoxic and cytostatic effects of adenosine and adenosine analogues are well established [e.g., Green & Chan (1973) and Ishii & Green (1973)]. Thus, the association of ADase with the plasma membrane would be advantageous for protecting the mammalian cell against toxic effect of these nucleosides entering from the extracellular space. In the human placenta this function would be of particular importance since it would provide protection for the developing fetus. It may be relevant that the apparent causal relationship between the inherited absence of ADase and a form of severe combined immunodeficiency disease (Dissing & Knudsen, 1972; Giblett et al., 1972) supports a special role for adenosine metabolism in the development and differentiation of the fetal immune system.

Acknowledgments

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References

Agarwal, R. P., & Parks, R. E., Jr. (1975) *Biochem. Pharmacol. 24*, 547–550.

Akedo, H., Nishihara, H., Shinkai, K., & Komatsu, K. (1970) *Biochim. Biophys. Acta 212*, 189–191.

Akedo, H., Nishihara, H., Shinkai, K., & Komatsu, K. (1972) *Biochim. Biophys. Acta 276*, 257–271.

Andrews, P. (1964) *Biochem. J. 91*, 222–233.

Brady, T. G., & O'Donovan, C. I. (1965) *Comp. Biochem. Physiol. 14*, 101–120.

Bücher, T., & Pfleiderer, G. (1955) *Methods Enzymol. 1*, 435–440.

Carlson, R. W., Wada, G. H., & Sussman, H. (1976) *J. Biol. Chem. 251*, 4139–4146.

Constine, J., Glazer, R. I., & Johns, D. G. (1978) *Biochem. Biophys. Res. Commun. 85*, 198–202.

Dadonna, P. E., & Kelley, W. N. (1977) *J. Biol. Chem. 252*, 110–115.

Dadonna, P. E., & Kelley, W. N. (1978) *J. Biol. Chem.* 253, 4617–4623.

Dadonna, P. E., & Kelley, W. N. (1979) *Biochim. Biophys. Acta 580*, 302–311.

Dissing, K., & Knudsen, B. (1972) *Lancet 2*, 1316.

Drummond, G. I., & Yamamoto, M. (1971) *Enzymes, 3rd Ed. 3*, 337–352.

Ellis, G., & Goldberg, D. M. (1972) *Clin. Chem. (Winston-Salem, N.C.) 18*, 523–527.

Fox, I. H., & Marchant, P. J. (1976) *Can. J. Biochem. 54*, 462–469.

Giblett, E. R., Anderson, J. E., Cohen, F., Pollara, B., & Meuwissen, H. F. (1972) *Lancet 2*, 1067.

Green, H., & Chan, T.-S. (1973) *Science (Washington, D.C.) 182*, 836–837.

Hunter, W. M. (1973) in *Handbook of Experimental Immunology* (Weir, D. M., Ed.) Chapter 17, pp 1–36, Davis, Philadelphia, PA.

Ishii, K., & Green, H. (1973) *J. Cell Sci. 13*, 429–439.

Kornberg, A. (1955) *Methods Enzymol. 19*, 441–443.

Leighton, F., Poole, B., Beaufay, H., Baudhuin, P., Coffey, J. W., Fowler, S., & de Duve, C. (1968) *J. Cell Biol. 37*, 482–513.

Li, C.-C., & Hochstadt, J. (1976) *J. Biol. Chem. 251*, 1181–1187.

Lloyd, K. O., Kabab, I. A., & Beychok, S. (1969) *J. Immunol. 102*, 1354–1362.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J.(1951) *J. Biol. Chem. 193*, 265–275.

Luck, H. (1965) in *Methods of Enzymatic Analysis* (Bergmeyer, H.-U., Ed.) pp 885–888, Academic Press, New York.

Ma, P. F., & Fisher, J. R. (1968) *Comp. Biochem. Physiol. 27*, 687–694.

Ma, P. F., & Fisher, J. R. (1969) *Comp. Biochem. Physiol. 31*, 771–781.

Ma, P. F., & Fisher, J. R. (1972) *Comp. Biochem. Physiol. B 41*, 469.

Ma, P. F., & Magers, T. A. (1975) *Int. J. Biochem. 6*, 281–286.

Mahler, H. R., Sarkar, N. K., Vernon, L. P., & Alberty, R. A. (1952) *J. Biol. Chem. 199*, 585–597.

Mustafa, S. J., & Tewari, C. P. (1970) *Biochim. Biophys. Acta 220*, 326–337.

Nagata, Y., & Burger, M. M. (1974) *J. Biol. Chem. 249*, 3116–3122.

Neville, D. M. (1971) *J. Biol. Chem. 246*, 6328–6334.

Nishihara, H., Ishikawa, S., Shinkai, K., & Akedo, H. (1973) *Biochim. Biophys. Acta 302*, 429–449.

Pull, I., & McIlwain, H. (1974) *Biochem. J. 144*, 37–41.

Scatchard, G. (1949) *Ann. N.Y. Acad. Sci. 41*, 660–672.

Schrader, W. P., & Stacy, A. R. (1977) *J. Biol. Chem. 252*, 6409–6415.

Schrader, W. P., & Pollara, B. (1978) *J. Lab. Clin. Med. 92*, 656–662.

Schrader, W. P., Stacy, A. R., & Pollara, B. (1976) *J. Biol. Chem. 251*, 4026–4032.

Schrader, W. P., Woodward, F. J., & Pollara, B. (1979) *J. Biol. Chem. 254*, 11964–11968.

Shobe, C. R., & Campbell, J. N. (1973) *Can. J. Microbiol. 19*, 1275–1284.
Sim, M. K., & Maguire, M. H. (1970) *Biol. Reprod. 2*, 291–298.
Sim, M. K., & Maguire, M. H. (1971) *Eur. J. Biochem. 23*, 17–21.
Smith, C. H., Nelson, D. M., King, B. F., Donohue, T. M., Ruzcki, S. M., & Kelley, L. K. (1977) *Am. J. Obstet. Gynecol. 128*, 190–196.
Steck, T. L., & Wallach, D. F. H. (1970) in *Methods in Cancer Research* (Busch, H., Ed.) pp 93–153, Academic Press, New York.
Sussman, H. H., Small, P. A., Jr., & Cotlove, E. (1968) *J. Biol. Chem. 243*, 160–166.
Trams, E. G., & Lauter, C. J. (1975) *Biochem. J. 152*, 681–687.
Trotta, P. P., & Balis, M. E. (1978) *Biochemistry 17*, 270–278.
Trotta, P. P., Peterfreund, R. A., Schonberg, R., & Balis, M. E. (1979) *Biochemistry 18*, 2953–2959.
Trotta, P. P., Tedde, A., Ikehara, S., Pahwa, R., Good, R. A., & Balis, M. E. (1981) *Cancer Res. 41*, 2189–2197.
Van der Weyden, M. B., & Kelley, W. N. (1976) *J. Biol. Chem. 251*, 5448–5456.

Kinetics of Transfer of Pyrene and *rac*-1-Oleyl-2-[4-(3-pyrenyl)butanoyl]glycerol between Human Plasma Lipoproteins†

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ABSTRACT: Control of lipid transfer between plasma lipoproteins has been studied with pyrene and a pyrene-containing diglyceride analogue, *rac*-1-oleyl-2-[4-(3-pyrenyl)butanoyl]-glycerol. The rate constants for transfer of the fluorescent probes between high-density lipoproteins in dilute solutions containing ions of the Hofmeister series are correlated with the molal surface tension increments produced by this lyotropic series. Moreover, the rates of transfer correlate well with the aqueous solubilities of the hydrophobic compounds, which were increased in dilute solutions containing organic solvents. The rates of transfer are faster with an increase in the distribution coefficients of the probes between the lipoprotein donor and the aqueous phase, suggesting that the rate-determining process can be considered simply as a partitioning between two immiscible solvents. Rate constants for transfer of pyrene between high-density, low-density, and very low density lipoproteins depend on the ratio of the donor/acceptor lipoprotein concentrations and agree with those predicted from the distribution coefficients. In addition, the rate of transfer is inversely related to the lipoprotein radius, with a linear correlation observed between the log of the rate constant and 1/radius. By analogy with the evaporation of liquid droplets described by Kelvin's law, the transfer of a lipid from a lipoprotein or a membrane surface can be described as $k_d \alpha e^{-K\pi/r}$ where K is a constant, π is the interfacial surface pressure of a curved surface of a radius r, and k_d is the dissociation constant. These results show that the rate of transfer of lipids can be modified by changes in the solubility of the transferring species in the aqueous phase and imply that other factors, such as solubility of the transferring species in the membrane and the properties of the lipid–protein surface, may have important roles in regulating the kinetics of lipid transfer in biological systems.

Plasma lipoproteins function as circulating reservoirs for a wide variety of hydrophobic substances that have extremely low solubility in aqueous solutions (Smith et al., 1978; Scanu et al., 1979). These compounds include triglyceride, cholesterol, cholesteryl ester, and phosphatidylcholine as major components and minor amounts of lipid-soluble vitamins and environmentally derived contaminants.

The relatively constant compositions of the circulating lipoproteins result from five simultaneous processes: (1) synthesis and secretion, (2) passive transfer and exchange of lipid and apoprotein components, (3) active transfer involving specific plasma exchange proteins, (4) enzymatic changes in composition involving lecithin–cholesterol acyltransferase and lipoprotein lipase, and (5) removal from circulation by cellular uptake of lipid and apoprotein components. The dynamics and the contribution of each process to overall lipoprotein metabolism are poorly defined.

All lipoproteins contain proteins, cholesterol, and phospholipids in a surface film that surrounds a neutral lipid core of cholesteryl ester and triglyceride (Shen et al., 1977). Lipoprotein surfaces have the dynamic properties of a two-dimensional fluid, in which there are rapid noncovalent interactions between individual lipid and protein components, involving both rotational motion and translational diffusion in the surface (Lee, 1975; Thompson & Huang, 1978). Because the lipid components of lipoproteins are soluble in the aqueous solution to some extent, they also partition between the lipoprotein surface and the aqueous solution (Charlton et al., 1976, 1978a). Since some lipoprotein components transfer on time scales (milliseconds to hours) that are much shorter than the

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