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A Single Rainbow Trout Cobalamin-binding Protein Stands in for Three Human Binders[S]

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Background: Three cobalamin-binding proteins are expressed in most mammals. The expression in lower vertebrates is poorly explored.
Results: We identified a single cobalamin-binding protein in the rainbow trout. The protein behaves as a structural hybrid between the three known mammalian binders.
Conclusion: A novel cobalamin-binding protein present in fish has been identified.
Significance: This study provides insights into the phylogenetics of cobalamin-binding proteins.

Cobalamin uptake and transport in mammals are mediated by three cobalamin-binding proteins: haptocorrin, intrinsic factor, and transcobalamin. The nature of cobalamin-binding proteins in lower vertebrates remains to be elucidated. The aim of this study was to characterize the cobalamin-binding proteins of the rainbow trout (*Oncorhynchus mykiss*) and to compare their properties with those of the three human cobalamin-binding proteins. High cobalamin-binding capacity was found in trout stomach (210 pmol/g), roe (400 pmol/g), roe fluid (390 nmol/liter), and plasma (2500 nmol/liter). In all cases, it appeared to be the same protein based on analysis of partial sequences and immunological responses. The trout cobalamin-binding protein was purified from roe fluid, sequenced, and further characterized. Like haptocorrin, the trout cobalamin-binding protein was stable at low pH and had a high binding affinity for the cobalamin analog cobinamide. Like haptocorrin and transcobalamin, the trout cobalamin-binding protein was present in plasma and recognized ligands with altered nucleotide moiety. Like intrinsic factors, the trout cobalamin-binding protein was present in the stomach and resisted degradation by trypsin and chymotrypsin. It also resembled intrinsic factor in the composition of conserved residues in the primary cobalamin-binding site in the C terminus. The trout cobalamin-binding protein was glycosylated and displayed spectral properties comparable with those of haptocorrin and intrinsic factor. In conclusion, only one soluble cobalamin-binding protein was identified in the rainbow trout, a protein that structurally behaves like an intermediate between the three human cobalamin-binding proteins.

Cobalamin (vitamin B_{12}) absorption and transport in humans and most other mammals rely on three structurally kindred proteins. Haptocorrin (HC)[2] carries most of the circulating cobalamin as well as inactive cobalamin analogs. HC is also present in exocrine secretions such as saliva. Intrinsic factor (IF) is synthesized in the gastric mucosa and promotes intestinal uptake of cobalamin by receptor-mediated endocytosis. This glycoprotein is resistant to intestinal degradation. Finally, transcobalamin (TC) transports cobalamin in the blood and ensures its cellular uptake by binding to a specific receptor (1–3).

All three cobalamin-binding proteins have a primary structure with several highly conserved regions (the cobalamin-binding signature) (4), and they apparently evolved from a common ancestral gene by duplication (4–6). Although human HC and IF are glycoproteins, human TC is not (1).

Phylogenetically, the investigation of cobalamin-binding proteins has been mostly restricted to mammals. The three cobalamin-binding proteins have been found in all investigated mammalian species (7), except for mice (and possibly rats and opossum), which lack HC (8). Very little is known concerning the expression of cobalamin-binding proteins in lower vertebrates. Recently, we found that the zebrafish (*Danio rerio*) express only one cobalamin-binding protein that resembles an intermediate form of HC, IF, and TC (7). We proposed the protein to be named zebrafish HIT as an abbreviation of HC, IF, and TC (7). The difference in the nature of cobalamin-binding proteins from higher and lower vertebrates is indirectly supported by unmatched DEAE chromatography patterns (9).

In this study, we searched for cobalamin-binding proteins in the rainbow trout (*Oncorhynchus mykiss*) and found only one, a protein abundantly expressed in plasma, stomach, roe, and roe fluid. This novel protein resembles in its features all three human cobalamin-binding proteins, and therefore, we propose the protein to be named trout HIT.

EXPERIMENTAL PROCEDURES

Sample Preparations and Protein Extractions—Female rainbow trout ($n = 5$) from an authorized fish farm (Volk Trout

[S] This article contains supplemental Fig. 1.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) JX313059.

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[2] The abbreviations used are: HC, haptocorrin; IF, intrinsic factor; TC, transcobalamin; $UB_{12}BC$, unsaturated cobalamin-binding capacity.

Farm, Bredsten, Denmark) were sacrificed by inflicted trauma to the head and eviscerated. Roe and roe fluid were collected by cutting of the roe sac; the liver, kidney, stomach, intestine, and muscle were collected by traditional dissection techniques. Organs, roe, and roe fluid were stored at −20 °C. Blood samples were collected from the caudal tail vein into lithium heparin tubes, and plasma was removed after centrifugation at 2000 × *g* for 10 min and stored at −20 °C until analysis. Protein extractions of roe and tissue samples (~1 ml of buffer per 1 g of tissue) were carried out by homogenization on ice as described previously (7).

Human Samples—For comparative analysis, samples containing human cobalamin-binding proteins were employed. Human HC was obtained from a pool of saliva collected during our previous research (10). Human IF was obtained from gastric juice, and human TC was obtained from partly purified spermal plasma. Both fluids were collected by the medical staff as part of diagnostic tests for acidity (gastric juice, Bispebjerg Hospital, Copenhagen, Denmark) and infertility (sperm fluid, Hillerod Hospital, Hillerod, Denmark) (11). Excess samples were collected without information that would allow the samples to be traced back to the donor.

Recombinant human HC (a kind gift from Evelyne Furger and Eliane Fischer, Paul Scherrer Institute, Switzerland (12)), recombinant human IF (Cobento, Aarhus, Denmark), and recombinant human TC (synthesized and described elsewhere (13)) were also used for comparison.

Unsaturated Cobalamin-Binding Capacity and Total Cobalamins—Unsaturated cobalamin-binding capacity ($UB_{12}BC$) was measured by charcoal precipitation as described by Gottlieb *et al.* (14), and the supernatants were measured for radioactivity in the Wizard Automatic Gamma Counter (PerkinElmer Life Sciences). Total cobalamins were measured using Cobas 6000 E immunoassay system (Roche Diagnostics) with a detection range of 55–1476 pM.

Corrinoid Binding Assay—The ability of trout cobalamin-binding protein to bind the corrinoids cyano-cobalamin (Sigma), dicyano-cobinamide (Sigma), and adenosyl-pseudo-cobalamin (synthesized and described elsewhere (15)) was explored using a competitive assay previously described (16) with corrinoid concentrations ranging from 0 to 355 nM.

Size Exclusion Chromatography—For size exclusion chromatography, the $UB_{12}BC$ of protein extracts (roe and stomach) and fluids (roe fluid and plasma) were adjusted to 0.5 nM. 75 μl (0.039 pmol) of protein extract/fluid was then incubated with 30 μl (0.15 pmol) of [^{57}Co]cobalamin (5 nM)) (Kem-En-Tec, Taastrup, Denmark) and 75 μl of buffer containing 0.1 M Tris, 1 M NaCl, 0.02% sodium azide, and 0.05% bovine serum albumin (Sigma), pH 8, for 15 min at room temperature. From this mixture, 90 μl was applied to a Superdex 200 column (GE Healthcare) attached to a Dionex ICS-3000 HPLC system. Collected fractions were measured for radioactivity and for cobalamin content. Blue dextran (Sigma) and ^{22}Na (GE Healthcare) were used for determination of void volume (V_0) and total volume (V_t), respectively. Human TC (2.55 nM) (17) was used as reference for calculation of Stokes radii according to the method of Ackers (18).

Binding to Concanavalin A—The presence of carbohydrates on the trout cobalamin-binding protein was examined by precipitation with concanavalin A-Sepharose (Amersham Biosciences), a lectin known to bind α-D-mannopyranosyl, α-D-glucopyranosyl, and kindred compounds. Protein extracts of roe, roe fluid, human HC, IF, and TC were investigated. The study was carried out as described previously (7).

Purification of the Trout Cobalamin-binding Protein—Trout roe fluid was collected as a leftover during roe harvest at the trout farm and stored at −20 °C until used. Altogether we used ~36 liters of roe fluid. The unsaturated cobalamin-binding protein was purified from roe fluid using affinity chromatography employing hydroxo-cobalamin coupled to a Sepharose matrix as earlier described (19). To improve outcome, the purification procedure was optimized with several additional steps. In brief, EAH Sepharose 4B (GE Healthcare) was washed with 20 volumes of 0.1 M NaH_2PO_4, pH 7.5, before addition of hydroxo-cobalamin (1 mg/ml) (GEA, Copenhagen, Denmark) in 0.1 M NaH_2PO_4, pH 7.5. The mixture was incubated at 57 °C for 2 h, while gently mixing the solution every 20–30 min, and placed at room temperature for 18 h to stabilize the thermo-labile bond between the cobalt atom of cobalamin and the amino group of the Sepharose. The prepared matrix was stored at 4 °C after addition of 0.02% sodium azide.

Binding of the trout cobalamin-binding protein to the affinity column was carried out at 4 °C. Prior to use, the cobalamin matrix (50 ml) was washed with 10 volumes of 0.05 M Tris, pH 7.5, with 0.02% sodium azide. Rainbow trout roe fluid (3–4 liters per purification scheme) was filtered through ordinary coffee filters and applied to the affinity column with a flow rate of ~5 ml/min. The column was washed with 8 volumes of 0.05 M Tris, pH 7.5, with 0.02% sodium azide and 2 volumes of 0.05 M Tris, pH 7.5, with 0.02% sodium azide and 5 M NaCl. The flow was stopped for 30 min before washing was continued with 8 volumes of demineralized water. Finally, the column was washed with 2 volumes of 0.1 M sodium acetate, pH 4.0. Absorbed cobalamin-binding protein was eluted from the column after incubation at 37 °C for 18 h using 2–3 volumes of 0.1 M sodium acetate, pH 4.0, warmed to 37 °C. The eluate was subjected to 18 h of dialysis against demineralized water using 14-kDa cut-off dialysis tubes (Medicell International, London, UK). Finally, the solvents were evaporated in a Hetovac vacuum centrifuge (HETO, Allerod, Denmark), and the pellet was resuspended in 100 μl of demineralized water/liter of roe fluid applied to the column.

The cobalamin-binding protein was further purified by size exclusion chromatography (as described above). The red fractions with bound cobalamin were pooled, and the purity of the sample was evaluated by 10% SDS-PAGE (Bio-Rad) stained with Coomassie Brilliant Blue according to the standard procedure.

Sequence Analysis—The amino acid sequence of the trout cobalamin-binding protein was determined by a three-step method of LC-MS, RT-PCR, and repeat LC-MS. First, protein gel bands of purified protein were cut out of a Coomassie Brilliant Blue-stained SDS-polyacrylamide gel, and in-gel digested using trypsin as described (20). Mass spectrometry was performed using an EasyLC nanoflow HPLC connected to an LTQ-

Orbitrap XL mass spectrometer equipped with a nano-ESI ion source (Thermo Fisher Scientific). Separation was performed on a fused silica emitter (100 μm inner diameter) packed in-house with RP ReproSil-Pur C18-AQ 3-μm resin (Dr. Maisch GmbH, Germany). The mass spectrometer was run at a resolution of 60,000 with the data-dependent top five ions fragmented by CID as described (21). The obtained MS/MS data were searched against the salmon TC precursor (accession number NP_001133733) using the X!Tandem search engine as implemented by GPMAW version 9.12. The main parameters were s follows: tryptic cleavage, parent ion precision 5 ppm, fragment error 0.5 Da, maximum expect value 0.01, and refinements enabled with semi-cleavage and point mutations. Identified peptides were manually validated and based on the overlapping sequence areas, and RT-PCR primers were designed (based on the sequence of the phylogenetically related salmon (*Salmo salar*) sequence, Gene ID 100195232) to cover the sequence gaps of the trout sequence.

For the RT-PCR, total RNA was purified from trout muscle by the use of QIAamp RNA purification kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The concentration of RNA in the sample was measured by absorbance at wavelength 260 nm and adjusted to 0.1 μg/μl in RNase-free H_2O. Trout cDNA was synthesized by mixing 1 μl (0.1 μg) of trout RNA with 5 μl of 25 mM MgCl (Applied Biosystems), 8 μl of 10 mM dNTP mix (dATP, dGTP, dTTP, and dCTP, GE Healthcare), 2 μl of 10× PCR buffer (Applied Biosystems), 1 μl of 50 units/μl reverse transcriptase (Applied Biosystems), 1 μl of 20 units/μl RNase inhibitor (Applied Biosystems), and 1 μl of 50 μM 16-mer d(T) oligonucleotide primers (DNA Technology, Risskov, Denmark), reaching a total volume of 20 μl. The sample was incubated for 30 min at 42 °C and stored at −20 °C.

The PCR was carried out using 1 μl of trout cDNA mixed with 5 μl of SYBR Green (Light Cycler 480 SYBR Green 1 Master, Roche Diagnostics), 0.5 μl of 5 pmol/μl forward and reverse primers, and RNase-free H_2O to a volume of 10 μl. The PCR program was conducted as follows: preincubation for 10 min at 95 °C followed by 50 cycles of 10 s at 95 °C, 20 s at 60 °C, and 5 s at 72 °C. The PCR products were examined by 1.5% agarose gel electrophoresis, and the DNA bands were purified as described previously (22) and sequences by Eurofins MWG Operon, Ebersberg, Germany.

As a final step, the LC-MS data were compared with the trout sequence obtained by RT-PCR. The final protein sequence (413 amino acids) encoding the trout cobalamin-binding protein was scanned against the NCBI Database before depositing in GenBank™ (accession number JX313059).

Absorbance Spectrum—The absorbance spectra of the purified trout cobalamin-binding protein in complex with hydroxo- and azido-cobalamins (≈24 μM) were recorded in 0.2 M sodium phosphate buffer, pH 7.5, on a Varian Cary 50 spectrophotometer (Varian A/S, Sydney, Australia).

Treatment with Trypsin and Chymotrypsin—The sensitivity of the cobalamin-binding protein from trout toward enzymatic cleavage was explored using increasing concentrations of trypsin and chymotrypsin. The $UB_{12}BC$ of the purified trout protein was adjusted to 1 nM by dilution with 0.1 M Tris-HCl, pH 7.4. Then 0.4 pmol of apoprotein (400 μl) was incubated with 25 μl of [^{57}Co]cobalamin (5 nM) in 0.1 M Tris-HCl, pH 7.4, in a total volume of 900 μl for 30 min at room temperature. Increasing concentrations (0–100 units) of trypsin (Sigma) or chymotrypsin (Sigma) in 100 μl of 0.1 M Tris-HCl, pH 7.4, were added (total volume adjusted to 1 ml), and the mixture was incubated for 18 h at 37 °C. Unbound cobalamin was removed by charcoal precipitation in a manner described previously for determination of $UB_{12}BC$. The supernatants were measured for residual radioactivity. For comparison, the enzymatic sensitivity of recombinant human HC, IF, and TC was measured employing the same protocol.

Sensitivity toward Low Gastric pH—The stability of the trout cobalamin-binding protein at pH 2 was investigated and compared with the human cobalamin-binding proteins. For this study, the $UB_{12}BC$ of the purified trout protein was adjusted to 1 nM by dilution in 0.9% NaCl, and 0.4 pmol of apoprotein (400 μl) was incubated with 25 μl of [^{57}Co]cobalamin (5 nM) for 30 min at room temperature. Then either 400 μl of Tris-HCl, pH 2, or 400 μl of Tris-HCl, pH 7, was added, and the mixture was incubated for 2 h at room temperature. pH was measured in each sample to ensure that it was pH 2 or pH 7, respectively. Excess of the unbound [^{57}Co]cobalamin was removed by charcoal precipitation, and the protein-bound [^{57}Co]cobalamin was measured in the supernatant. The results at pH 2 were compared with those at pH 7 and with the data obtained for recombinant human HC, IF, and TC tested according to the same protocol.

Antibody-based Examinations—A monoclonal antibody against the purified trout cobalamin-binding protein was produced by a standard method (kindly made by Claus Koch, Department of Cancer and Inflammation Research, University of Southern Denmark). In brief, three female NMRI mice (Harlan Laboratories) were immunized three times with trout cobalamin-binding protein. Antigen was diluted in 0.9% NaCl and adsorbed onto $Al(OH)_3$, and an emulsion was made with incomplete Freund's adjuvant (State Serum Institute, Copenhagen, Denmark). For each immunization, 20 μg of antigen was given subcutaneously in a total volume of 150 μl. Mice were bled 10 days after the second and third immunization. Titer in two mice was >1:10,000, and a final immunization was given intravenously in the tail vein 3 days before fusion. The fusion protocol was essentially as described by Galfreé and Milstein (23). Culture supernatants were assayed in ELISA on microtiter plates coated with antigen diluted to 1 μg/ml. Positive wells were cloned, and after three reclonings, cells were grown, and antibody-containing culture media from five clones were selected based on antibody affinities and were purified by protein A chromatography (24). From these five antibodies, the one exerting the highest binding affinity toward trout cobalamin-binding protein was employed in the study.

The monoclonal mouse antibody was employed to investigate the immunoreactivity of the cobalamin-binding protein from different compartments of the trout. Unsaturated trout cobalamin-binding protein (0.130 pmol) from trout roe, roe fluid, stomach, or plasma was incubated for 30 min at room temperature with 25 μl of [^{57}Co]cobalamin (5 nM) and 70 μl of precipitation buffer containing 0.16 g/liter NaH_2PO_4, H_2O, 0.98 g/liter Na_2HPO_4, $2H_2O$, 8.1 g/liter NaCl, and 0.1%

Immuno I human albumin (Life Science, Holte, Denmark), pH 7.4. After incubation, an anti-trout antibody dilution series from 1:1000 to 1:80,000 was added, and the mixture was incubated for 1 h at room temperature. Magnetic anti-mouse IgG beads (Dynabeads® anti-mouse IgG, Invitrogen) were washed three times in precipitation buffer, and 25 μl was added to the mixture. After 1 h of incubation under rotation at room temperature, beads were separated from the supernatants by a magnetic field, and radioactivity of the beads was measured.

For immunohistochemistry, stomach, intestine, heart, liver, kidney, pancreas, skin, gills, and roe sac containing roe and roe fluid in lamellae were removed from two female trout and fixed in 10% formalin. For the immunohistochemical analysis, the tissues were embedded in paraffin and cut into 5-μm sections using a microtome. The sections were deparaffinized and for antigen retrieval were boiled in a microwave oven for 15 min in citrate buffer, pH 6. Nonspecific binding was blocked by preincubation for 5 min in 2% bovine serum albumin followed by 18 h at 4 °C with the primary antibody diluted 1:1000. The immunoreaction was visualized by means of biotinylated horse anti-mouse immunoglobulins (Vector BA-2000) diluted 1:200, as the second layer, followed by streptavidin-peroxidase complex, Vectastain PK-4000, diluted 1:100, for 30 min as the third layer. The sections were finally developed by means of 3,3-diaminobenzidine for 30 min and counterstained with hematoxylin.

In Situ Sequence Analyses—Protein sequences of salmon cobalamin-binding protein (NP_001133733), zebrafish cobalamin-binding protein (NP_001116703), human HC (P20061), human IF (P27352), and human TC (P20062) were found in the protein database of the National Center for Biotechnology Information (NCBI) website. Protein sequence alignments were performed using the ClustalW alignment method at the home page of the European Molecular Biology Laboratory-European Bioinformatics Institute (EMBL-EBI) using the default settings.

Ethical Considerations—According to the "European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes," no approval was necessary for the sacrifice of trout used in this study.

RESULTS

Cobalamin and Cobalamin-binding Capacities in Rainbow Trout Tissues and Fluids—Total cobalamins and $UB_{12}BC$ were measured in protein extracts of trout roe, stomach, kidney, liver, intestine, and muscle (Table 1A), as well as in trout plasma and roe fluid (Table 1B). High cobalamin concentrations were found in trout roe, kidney, and liver, which is in agreement with previously published data (25, 26). The finding of a high $UB_{12}BC$ in the stomach suggests the presence of a protein that resembles human IF in its function to mediate intestinal cobalamin uptake (3). Likewise, the occurrence of a high $UB_{12}BC$ in plasma suggests the presence of a protein that resembles human TC in its function to distribute cobalamin between different tissues (3). In addition, we observed a high $UB_{12}BC$ in roe and roe fluid (Table 1B).

Characterization of Trout $UB_{12}BC$—We tested how cobalamin and its analogs competed with labeled cobalamin for binding to the cobalamin-binding proteins present in trout tissues (roe and stomach) and fluids (plasma and roe fluid), and we obtained comparable results for all of the trout cobalamin-binding proteins (Fig. 1). Cobalamin-binding curves were comparable with those of human HC, IF, and TC (Fig. 1*A*), thus suggesting that the affinity of the trout cobalamin-binding protein is of the same magnitude as for the human proteins (16). Both cobinamide (Fig. 1*B*) and adenosyl-pseudo-cobalamin (Fig. 1*C*) efficiently competed with labeled cobalamin for the trout cobalamin-binding proteins. This is also the case for human HC (cobinamide) and human HC and TC (adenosyl-pseudo-cobalamin) (16).

TABLE 1
Cobalamin content and unsaturated cobalamin-binding capacity ($UB_{12}BC$) in rainbow trout tissues and fluids
The number (*n*) indicates the number of fish that donated samples.

	Cobalamin	$UB_{12}BC$
A, Results on tissues[a]		
Roe ($n = 5$)	32 (27–37)	400 (350–470)
Stomach ($n = 3$)	6 (4.6–8)	210 (200–220)
Kidney ($n = 3$)	58 (53–64)	0.12 (0.11–0.14)
Liver ($n = 3$)	20 (16–24)	0.32 (0.14–0.44)
Intestine ($n = 3$)	11 (10–13)	1.6 (0.7–2.4)
Muscle ($n = 3$)	0.9 (0.8–1.1)	30 (24–34)
B, Results on fluids[b]		
Plasma ($n = 5$)	5.7 (3.4–8.0)	2500 (2400–2500)
Roe fluid ($n = 5$)	5.1 (2.6–7.9)	390 (320–430)

[a] The values for the results on tissues are given in mean (range) pmol/g.
[b] The values for the results on fluids are given in mean (range) nM.

Size exclusion chromatography profiles of the cobalamin-binding proteins from trout roe fluid, plasma, and stomach displayed similar elution profiles (Fig. 2) with a Stokes radius of 4.4 nM. The cobalamin-binding protein from roe eluted as a molecule of a slightly smaller size (3.3 nM).

The trout cobalamin-binding proteins were glycosylated like HC and IF, as judged from their ability to interact with concanavalin A (data not shown). We speculate that a difference in glycosylation may explain the differences observed in the elution profiles of the proteins.

Purification of the Trout Cobalamin-binding Protein—To further explore the nature of the cobalamin-binding proteins in trout, we purified the protein. Roe fluid was selected as starting material based on a high $UB_{12}BC$ and the ease of getting a sufficient amount of starting material.

The cobalamin-binding protein was purified by affinity chromatography followed by size exclusion chromatography. In total, 36 liters of roe fluid ($UB_{12}BC$ ~390 nmol/liter) was used for purification giving a total of ~70 mg (1.2 μmol) of the isolated product. Subjected to native gel electrophoresis, the purified protein showed one major band corresponding to a mass of 60 kDa (Fig. 3*A*). The reduced samples resolved in two bands with the approximate sizes of 25 and 20 kDa. A similar pattern of bands has been observed for other cobalamin-binding proteins, where it reflected cleavage to α and β domains (27).

Amino Acid Sequence and Alignment of the Trout Cobalamin-binding Protein—The amino acid sequence of the purified trout cobalamin-binding protein was determined by a three-step procedure of LC-MS, RT-PCR, and repeat LC-MS (Fig. 3*B*). The peptides identified by the first LC-MS analysis were compared with cDNA sequence data for the salmon cobalamin-

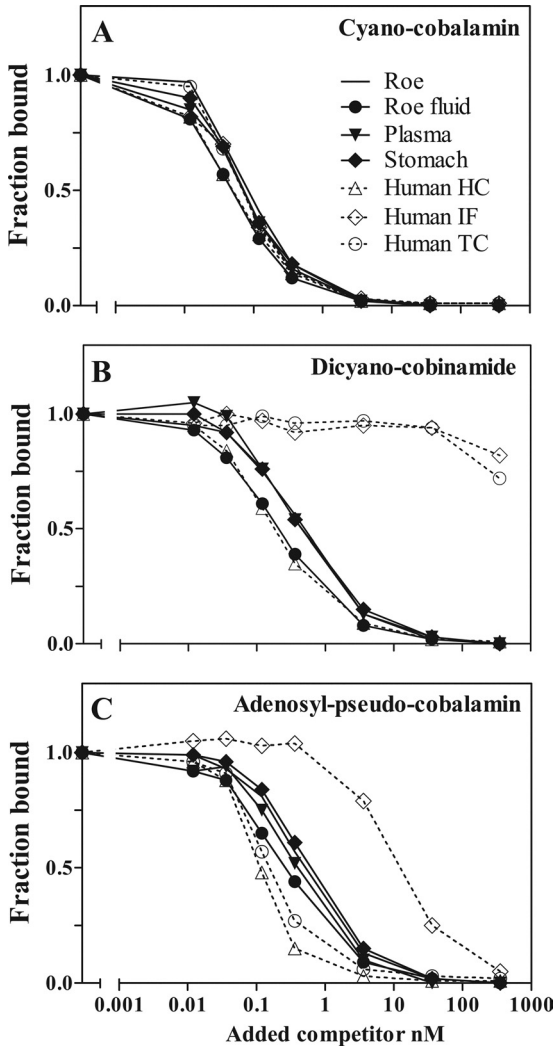

FIGURE 1. **Binding of the trout cobalamin-binding protein to cobalamin and cobalamin analogs.** The trout cobalamin-binding protein found in roe, roe fluid, plasma, and stomach was incubated for 18 h with [^{57}Co]cobalamin and increasing concentrations of unlabeled cyano-cobalamin (*A*), dicyano-cobinamide (*B*), and adenosyl-pseudo-cobalamin (*C*). Protein-bound [^{57}Co]cobalamin was measured after removal of free cobalamin by charcoal precipitation, and the amount of [^{57}Co]cobalamin bound was calculated relative to the amount when only [^{57}Co]cobalamin was present. Binding characteristics of the human cobalamin-binding proteins are shown for comparison.

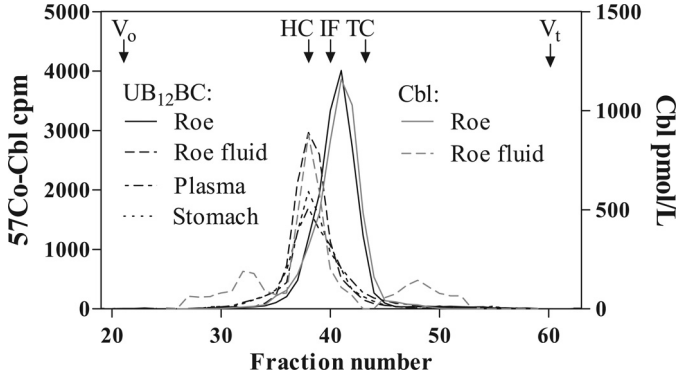

FIGURE 2. **Size exclusion chromatography of the trout cobalamin-binding protein.** Size exclusion chromatography performed on a Superdex 200 column of the cobalamin-binding proteins found in trout roe, roe fluid, plasma, and stomach measured as $UB_{12}BC$, [^{57}Co]cobalamin (*57Co-Cbl*) (*black lines*), and as amount of cobalamin (*Cbl*) in protein extract (roe and roe fluid) (*gray lines*). The *x* axis indicates the fraction number. Elution volume for void volume (V_0), human HC, IF, TC, and total volume (V_t) are shown by *arrows*.

binding protein, because no sequence data exist for trout. Based on these data, we designed primers for RT-PCR. From three overlapping PCRs, we constructed the full sequence of the protein and aligned the LC-MS data against the obtained trout cDNA sequence. The protein sequence covered 43% of the total structure. The fact that LC-MS data were obtained from a protein purified from trout roe fluid, whereas the cDNA sequence was based on RNA isolated from muscles, points to the presence of only one cobalamin-binding protein in the trout.

The LC-MS data consistently showed peptides starting from position 4 of the trout sequence (*i.e.* prior to KPCD . . .) (supplemental Fig. S1). This indicates that this is the N terminus of the mature protein after cleavage of the signal peptide. The finding is in agreement with the observed N termini of human HC and TC, as well as with the predicted N termini of salmon and zebrafish cobalamin-binding proteins. The aspartic acid in position 208 was observed as a glutamic acid by LC-MS, but as this is situated in a nonconserved region (supplemental Fig. S1), it is unlikely to have any biological significance. Altogether, the trout cobalamin-binding protein is predicted to consist of a single amino acid chain (413 amino acids) with a backbone size of 45 kDa. Surprisingly, no apparent *N*-glycosylation sites were found in the protein sequence.

The amino acid sequence of the trout cobalamin-binding protein was aligned with the corresponding sequences of salmon, zebrafish, and the three human cobalamin-binding proteins, all from the NCBI database. The pairwise sequence identities were as follows: trout binder/salmon binder = 91.3%; trout binder/zebrafish binder = 58.2%; trout binder/human TC = 28.6%; trout binder/human HC = 26%; trout binder/human IF = 22.1%. The full-length nucleotide and amino acid sequences of the trout binder and the mentioned alignments are presented in supplemental Fig. S1.

Fig. 3*C* shows a partial protein sequence alignment of the primary cobalamin-binding site containing conserved residues important for ligand specificity for cobalamin and cobalamin analogs. As the salmon and zebrafish cobalamin-binding proteins, the trout cobalamin-binding site contains a Trp^{362} residue present in IF (Trp^{366}) and HC (Trp^{382}) but absent in TC (27). Lack of a Tyr residue (Tyr^{385} in HC and Tyr^{380} in TC) is a characteristic trait of IF (27).

Absorption Spectra—The absorbance spectrum of the purified cobalamin-binding protein in complexes with hydroxo- and azido-cobalamins (≈24 μM) were examined and compared with those of human/bovine TC and human HC/IF, described earlier (13, 28, 29). For hydroxo-cobalamin, the spectrum (Fig. 4) displayed a γ-peak at 357 nm and an α-peak at 532 nm showing resemblance to the data for human HC (γ = 356 nm and α = 528 nm) and IF (γ = 356 nm and α = 531 nm), but differing from the spectra of human and bovine TCs (γ = 362 nm and α = 546 nm) (29). If all spectral features of the trout binder-hydroxy-cobalamin complex are indeed similar to those of human HC and IF (29), then its molar absorbances can be evaluated as ϵ_{280} = 26,200, ϵ_{357} = 28,700, and ϵ_{532} = 10,500 M^{-1} cm^{-1}. This estimate is based on the assumption of 24.3 μM

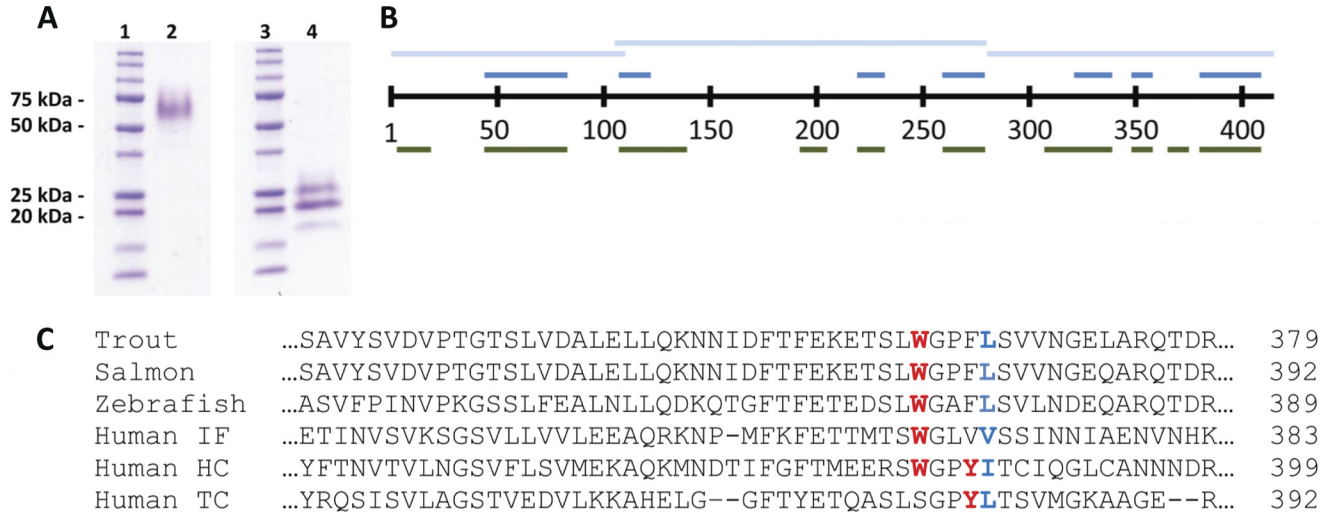

FIGURE 3. **Protein sequence of the trout cobalamin-binding protein.** *A,* gel electrophoresis of purified trout cobalamin-binding protein. The purified protein (15 μg) is shown as a major band with an apparent molecular size of approximate 60 kDa (*lane 2*), whereas the reduced samples resulted in two major bands (*lane 4*). *Lanes 1* and *3* are markers. *B,* schematic presentation of peptide fragments achieved by the initial LC-MS analysis of the protein purified from roe fluid and based on expected sequences of the salmon cobalamin-binding protein (*dark blue*). Results obtained by RT-PCR on RNA isolated from trout muscle are shown (*light blue*). The LC-MS data were aligned against the trout sequence obtained by RT-PCR yielding a sequence coverage of 43% (*green*). *C,* partial protein sequence alignment of the primary cobalamin-binding site in the C terminus. The sequence alignment of trout, salmon, zebrafish cobalamin-binding proteins, and the human cobalamin-binding proteins (HC, IF, and TC) was done in ClustalW2 using default settings. The residues highlighted in *red* are the ones that form hydrophobic interactions with cobalamin and that are characteristic for the specificity for cobalamin (HC, IF, and TC) and other corrinoids (only HC) (27). The residues highlighted in *blue* form hydrogen bonds to the corrinoid (27). The *numbers* in the *right margin* refers to the specific amino acids in the full-length protein, including signal peptides. The full-length alignments are listed in supplemental Fig. S1.

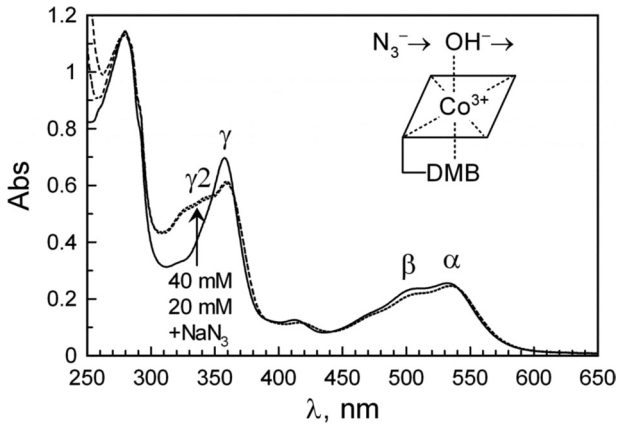

FIGURE 4. **Absorbance spectra of cobalamin bound to the purified trout cobalamin-binding protein.** Spectra of hydroxo- and azido-cobalamin in complexes with the purified trout cobalamin-binding protein are indicated by *solid* and *dashed lines*, respectively. The original complex of hydroxo-cobalamin (24.3 μM) was exposed to 20 and 40 mM NaN_3 (0.2 M phosphate buffer, pH 7.5, 22 °C), which showed virtually no difference. Position of the major γ (γ2) peak is indicated. Two smaller peaks α and β are situated at longer wavelengths. The scheme depicts substitution of water at the upper surface of cobalamin by the external azide anion.

protein concentration in Fig. 4 (deduced from the absorbance coefficient of IF and HC).

Addition of sodium azide converted cobalamin to its azido-form in a few seconds, which points to a nearly free access of substituting ligands to the upper surface of the corrin ring. The record for azido-cobalamin complex (Fig. 4) displayed a γ-peak at 358 nm with some hint of a γ2-peak at 342 nm. This pattern was also seen for azido-cobalamin when bound to human TC and IF (28). On the contrary, azido-cobalamin in complexes with human HC and bovine TC showed a remarkable γ2-peak (28, 29), whose amplitude exceeded the "typical" γ-peak at 350–370 nm.

Stability of Trout Cobalamin-binding Protein toward Enzymatic Degradation and Low pH—We investigated the purified trout cobalamin-binding protein and human recombinant HC, IF, and TC concerning their resistance to proteolysis by trypsin and chymotrypsin. The trout cobalamin-binding protein and IF remained intact in terms of cobalamin retention, whereas HC and TC were easily degraded (as judged by liberated cobalamin) (Fig. 5, *A* and *B*). The trout cobalamin-binding protein, like HC, bound cobalamin equally well at pH 2 and pH 7 (Fig. 5*C*), although IF and TC had no binding capacity at pH 2.

Immunohistochemical Localization of Trout Cobalamin-binding Protein—We used a monoclonal antibody raised from trout cobalamin-binding protein purified from roe fluid. The antibody efficiently bound and precipitated $UB_{12}BC$ present not only in roe fluid but also in plasma, as well as extracts of roe and stomach (Fig. 6). This strongly supports our conjecture that the same cobalamin-binding protein is present in all of these sources.

The expression of trout cobalamin-binding protein was examined by immunohistochemistry of the tissues from stomach, intestine, heart, liver, kidney, pancreas, skin, gills, and roe sac containing roe and roe fluid in lamellae. Cobalamin-binding protein stained positive in exocrine cells of the glands in the stomach, plasma, roe, and in the roe fluid of the roe sac (Fig. 7), which supports the findings in tissue protein extracts. No immunoreactivity was detected in any of the other tissues investigated apart from the blood vessels where plasma stained strongly.

DISCUSSION

We have investigated the soluble cobalamin-binding proteins from rainbow trout (*O. mykiss*), and we report that the trout contains only one cobalamin-binding protein. This con-

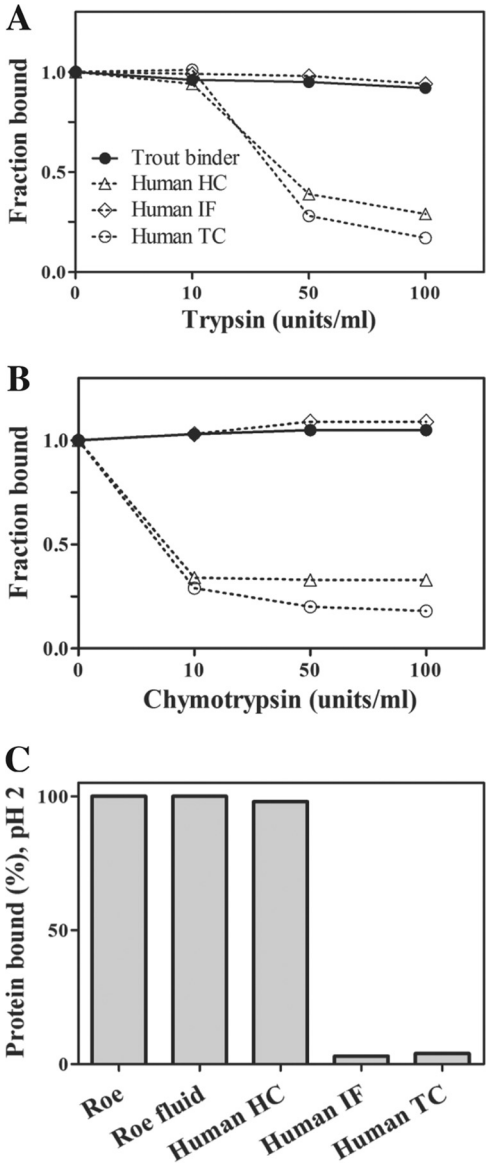

FIGURE 5. **Sensitivity of the trout cobalamin-binding protein toward degradation with trypsin, chymotrypsin, and low gastric pH.** Purified trout cobalamin-binding protein and recombinant human HC, IF, and TC were incubated with [^{57}Co]cobalamin for 30 min before addition of increasing concentrations of trypsin (*A*) and chymotrypsin (*B*) and incubation for 18 h at 37 °C. Protein-bound [^{57}Co]cobalamin was measured after removal of free cobalamin by charcoal precipitation, and the amount of [^{57}Co]cobalamin bound was expressed relative to the amount bound when only [^{57}Co]cobalamin was present. *C,* stability of the purified trout cobalamin-binding protein toward low gastric pH was determined by incubation of [^{57}Co]cobalamin-bound protein for 2 h at pH 2. The percentage of [^{57}Co]cobalamin bound at pH 2 in relation to [^{57}Co]cobalamin bound at pH 7 was calculated.

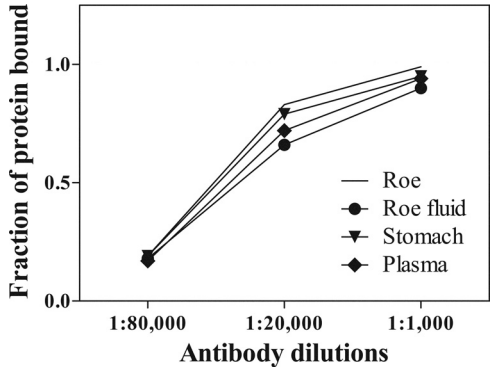

FIGURE 6. **Antibody precipitation of cobalamin-binding protein in trout tissues.** Monoclonal antibody raised toward the trout cobalamin-binding protein purified from roe fluid was used to precipitate $UB_{12}BC$ in roe, roe fluid, stomach, and plasma. The fraction of [^{57}Co]cobalamin precipitated with different antibody dilutions in relation to [^{57}Co]cobalamin precipitated when no antibody was present was calculated.

clusion is based on the findings that the cobalamin-binding proteins from different body fluids and tissues of trout display similar characteristics, that all are precipitated by a monoclonal antibody raised against the purified protein from roe fluid, and that the protein isolated from roe fluid shows the same sequence as translated RNA from muscles. Because this protein showed features comparable with mammalian HC, IF, and TC, we propose the name trout HIT for this new cobalamin-binding protein.

Trout HIT was present in fluids and tissues that in humans express one or the other of the cobalamin-binding proteins, notably in the stomach, the production site for human IF, and in plasma, where human TC transports the vitamin from the gut and into all the cells of the body. Surprisingly, the absolute concentration of trout HIT in plasma is 2–3 orders of magnitude higher than all previously published values for mammalian TC (30). We have no explanation to offer for this observation. Interestingly, trout HIT was also found in high concentrations in roe and roe fluid. Its physiological role in these tissues remains to be clarified.

Trout HIT was glycosylated (like HC and IF) and had a high affinity toward the baseless cobalamin analog cobinamide (like HC). The purified protein was resistant toward degradation by trypsin and chymotrypsin (like IF) as well as pH (like HC).

Spectral properties of trout HIT in complex with hydroxocobalamin clearly distance it from human and bovine TCs, where the cobalt ion is protected by a His residue (29). Substitution of Co^{3+}-coordinated water by azide at the upper face of cobalamin in the complex with the trout HIT revealed no γ2-peak (a shift observed in human/rabbit HCs and bovine TC). All in all, the spectrum of trout HIT resembled a structural hybrid of HC and IF in higher vertebrates.

The presence of just one soluble cobalamin-binding protein in trout is in accord with our previous study on zebrafish (7) and the database prediction for salmon. Trout HIT shares sequence identity with the zebrafish HIT protein (58.2%) and even more with its close relative, the salmon (91.3%). In the database, the salmon cobalamin-binding protein is called a TC-like protein based on its high sequence identity to TC, but based on our results, we find it more likely that it is an intermediate of HC, IF, and TC. Although the proteins of trout, salmon and zebrafish have the highest overall amino acid identity to TC, they show a better resemblance to IF in the composition of the conserved amino acids involved in cobalamin binding (27).

The finding of a single cobalamin-binding protein in each fish suggests that the three kindred proteins of higher vertebrates have descended from a common ancestral gene after divergence of the bony fish (Osteichthyes). All three fish cobalamin-binding proteins seem to be intermediates between the mammalian HC, IF, and TC, which recently led us to suggest the name HIT for the zebrafish cobalamin-binding protein (7).

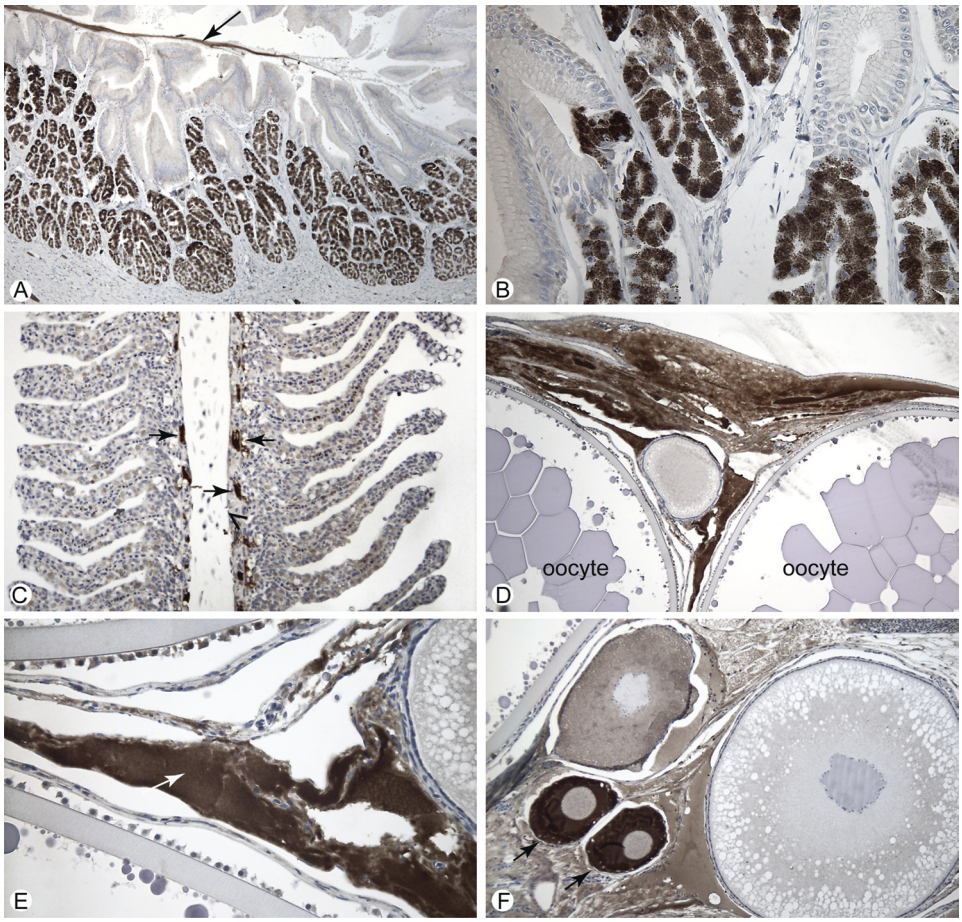

FIGURE 7. **Immunohistochemical localization of the cobalamin-binding protein in trout tissues.** A monoclonal antibody raised against the cobalamin-binding protein purified from roe fluid was employed in a final dilution of 1:1000 for immunohistochemical staining of selected trout organs. *A,* strong and selective immunoreactions localized to the mucosal gastric glands and a little to the luminal secretions (*arrow*). The surface epithelium was completely negative. *B,* larger magnification showing the staining localized to the secretory granules of all cells in the gastric glands. *C,* in all the tissues investigated, blood stained positive for the cobalamin-binding protein, which is shown here by small blood vessels in the gills (*arrows*). *D,* in the roe sac, a strong immunoreaction was observed in the stroma. The immunoreactions were localized both to the lamella and the plasma of the blood vessels. *E,* larger magnification showing immunoreaction in the lamella (*arrow*). *F,* roe (oocytes) stained positive in small immature oocytes (*arrows*) but not in large mature oocytes. Data are shown here by oocytes in different developmental stages and sizes. An inverse relation between size and staining intensity was observed. No cobalamin-binding protein was found in intestine, heart, liver, kidney, pancreas, skin, and gills (data not shown). Magnifications are as follows: *A, C,* and *D,* ×40; *B* and *E,* ×160; *F,* ×100.

Interestingly, the cobalamin-binding protein of trout and salmon does not contain the alkaline residues Lys-159 or Arg-323 necessary for the binding of the IF-cobalamin complex to cubilin in the intestine. This suggests that other receptors are involved in the intestinal cobalamin uptake in these fishes.

Our study also presents data on the cobalamin content of trout tissues. Interestingly, the kidney contained a high level of cobalamin as compared with the liver. This is in opposition to findings in humans (31), but in agreement with the distribution in rodents (32). Cobalamin in the kidney and liver is unlikely to be attached to HIT, because this protein could not be identified in the two organs by immunohistochemistry. We observed $UB_{12}BC$ in both tissues, and because of that we conclude that all of the cobalamin was protein-bound. We do not know the nature of these cobalamin-binding proteins, but we find it likely that it is the cobalamin-dependent enzymes, 5-methyltetrahydrofolate-homocysteine methyltransferase and methylmalonyl coenzyme A, present not only in mammals but also in zebrafish and other vertebrates (7).

In conclusion, we report that one soluble extracellular cobalamin-binding protein is present in the rainbow trout (*O. mykiss*). Further studies are needed to clarify its role for the uptake and distribution of cobalamin in trout.

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