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CHARACTERIZATION OF THE PARTICULATE AND SOLUBLE ACCEPTOR FOR TRANSCOBALAMIN II FROM HUMAN PLACENTA AND RABBIT LIVER *

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Summary

We describe in both human placenta and rabbit liver membranes specific acceptors which bind the human transcobalamin II-vitamin B-12 (cobalamin) complex with an affinity of $2.3 \cdot 10^9$ (placenta) and $6.7 \cdot 10^9$ (liver) M^{-1} and which bind the rabbit transcobalamin II-cobalamin complex with an affinity of $1.1 \cdot 10^9$ (placenta) and $1.9 \cdot 10^9$ (liver) M^{-1}, respectively. The binding requires Ca^{2+} and is sensitive to both 1 M NaCl and acid pH. A new ligand binding assay, based on the ability of the acceptor, but not transcobalamin II, to bind to concanavalin A, is described and is used to characterize the solubilized acceptors. The solubilized acceptors bind human transcobalamin II-cobalamin with high affinity (about $2—9 \cdot 10^9$ M^{-1}) but do not bind free cobalamin; unsaturated transcobalamin II is bound with an affinity approximately one-third of that for transcobalamin II saturated with cobalamin. On gel filtration, the human acceptor saturated with transcobalamin II-cobalamin exhibits a Stokes radius of 6.7 nm, whereas the free acceptor has a Stokes radius of 5.1 nm. The rabbit liver acceptor either unsaturated or saturated with transcobalamin II-cobalamin exhibits a Stokes radius of 5.7 nm. Both acceptors bind to lectins such as concanavalin A, wheat germ agglutinin and phyto-

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hemagglutinin, indicating their glycoprotein nature, and both acceptors can be purified approximately 30-fold by affinity chromatography on wheat germ agglutinin-Sepharose columns. The concanavalin A assay, combined with lectin-Sepharose and transcobalamin II-cobalamin-Sepharose affinity chromatography will provide for the isolation and study of pure acceptors from a variety of tissue sources.

Introduction

Transcobalamin II is a 39 000 dalton plasma protein required for the cellular uptake of vitamin B-12 (cobalamin) [1—3]; the transcobalamin-cobalamin complex is internalized subsequent to its adsorption to a specific cell-surface binding site. Since the binding site acts to internalize the complex, rather than to cause cellular activation, it may be termed an acceptor, in contrast to the cellular receptors for activating agents such as insulin or acetylcholine [4]. Transcobalamin II, which has been detected in all mammals so far examined [5,6], exhibits a high degree of interspecies immunological cross-reactivity [7]. Both human and rabbit transcobalamin II have been isolated and studied in detail [8,9]. The present study describes the characterization of the membrane-associated and solubilized acceptors obtained from human placenta and rabbit liver.

Materials and Methods

Human and rabbit serum was collected by centrifugation of freshly obtained, clotted (30 min 24°C, then 17 h at 4°C) whole blood. Serum was incubated at 24°C for 30 min with ^{57}Co-labeled cobalamin (100—300 Ci/g cyanocobalamin from Amersham Radiochemicals, Arlington Heights, IL). From 70 to 90% of the added [^{57}Co]cobalamin was incorporated into a transcobalamin II-cobalamin complex, as determined by gel filtration. The specific activity of the radioactive transcobalamin II-cobalamin complex so formed was corrected for the endogenous transcobalamin II-cobalamin already present in the samples determined either as described previously [10] (human) or by analyzing the cobalamin content of the transcobalamin II-cobalamin fraction isolated by gel filtration. The cobalamin analysis was performed as described [11].

Transcobalamin II for binding competition studies. Rabbit transcobalamin II-cobalamin complex, purified by labile ligand affinity chromatography [9] was a gift from Dr. Henrik Olesen Rigshospitalet, Copenhagen; human transcobalamin II-cobalamin complex was obtained by incubating serum with 1.8 nM hydroxocobalamin (GEA a/s, Copenhagen); nonspecific binding of the ^{57}Co-labeled transcobalamin II-cobalamin complexes was determined in the presence of an excess of unlabeled rabbit transcobalamin II-cobalamin complex (0.6—0.8 μM).

Preparation of membranes. Human term placenta was stored at −20°C until used for the preparation of membranes. Fresh rabbit livers were obtained for the preparation of membranes from anesthetized (pentobarbital) male white New Zealand rabbits. The membranes ('microsomal' fraction) were prepared

by differential centrifugation as described [12] and stored at −20°C in 50 mM Tris-HCl buffer (pH 7.4) until used.

Membranes (1—3 mg protein/ml, as determined by Lowry et al. [13] using bovine serum albumin as a standard) were solubilized using 0.5% (w/v) Ammonyx-LO (Onyx Chemical Co., Hoboken, NJ, U.S.A.) and the extract was clarified by ultracentrifugation (200 000 × *g* for 30 min at 4°C). The supernatant was used as a source of soluble acceptor.

Assay for binding to membranes. Membranes (50 μl) were incubated with 50 μl of serum containing the transcobalamin II-[^{57}Co]cobalamin complex together with 100 μl of Tris buffer (0.025 M Tris-HCl, 150 mM NaCl, 1 mM $CaCl_2$) at room temperature for 60 min. The mixture was transferred to 400-μl plastic conical centrifuge tubes (Beckman) containing 100 μl of a 1 : 2 (v/v) mixture of dinonyl and dibutyl phthalate (Eastman) and centrifuged at 10 000 × *g* for 1 min at 24°C in a Beckman Microfuge. By this procedure, membrane-bound transcobalamin II-cobalamin complex was pelleted below the oil/water interface, free from unbound ligand and was collected by cutting off the tube tips; radioactivity in the pellet was measured by crystal scintillation counting.

Preparation of Sepharose derivatives. CnBr-activated Sepharose 4BCL (Pharmacia) was prepared as described [14]. The lectins concanavalin A (Pharmacia), wheat germ agglutinin (a gift from Dr. J. Shaper, Johns Hopkins Medical School) and phytohemagglutinin (Wellcome) were coupled (18 h at 4°C) using a concentration of approximately 2 mg/ml lectin in 0.2 M sodium bicarbonate, pH 8.0. Concanavalin A and wheat germ agglutinin were coupled in the presence of 0.2 M α-methyl-D-mannoside (Sigma) and *N*-acetyl-D-glucosamine (Sigma), respectively. After the coupling of the lectins was complete, unreacted sites were blocked by incubating the mixture for 2 h more at room temperature with 1 M glycine. The Sepharose was washed with three cycles of 5 vols. each of 0.1 M acetate buffer, pH 4.5, 0.5 M NaCl and 0.05 M borate buffer, pH 8, containing 0.5 M NaCl. The derivatives (approx. 1.2 mg lectin/ml packed beads) were stored in 0.2 M sodium bicarbonate, pH 8, containing 1 mM $CaCl_2$ and 1 mM $MnCl_2$. The Sepharose was washed with an appropriate buffer just prior to use. Transcobalamin II-cobalamin-Sepharose was prepared by adsorbing unsaturated pure rabbit transcobalamin II to cobalamin-Sepharose prepared as described [15]. Unsaturated transcobalamin II was obtained after incubation of 10 mmol transcobalamin II-cobalamin in 7.5 M guanidine-HCl (Schwartz/Mann)/0.1 M Tris-HCl, pH 7.4, for 30 min followed by gel filtration on a 0.5 × 9 cm Sephadex G-25 (Pharmacia) column in 25 mM Tris-HCl, 1 M NaCl, pH 7.4.

Assay for the solubilized acceptor. The soluble acceptor, but not free transcobalamin II-[^{57}Co]cobalamin binds to concanavalin A. Furthermore, when bound to concanavalin A-Sepharose, the solubilized acceptors were able to bind the ^{57}Co-labeled transcobalamin complex. Thus, concanavalin A-Sepharose could be used to separate the soluble transcobalamin II-cobalamin-acceptor complex from free transcobalamin II-cobalamin, according to a new ligand binding assay described in detail elsewhere [16]. Briefly, replicate soluble acceptor samples were adsorbed to 50 μl aliquots of concanavalin A-Sepharose (1.2 mg concanavalin A/ml packed beads) and were incubated in 12 × 75 mm

glass tubes in a total volume of 300 μl with ^{57}Co-labeled transcobalamin II-cobalamin complex, both in the presence and absence of an excess of unlabeled transcobalamin II-cobalamin complex. After equilibration (usually 60 min at 24°C), the beads were washed with 4 ml ice-cold buffer and the bead-bound complex was collected by centrifugation for the estimate of bead-bound radioactivity.

Purification of the solubilized acceptors. Between 5 and 30 mg of solubilized membrane protein in 5—10 ml buffer was adsorbed at 4°C to 200—400 μl wheat germ agglutinin-Sepharose columns, packed in Pasteur pipettes (flow rate, 1 ml/h). The columns were washed with 50 column vols. of Tris/Ammonyx buffer (50 mM Tris, 0.1% (w/v) Ammonyx, 1 mM $CaCl_2$, 1 mM $MnCl_2$, 150 mM NaCl, pH 7.4). The transcobalamin II acceptor adsorbed to wheat germ agglutinin-Sepharose could be complexed with ^{57}Co-labeled transcobalamin II-cobalamin by incubation for 1 h at 24°C with 100 μl [^{57}Co]-cobalamin-equilibrated human serum, followed by washing the bead-bound complex with 1 ml Tris/Ammonyx buffer. Either the free acceptor or the acceptor-transcobalamin II-cobalamin complex were recovered from the column by elution with 0.2 M *N*-acetylglucosamine, and were stored at 0°C until used.

Results

Both human placenta and rabbit membranes possess high-affinity saturable binding sites for the transcobalamin II-cobalamin complexes (Fig. 1). The binding data, when analyzed according to the method of Scatchard [17], yielded linear plots, indicating one class of high-affinity binding sites (Table I) with a maximum binding capacity of approximately 150 fmol/mg and 50 fmol/mg for placenta and liver membranes, respectively.

Because of the lower acceptor affinity exhibited by rabbit transcobalamin II-cobalamin and because of the comparatively low concentration of the transcobalamin II-acceptor in liver membranes, kinetic studies were done using ^{57}Co-labeled human transcobalamin II-cobalamin complex and human placenta membranes. The rates of association and dissociation of the transcobalamin II-cobalamin-membrane complex are consistent with a bimolecular process (Fig. 2). From the dissociation rate data the off-rate constant (k_{-1}) for the binding of human transcobalamin II-cobalamin to human placenta membranes was calculated to be $6.7 \cdot 10^{-4}$ s^{-1}. Based on the off-rate constant and the estimate of the equilibrium association constant (Table I), the on-rate constant (k_1) can be calculated to be approximately $0.15 \cdot 10^7$ M$^{-1} \cdot$ s^{-1}. Dissociation between the transcobalamin II-cobalamin complex and the acceptor occurred equally well, either upon adding pure rabbit transcobalamin II-cobalamin or upon adding 10 mM EDTA subsequent to the binding of the [^{57}Co]cobalamin-labeled complex (Fig. 2). This observation indicated that a divalent cation was needed for the formation of the complex between transcobalamin II-cobalamin and the acceptor. Thus, no binding was observed to either the liver or placenta acceptors if 10 mM EDTA was included in the incubation mixture; however, the binding was restored if 15 mM $CaCl_2$ was added.

The transcobalamin II acceptor can be solubilized with a number of commonly used detergents such as Triton X-100, Lubrol and Ammonyx-LO. The

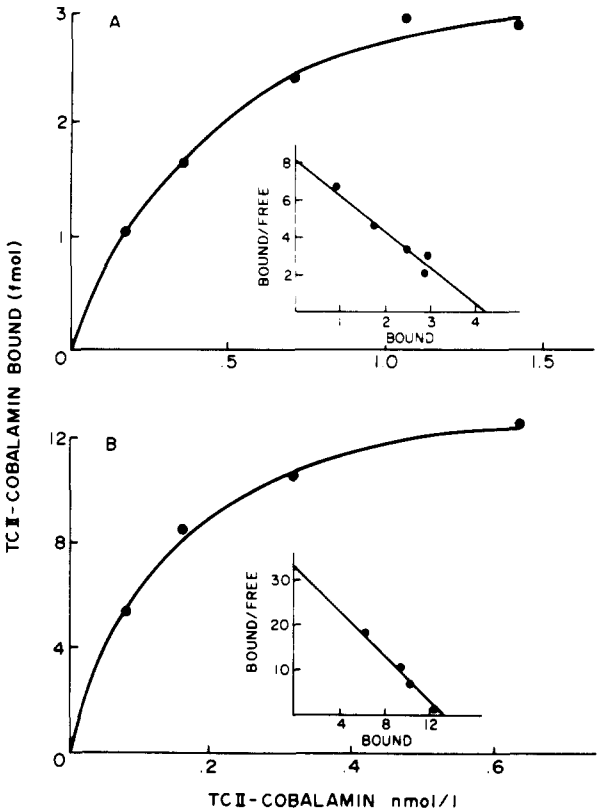

Fig. 1. Binding of transcobalamin II-cobalamin by rabbit liver membranes and by solubilized acceptor from human placenta membranes. Upper. The binding at increasing concentrations of rabbit transcobalamin II-cobalamin by rabbit liver membranes was measured by the centrifugation method (see text). Lower. The binding at increasing concentrations of human transcobalamin II-cobalamin was measured for solubilized placenta acceptor that was adsorbed to concanavalin A-Sepharose. The insets in both figures show the analysis of the binding data according to Scatchard [17]. Abscissa: Transcobalamin II-cobalamin added (nM). Ordinate: Transcobalamin II-cobalamin bound (fmol). Inset abscissa: bound (fmol). Inset ordinate: 10^6 X (bound/free) (l).

latter detergent solubilizes the acceptor equally well in concentrations between 0.5 and 2% (w/v) and has the advantage that it can be removed by dialysis. More than 90% of the acceptor was recovered upon solubilization. After solubilization, both the human and the rabbit acceptors bound to concanavalin A-wheat germ agglutinin- and phytohemagglutinin-Sepharose suggesting that they are glycoproteins possessing mannose, *N*-acetylglucosamine and *N*-acetylgalactosamine residues.

When adsorbed to concanavalin A-Sepharose columns, the solubilized acceptors, like the membrane-associated acceptors, exhibit high-affinity, EDTA-sensitive (i.e. calcium-dependent) specific saturable binding of the transcobalamin II-cobalamin complex. Solubilization does not appear to change the affinity of the acceptors for human transcobalamin II-cobalamin (Table I). Interestingly, 10 mM EDTA, which permits a dissociation of the transcobalamin II-cobalamin complex from the concanavalin A-Sepharose-bound

TABLE I

ASSOCIATION CONSTANTS FOR THE BINDING OF HUMAN AND RABBIT TRANSCOBALAMIN II-COBALAMIN TO HUMAN PLACENTA AND RABBIT LIVER ACCEPTORS

TC II, transcobalamin II; n.d., not determined.

	K_A ($\times 10^9$) (M^{-1})	
	Human TC II	Rabbit TC II
Placenta membranes		
Particulate	2.3	1.1
Solubilized	2.4	n.d.
Liver membranes		
Particulate	6.7	1.9
Solubilized	8.7	n.d.

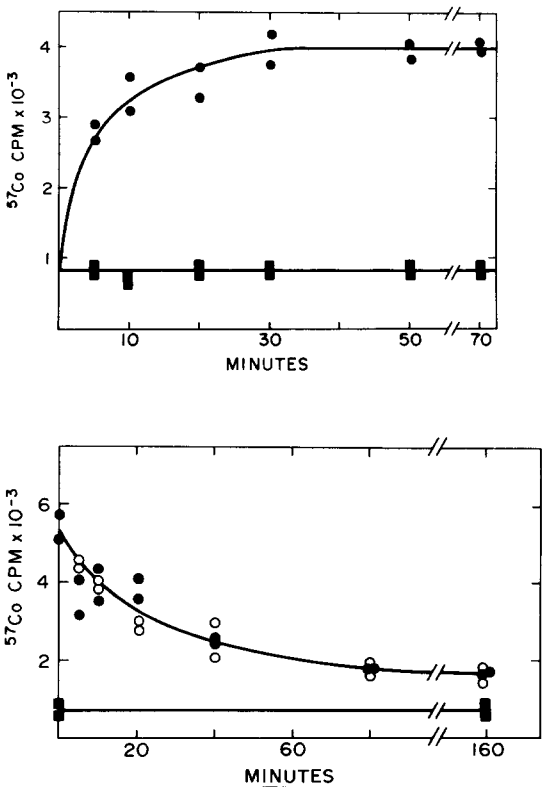

Fig. 2. Rates of association and dissociation between human transcobalamin II-cobalamin and placenta membranes. Upper. Radiolabeled transcobalamin II-colobamin complex was added to placenta membranes either with (■) or without (●) an excess of unlabeled transcobalamin II-cobalamin. The total binding at timed intervals was then measured by the centrifugation method. Lower. Placenta membranes were equilibrated (1 h, 24°C) with radiolabeled transcobalamin II-cobalamin either with (■) or without (●, ○) an excess of unlabeled transcobalamin II-cobalamin. Dissociation was initiated by the addition of either pure rabbit transcobalamin (●) or 10 mM EDTA (○). The radioactivity remaining bound at timed intervals was then determined.

acceptor, leaves the acceptor adsorbed to the column, capable of binding transcobalamin II-cobalamin again when the EDTA is removed. The transcobalamin II-cobalamin complex that adsorbs to the concanavalin A-Sepharose-immobilized acceptor and that dissociates in the presence of EDTA coelutes with native transcobalamin II-cobalamin, upon Sephacryl S-200 chromatography. Likewise, 1 M NaCl and 50 mM acetate buffer, pH 4.5, cause a reversible dissociation of transcobalamin II-cobalamin from the acceptor, leaving the acceptor adsorbed intact to concanavalin A-Sepharose. The soluble acceptor was also adsorbed by Sepharose-transcobalamin II-cobalamin and could be eluted with either 1 M NaCl or 10 mM EDTA. Like the particulate acceptors, the solubilized acceptor binds transcobalamin II-cobalamin with comparative selectivity, in that free cobalamin is not bound at all and free transcobalamin II is bound with a much lower affinity (about 1/3) than that of the transcobalamin II-cobalamin complex (Fig. 3).

The solubilized acceptors could be purified by adsorption to the lectin-Sepharose derivatives (concanavalin A, wheat germ agglutinin, phytohemagglutinin) and could be eluted with the appropriate sugar-containing buffers. The wheat germ agglutinin-Sepharose columns provided the highest yield of acceptor with about a 30-fold purification upon elution with 0.2 M *N*-acetylglucosamine. Therefore, after membrane solubilization, acceptors were first adsorbed to wheat germ agglutinin-Sepharose, washed free of contaminating protein and, where appropriate, were exposed to ^{57}Co-labeled transcobalamin II-cobalamin complex. Either the washed, acceptor transcobalamin II-[^{57}Co]-cobalamin complex so formed or the free acceptor was then desorbed from the lectin column in the presence of 0.2 M *N*-acetylglucosamine and analyzed further by gel filtration on Sephacryl S-200.

The complexes formed between ^{57}Co-labeled human transcobalamin II-cobalamin and both the human and rabbit acceptors behaved as single components upon Sephacryl S-200 chromatography (Fig. 4), with apparent Stokes

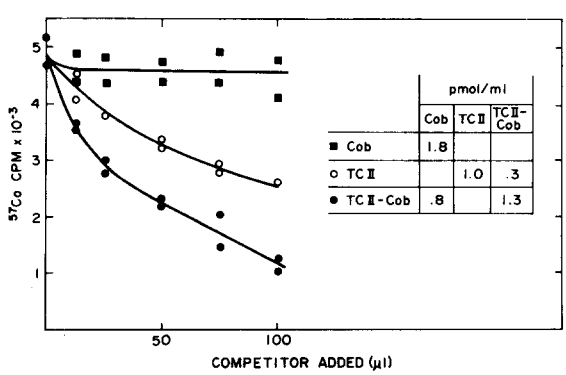

Fig. 3. Binding competition curves for solubilized placenta membrane acceptor and transcobalamin II-[^{57}C]cobalamin. Unpurified soluble acceptor from placenta membranes was adsorbed to concanavalin A-Sepharose and the binding of human transcobalamin II-[^{57}Co]cobalamin complex was measured as described in the text. The ability of increasing concentrations of cobalamin (Cob), transcobalamin II (TC II) and transcobalamin II-cobalamin complex (TCII-Cob) to compete for the binding of radiolabeled complex was then determined. The inset shows the unavoidable content of TCII-Cob and free Cob present in the solutions of TCII (○) and TCII-Cob (●) used for the competition studies.

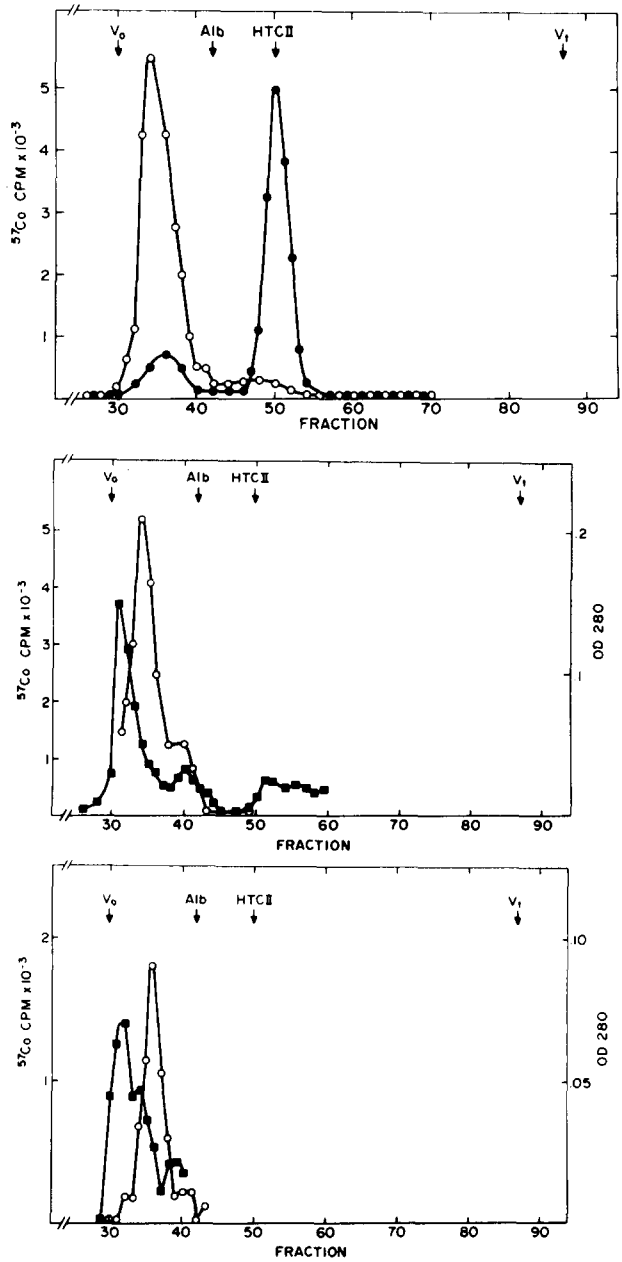

Fig. 4. Gel filtration of solubilized acceptor. Solubilized acceptor from either human placenta or rabbit liver membranes was first partially purified by adsorption to wheat germ agglutinin-agarose followed by elution with *N*-acetylglucosamine (0.2 M^{-1}). Upper. Rabbit liver acceptor was incubated with transcobalamin II-[^{57}Co]cobalamin for 1 h at 4°C prior to elution with the specific sugar. The eluate was analysed either directly (○) or after incubation for 1 h at room temperature with 100 pmol pure unlabeled rabbit transcobalamin II (●) on columns (1.5 × 87 cm) of Sephacryl S-200 in 50 mM Tris-HCl, pH 7.4, containing 150 mM NaCl, 1 mM $CaCl_2$, 1 mM $MnCl_2$ and 0.1% (w/v) Ammonyx-LO (Onyx, NJ), at a flow rate of about 7 ml/h. 1.67-ml fractions were collected. Middle. Crude soluble rabbit acceptor (about 2 mg protein) was analyzed by gel filtration, as above, and the transcobalamin II-cobalamin binding activity of the effluent fractions (○) was determined using concanavalin A-Sepharose as outlined in the text. The absorbance at 280 nm (■) of effluent fractions was also monitored. Column calibration: V_0, void volume; V_t, total volume; HTCII, human transcobalamin II-cobalamin complex, and Alb, bovine serum albumin. Lower. Soluble human placenta acceptor (about 0.9 mg protein) was subjected to gel filtration and effluent fractions were monitored for both transcobalamin II-cobalamin binding activity (○) and absorbance at 280 nm (■).

radii of 6.7 nm (human acceptor) and 5.7 nm (rabbit acceptor). In some acceptor preparations that had been stored for more than 1 week at 0°C, a component of smaller size (about 3.5 nm) could also be detected. Incubation of the acceptor-transcobalamin II-cobalamin complexes with an excess of unlabeled pure rabbit transcobalamin II-cobalamin, prior to chromatography, resulted in a marked reduction in the amount of radioactivity migrating with the acceptor, and in the appearance of radioactivity in the position of free transcobalamin II-cobalamin (apparent Stokes radius, 2.4 nm).

The soluble uncomplexed acceptors from human and rabbit membranes could both be detected in the effluent fractions subsequent to chromatography with the use of the concanavalin A-Sepharose binding method (Fig. 4). As expected, the uncomplexed human acceptor exhibited a molecular size (apparent Stokes radius, 5.1 nm) smaller than that of the acceptor saturated with transcobalamin II-cobalamin. Surprisingly, the apparent Stokes radius of the uncomplexed rabbit acceptor was the same (5.7 nm) as that of the entire rabbit acceptor-transcobalamin II-cobalamin complex.

Discussion

The major findings of this study comprise the characterization both in the membrane-associated and soluble state of specific high-affinity acceptors for transcobalamin II-cobalamin that can be detected in both human placenta and rabbit liver membranes. Our studies expand upon previous work done with human placenta membranes [18,19] and can be compared with data obtained previously with rat liver membranes [20]. Our data are also in excellent agreement and complement a concurrent study of the solubilized transcobalamin II acceptor from human placenta that appeared upon completion of our studies [21]. Further, our work provides a new, rapid and reliable method for the detection of the transcobalamin II-cobalamin acceptor, subsequent to membrane solubilization.

In close agreement with the work of Seligman and Allen [21], we estimate an affinity constant of $2.3 \cdot 10^9$ M^{-1} for the particulate acceptor (a value about 3-fold higher than that reported by Friedman et al. [18]) and a large molecular size (6.7 nm) for the human acceptor-transcobalamin II-cobalamin complex. Our data with the lectin-agarose derivatives substantiate the chromatographic identification of a number of carbohydrate residues in the acceptor by Seligman and Allen [21]. The binding of the acceptors to both wheat germ agglutinin and phytohemagglutinin with desorption by *N*-acetylglucosamine (for wheat germ agglutinin) and *N*-acetylgalactosamine (phytohemagglutinin) suggests further the N-acetylation of the glucosamine and galactosamine residues in the oligosaccharide portion of the acceptor.

In contrast to the study of Seligman and Allen [21], we observe a high affinity of the solubilized acceptor for transcobalamin II-cobalamin, equal to that of the membrane-associated acceptor. Further, we observe a more pronounced difference in the Stokes radius of the uncomplexed human acceptor (30% smaller in this study as opposed to 3% previously reported [21]) compared with the complete acceptor transcobalamin II-cobalamin complex. Also, only occasionally, and only in old preparations have we observed a

smaller molecular size form of the acceptor (apparent Strokes radius, 3.5 nm) possibly similar to the one described by Seligman and Allen [21].

In many respects, the acceptors obtained from human placenta and rabbit liver appear to be similar membrane glycoproteins possessing comparable ligand affinities and exhibiting similar molecular sizes of the acceptor-transcobalamin II-cobalamin complexes upon chromatography. There does, however, appear to be a difference in the two acceptors in the molecular size change caused by the binding of transcobalamin II-cobalamin. Whereas the apparent Stokes radius of the human acceptor increases by about 30%, the size of the rabbit acceptor remains unchanged subsequent to complex formation. It is as though transcobalamin II-cobalamin complex fits into a cleft in the rabbit membrane acceptor. It will be of interest to determine whether this difference in size change can be attributed to interspecies differences or, more interestingly, whether the differences may relate to differences in the tissue used as a source (i.e., placenta versus liver) of the acceptor.

Whereas the acceptor displayed considerable selectivity for transcobalamin II-cobalamin (i.e., cobalamin alone was unable to compete for binding) the selectivity was not absolute, in that uncomplexed transcobalamin II still possesses an appreciable affinity (about 30% that of the complete complex) for the acceptor site. These data are essentially in agreement with those of Seligman and Allen [21] and do not substantiate the presence in liver membranes of a specific acceptor for uncomplexed cobalamin, as suggested by the work of Fiedler-Nagy et al. [20]. Given the excess (about 3-fold) of uncomplexed transcobalamin II relative to transcobalamin II-cobalamin that occurs in plasma [10], one might have expected a higher degree of selectivity of the acceptor for the transcobalamin II-cobalamin complex. The consequences of this relative lack of specificity remain to be determined.

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