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HYDROPHOBIC INTERACTIONS OF THE APO AND HOLO FORMS OF HUMAN COBALAMIN BINDING PROTEINS

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SUMMARY

The interactions of transcobalamin II (TC II), intrinsic factor (IF) and R-type binding protein of cobalamin (Cbl, vitamin B_{12}) with the hydrophobic chromatography matrix Phenyl-Sepharose CL-4B were investigated. IF-Cbl and R-Cbl complexes were not adsorbed on Phenyl-Sepharose at room temperature or at 4°C with buffer containing 50 mM sodium phosphate, pH 7.4 containing 150 mM sodium chloride. The TC II-Cbl complex adsorbed and could be eluted with buffer containing 50% v/v glycerol. IF without Cbl adsorbed and was eluted with 50% glycerol at room temperature and 4°C. At room temperature, R binder without Cbl eluted with buffer, but later than the R-Cbl complex. At 4°C, R binder completely adsorbed to the matrix. TC II-without Cbl bound to the matrix at 4°C and room temperature and could not be eluted with glycerol. These results suggest that Cbl binding proteins can be separated and identified based on their hydrophobic properties. In addition, upon binding Cbl, TC II, IF and R-type binders undergo a conformational change such that the protein-Cbl complex shows reduced hydrophobicity.

Transcobalamin II (TC II), intrinsic factor (IF) and R-type binders (R) are vitamin B_{12} (cobalamin, Cbl) binding proteins whose function and physicochemical properties have been recently reviewed (1). These Cbl binders are found in a variety of tissues and body fluids either bound (holo) or not bound (apo) to endogenous Cbl. All contain one binding site for Cbl. IF and TC II facilitate Cbl entry into cells via specific receptors on cell membranes. The function of R-type binders has not been conclusively established, although it does carry >80% of the endogenous serum Cbl.

It is now generally accepted that most proteins contain hydrophobic areas composed of groups or regions of non-polar amino acids in both the interior and

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exterior portions of the molecule (2). Their number, size and accessibility will vary depending on the primary and secondary structure of a given protein (3). Rose et al have suggested that hydrophobic regions are a contributing factor in the formation and stabilization of a protein's secondary structure (4). A number of biological processes depend on exposed hydrophobic regions, including interaction of enzymes with specific substrates and coenzymes (5), host defense mechanisms (6) and albumin binding and transport of metabolites and drugs (7).

We report here the interactions of the holo and apo forms of TC II, IF and R with the non-charged hydrophobic chromatography matrix Phenyl-Sepharose CL-4B. The results show that hydrophobic adsorption chromatography (8) can be used for the separation of apo from holo Cbl binding proteins and perhaps in an overall scheme for purification of these proteins. In addition, and perhaps more importantly, it suggests that Cbl binding proteins undergo a conformational change upon binding Cbl which results in a change in their hydrophobic nature.

MATERIALS AND METHODS

Phenyl-Sepharose CL-4B was purchased from Sigma Chemical Company and stored at 4°C. Radioactive cyanocobalamin ([^{57}Co] CN-Cbl) and human Cbl binding proteins were prepared as described (9). Glycerol was purchased from the Fisher Scientific Company.

The Phenyl-Sepharose CL-4B was equilibrated to room temperature and packed by gravity to a height of 6 cm (3 ml of packed material) in 10 ml plastic serological pipettes (Falcon Plastics) fitted with a plug of glass wool. Prior to use, columns were washed with 30 ml of buffered saline (50 mM sodium phosphate, pH 7.4 containing 150 mM NaCl). 0.5 ml of R, TC II, or IF (total Cbl binding ability of 9,750, 6,450 and 10,150 pg respectively) were labeled with 50 µl (338 pg) of [^{57}Co] CN-Cbl and incubated for 30 minutes at 37°C. The volume was adjusted to 3 ml with buffered saline and applied to separate columns. Unless otherwise indicated, columns were eluted with 20 ml of buffered saline followed by 40 ml of buffer containing 50% glycerol. 2 ml fractions were collected and the ^{57}Co radioactivity analyzed in each with a gamma well-type scintillation spectrometer. Apo binders were detected in each fraction by labeling aliquots (1 ml for buffered saline fractions and 0.2 ml for glycerol fractions) with [^{57}Co] CN-Cbl, incubation for 30 minutes at 37°C and separation of bound from free radioactivity with albumin-coated charcoal (10) or gel filtration on Sephadex G-200 (Pharmacia Fine Chemicals). Aliquots of fractions which bound >90% of the added [^{57}Co] CN-Cbl were reanalyzed after appropriate dilutions with buffer. Preliminary experiments showed that the resulting concentration of glycerol (<5%) did not affect either method of analysis. For experiments performed at 4°C, the columns were poured at that temperature, and all buffers were cold. After labeling the binding proteins with [^{57}Co] CN-Cbl and incubation at 37°C for 30 minutes, the samples were equilibrated at 4°C for 10 minutes prior to application.

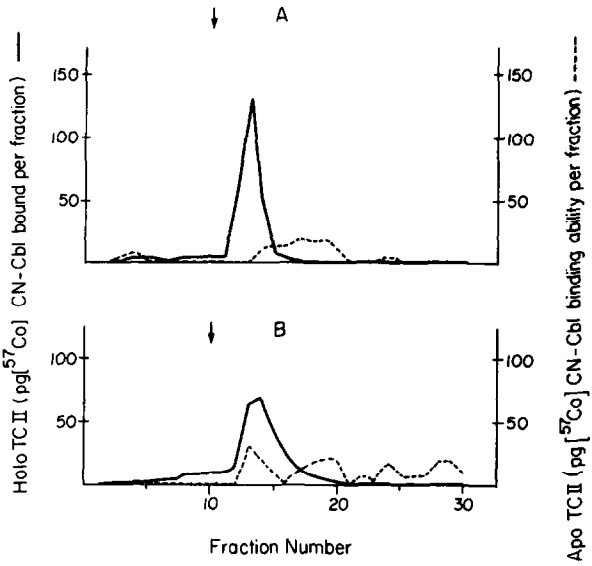

Figure 1. Hydrophobic adsorption chromatography of human transcobalamin II (TC II) at room temperature (A) and 4°C (B). 0.5 ml of sample (6,450 pg of total Cbl binding ability) was labeled with 50 µl (338 pg) of [^{57}Co] CN-Cbl and brought to a volume of 3 ml with buffered saline (50 mM $NaPO_4$ buffer, pH 7.4 containing 150 mM NaCl). The sample was applied and the column eluted with 20 ml of buffered saline. At fraction number 10 (arrow) the buffer was changed to buffered saline containing 50% v/v glycerol and an additional 20 fractions collected. All fractions were assayed for [^{57}Co] radioactivity and converted to pg [^{57}Co] CN-Cbl (holo TC II, ———). Apo TC II (-----) was determined by measuring the [^{57}Co] CN-Cbl binding ability in each fraction (10).

In order to determine the effect of complete saturation of the binders, 0.5 ml samples of R, TC II and IF were labeled with [^{57}Co] CN-Cbl at 20% in excess of their binding capacity and dialyzed for 17 hours against 100 volumes of buffered saline at 4°C. The samples were adjusted to 3 ml and applied to separate columns. Duplicate samples were chromatographed without the addition of [^{57}Co] CN-Cbl and assayed for apo binder.

RESULTS

Figure 1A shows the elution profile of TC II on Phenyl-Sepharose CL-4B at room temperature. Neither apo nor holo TC II eluted with buffered saline. Inclusion of 50% glycerol in the column buffer caused the [^{57}Co] CN-Cbl labeled TC II to elute. Recovery of the radioactive label (holo TC II) was 100%. Only 2% of the apo TC II was recovered. Gel filtration of the radioactive peak on Sephadex G-200 confirmed that the [^{57}Co] CN-Cbl was bound to TC II (data not shown). Temperature had little effect, since the elution

profile and the recovery of holo and apo TC II at 4°C (Figure 1B) were similar to that at room temperature. Attempts to elute apo TC II with 1% Triton X-100 or 3 M urea in buffered saline, or 1% Triton X-100 in 10 mM Tris-Hcl, pH 10 were unsuccessful. However, these buffers were shown to elute the [^{57}Co] CN-Cbl (holo TC II), which was stable to dialysis and not removed by albumin coated charcoal (data not shown). Incubations of apo TC II in these buffers equivalent to the column elution time failed to show an effect of these materials sufficient to account for the non-recoverable apo TC II. Attempts to convert the column bound apo TC II to holo TC II in order to elute it off with buffer containing 50% glycerol (Figure 1) were not successful since we could not demonstrate binding of [^{57}Co] CN-Cbl to the column bound apo TC II.

Figures 2A and B show the interaction of apo and holo IF on Phenyl-Sepharose CL-4B at room temperature and 4°C respectively. Holo IF was eluted with buffered saline, while apo IF did not elute until 50% glycerol was present in the buffer. The recovery of holo IF was >99% in both. Apo IF recovery was 84% at 4°C and 61% at room temperature. The lower recovery at room temperature was due to a total binding of the added [^{57}Co] CN-Cbl to a .2 ml aliquot of fraction number 13 (Figure 2A). Subsequent loss of this fraction did not permit reanalysis. It is expressed as greater than 2,970 pg [^{57}Co] CN-Cbl binding ability.

Figure 3 shows the interaction of holo and apo salivary R-type binder of Cbl. Holo R eluted with buffered saline (>99%) at both room temperature (Figure 3A) and at 4°C (Figure 3B). The interaction of apo R with Phenyl-Sepharose CL-4B varied with temperature. At room temperature, the apo R binder eluted as a broad tailing peak which did not coincide with the holo R binder peak (Figure 3A.) Addition of the 50% glycerol resulted in an apparent peak of apo R binder, but this was thought to be due to facilitated elution of the tailing area of the buffer region. At 4°C, holo R eluted with buffered saline while the apo R did not elute until 50% glycerol was added.

Figure 4 shows the elution profile of the apo and holo forms of IF, R,

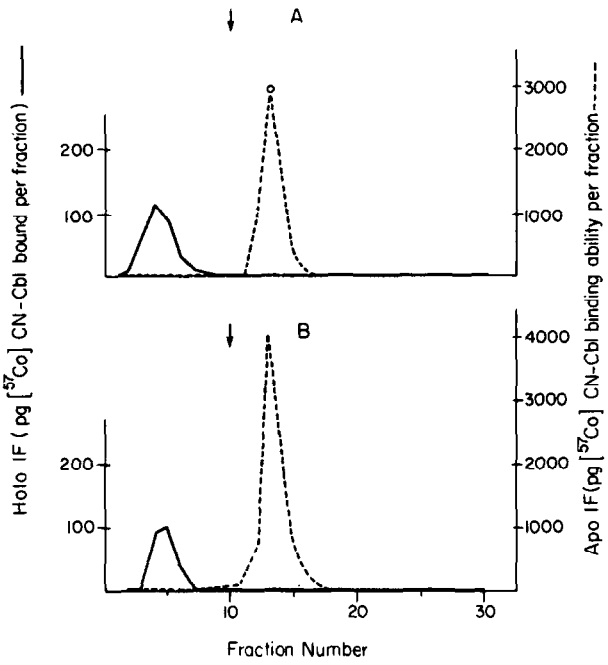

Figure 2. Hydrophobic adsorption chromatography of intrinsic factor (IF) on Phenyl-Sepharose CL-4B. 0.5 ml of sample (10,150 pg of Cbl binding ability) was labeled with 50 µl (338 pg) [^{57}Co] CN-Cbl, chromatographed and fractions analyzed for holo (———) and apo (-----) IF, (see Figure 1). Fraction number 13 (0) is expressed as greater than 2,970 pg of [^{57}Co] CN-Cbl binding ability.

and TC II at room temperature. Each section is a composite graph obtained from two separate columns of the respective binder either completely saturated with [^{57}Co] CN-Cbl or without added [^{57}Co] CN-Cbl. Complete saturation of IF (Figure 4A) or R (Figure 4B) did not affect the elution of either binder. The holo TC II eluted as two peaks of radioactivity (Figure 4B), one with buffered saline (24%) and one with glycerol (76%). Dialysis and rechromatography of the peaks resulted in greater than 90% of each eluting in the original position (data not shown).

DISCUSSION

It had been assumed that the hydrophobic regions of proteins were situated in the interior (core) of a protein molecule and were generally inaccessible to solvent (11). However, more recent evidence indicates that most proteins do, in fact, have accessible surface hydrophobic areas (2, 4, 12).

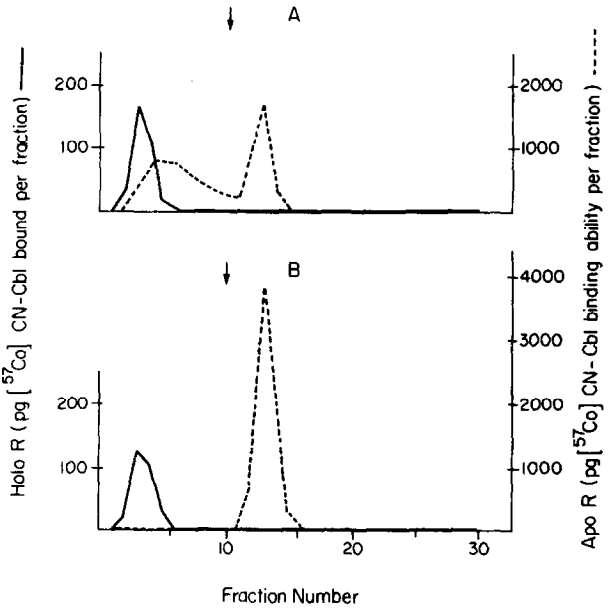

Figure 3. Hydrophobic adsorption chromatography of R binder on Phenyl-Sepharose CL-4B. 0.5 ml of sample (9,750 pg Cbl binding ability) was labeled with 50 µl (338 pg), [^{57}Co] CN-Cbl, chromatographed and fractions analyzed for holo (——) and apo (-----) R binder, (see Figure 1).

The present data indicates that Cbl binding proteins possess external hydrophobic regions and that the overall hydrophobicity of the molecule is modified by the binding of a Cbl molecule. TC II seems to be more hydrophobic than R or IF, since only holo TC II bound to Phenyl-Sepharose CL-4B with buffered saline. In addition, we could not elute apo TC II from the column with 50% glycerol (Figure 1) as did apo IF and R (Figure 2 and 3). The greater hydrophobicity of TC II might be partly responsible for the binding of serum TC II to Cibacron Blue F3GA (9), nitrocellulose (13) and Silica (14) since these substances have been implicated in hydrophobic interactions (9, 15, 16).

The holo form of all three proteins showed less interaction with Phenyl-Sepharose CL-4B than the corresponding apo form. Hippe et al (17) determined that the binding of Cbl to TC I (an R-type binder in serum) and TC II was endothermic while the reaction between IF and Cbl was exothermic. Based on these thermodynamic considerations, they postulated that hydrophobic interactions were involved in the binding of Cbl to TC II and R, although less important

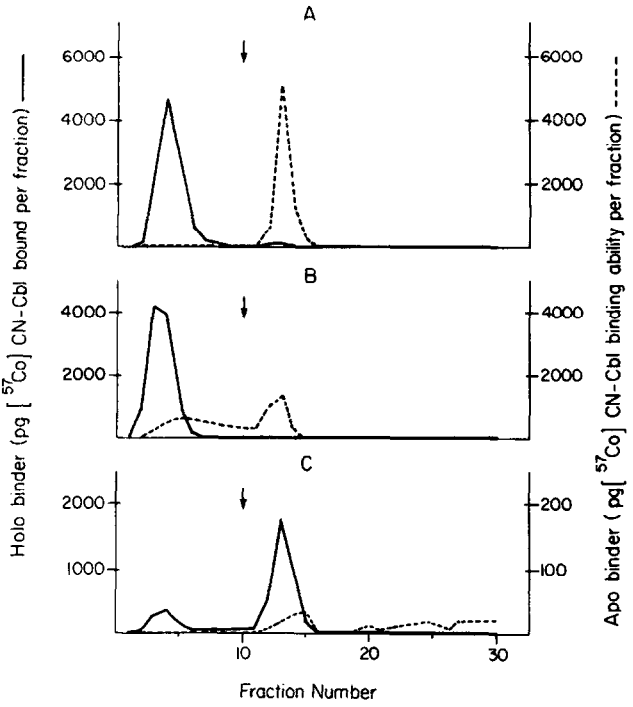

Figure 4. Hydrophobic adsorption chromatography of holo (———) and apo (-----) intrinsic factor (A), R-binder (B) and transcobalamin II (C) at room temperature. 0.5 ml of each binder was labeled with excess [^{57}Co] CN-Cbl, dialyzed against buffered saline and applied to separate columns. Each fraction was analyzed for [^{57}Co] and converted to pg [^{57}Co] CN-Cbl (holo binder). Duplicate samples were also chromatographed without the addition of [^{57}Co] CN-Cbl. Apo binders were determined by measuring the [^{57}Co] CN-Cbl binding ability in each fraction (10).

in IF binding of Cbl. In addition, Hippe has shown (18) that human IF and TC II, but not R-type binders, undergo a decrease in the Stokes radius after Cbl binding and this decrease was attributed to a conformational change resulting in a more compact structure.

The present results could be explained by the binding of Cbl to the apo binder and the subsequent burying of the Cbl molecule (18) with a resulting conformational change such that less surface area, including hydrophobic regions, is accessible to solvent (5). The decrease in Stokes radius of IF and TC II on binding Cbl may support this contention (18). Although the Stokes radius of R binder does not change when Cbl is bound (18), it is possible that the mechanism which produces a change in hydrophobicity is not the same as for

TC II and IF. The present observations have immediate as well as potential applications. In addition to its implementation in the purification and identification of Cbl binding proteins, it is unique among existing methods in that it is capable of separating total Cbl binding protein into its holo and apo fractions. The biological significance of the present observations need to be explored further. It is conceivable that the differential hydrophobicities of the apo and holo forms of these binders might play a role in the different affinities they exhibit for membrane receptors (1), facilitation of transmembrane transport, and subsequent intracellular processes.

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