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Sequence, S–S Bridges, and Spectra of Bovine Transcobalamin Expressed in *Pichia pastoris**

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Transcobalamin (TC) -encoding cDNA was isolated from a bovine mammary gland cDNA library. Hybridization of the cloned bovine TC-cDNA to RNA samples from bovine tissues showed that the most intensive synthesis of a TC positive 1.9-kilobase mRNA occurred in kidney, lymphatic nodes, and liver. Bovine TC was expressed in yeast *Pichia pastoris*, and the isolated recombinant protein showed cobalamin (Cbl) and receptor binding properties similar to TCs from other sources. Alignment of the related Cbl carriers (haptocorrins and intrinsic factors from other species) with bovine TC (414 residues) revealed four conservative clusters in the sequence (85–98, 137–147, 178–190, and 268–288), which may be responsible for Cbl binding. Three S–S bonds connected Cys residues 3–252, 98–294, and 147–190. Treatment with an S–S reducing agent caused liberation of Cbl from TC-Cbl. A significant change was observed in the TC-Cbl absorbance spectrum upon substitution of Co^{2+}-coordinated H_2O by azide. The reaction developed several orders of magnitude slower, and the spectral distortions were much stronger than those in free Cbl. This may be caused by significant deformation of the Cbl molecule and/or by its shielding when bound to TC.

Transcobalamin (TC)[1] is one of three proteins (TC, intrinsic factor (IF), and haptocorrin (HC)) involved in transportation and internalization of vitamin B_{12} (cobalamin, Cbl) in an organism (1–4). The vitamin, released from food in the intestine, binds to IF and enters the body by a receptor-mediated mechanism (3, 4). The IF-Cbl complex is degraded in lysosomes of epithelial cells of the intestine whereupon the vitamin enters portal blood associated with TC. This carrier delivers Cbl to different tissues where it binds to one or more specific receptors present in the cell membrane (2, 4). A significant amount of Cbl circulates in blood bound to HC (1, 4). The functional importance of this carrier is questionable because inherited HC deficiency does not provoke any visible pathological effect (5) in contrast to the cases of IF- and TC-deficiencies (6, 7). High amounts of apoHC (10–50 nM) are present in many body fluids like saliva, tears, and milk (8, 9). However, only holo-TC has been found in bovine milk (10).

Comparison of these proteins shows some common and some specific features. All of them have a protein core of approximately 46 kDa, which is heavily glycosylated for IF and HC but not for TC (1–4). The affinity toward Cbl is supposed to be highest for HC (11, 12), though the dissociation constants determined for Cbl binders by different methods vary considerably (1, 4, 11, 12). The selectivity for the "true" Cbl under competition with Cbl analogues declines in the range IF > TC > HC (11, 14). The Cbl-binding and receptor-binding sites are likely to be spatially separated (15, 16).

Purification of Cbl carriers is complicated by low concentration of these proteins in natural sources (1–4). Isolation of 1 mg of TC requires, for instance, 150–300 liters of human plasma (17, 18) or 100 liters of bovine milk (10). The available amount of pure TC is insufficient for investigations requiring high protein quantities. An expression system has been established for IF and TC in insect cells (19, 20), and the recombinant proteins were obtained at the level of 10–100 μg.

We have expressed and purified recombinant bovine TC from transformed yeast *Pichia pastoris*. The biological activity of the recombinant TC was confirmed by its binding to Cbl as well as to the specific receptor in the membranes of placenta. The protein sequence and the location of the intramolecular S–S bridges have been established. Spectral properties of TC-Cbl with different Co^{2+}-coordinated groups pointed to significant deformations in the Cbl molecule and/or to its shielding from the environment when bound to TC.

EXPERIMENTAL PROCEDURES

Materials

All salts and materials for media were purchased from Merck, Roche Molecular Biochemicals, Sigma, and Difco. The enzymes and kits for DNA handling were obtained from New England Biolabs, Stratagene, and Roche Molecular Biochemicals, and the kit for polymerase chain reaction was from HT Biotechnology LTD. Membranes for screening were from Amersham Pharmacia Biotech and Schleicher & Schuell. Oligonucleotides were synthesized by DNA technology. The yeast expression system was purchased from Invitrogen. The affinity matrix ProBond and anti-Myc antibody (Invitrogen) as well as Fast Flow CM-Sepharose and Sephacryl S-200 (Amersham Pharmacia Biotech) have been used during the protein purification.

Methods

Purification of TC and Sequence of Its Peptides—TC was isolated from bovine milk according to the method described earlier (10). 100 μg of protein was treated with 2 μg of trypsin in 100 μl of 0.04 M NH_3HCO_3 at 37 °C for 4 h. The peptides in 200 μl of 0.5% heptafluorobutyric acid were applied to an HPLC column and separated by elution with a linear gradient of 80% acetonitrile in 0.5% heptafluorobutyric acid. Sequence of the peptides was performed on Protein Sequencer 477A (Perkin-Elmer). Purification of rabbit TC was carried out according to the method described elsewhere (21).

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF121289.

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[1] The abbreviations used are: TC, transcobalamin; Cbl, cobalamin; Cbl*, [^{57}Co]cyanocobalamin; HC, haptocorrin; IF, intrinsic factor; P_i buffer, NaH_2PO_4/Na_2HPO_4 buffer; HPLC, high pressure liquid chromatography; Cbl-OH_2, Cbl-CN, Cbl-N_3, aquo-, cyano-, azidocobalamin; mHX-TC, fusion protein composed of Myc site, His_6 peptide, recognition site for factor X_a, and mature TC.

Signal peptide:

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ATGGGGCACCTCGGGGCCCTCCTCTTCCTGCTGGGGGGCCTGGGAGCGCTAGCC
M  G  H  L  G  A  L  L  F  L  L  G  G  L  G  A  L  A
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Mature bovine transcobalamin:

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AACATCTGCGAGATAACCGAGGTGGACAGCACGCTGGTGGAGAGGCTGGGCCAGCGCCTCTTGCCCTGGATGGAC
N  I  C* E  I  T  E  V  D  S  T  L  V  E  R  L  G  Q  R  L  L  P  W  M  D      25

CGGCTCTCCCAGGAGCAGCTGAACCCCAGTATCTACGTGGGCCTGCGCCTCTCGAGCCTGCAGGCTGGGGCCAAG
R  L  S  Q  E  Q  L  N  P  S  I  Y  V  G  L  R  L  S  S  L  Q  A  G  A  K      50

GAGGCCCACTACCTGCACAGCCTCAAGCTCAGCTACCAGCAGAGCCTCCTGAGGCCTGCCTCCAACAAGGATGAC
E  A  H  Y  L  H  S  L  K  L  S  Y  Q  Q  S  L  L  R  P  A  S  N  K  D  D      75

AATGACTCCGAGGCCAAGCCCTCTATGGGCCAGCTGGCCCTCTACCTGCTGGCTCTCCGGGCCAACTGCGAGTTC
N‡ D  S  E  A  K  P  S  M  G* Q  L* A  L* Y  L  L  A  L  R  A  N  C* E  F     100

ATCGGAGGCCGCAAGGGGGACAGGCTGGTCTCCCAGCTGAAGCGGTTCCTGGAGGACGAGAAGAGGGCCATCGGG
I  G  G  R  K  G  D  R  L  V  S  Q  L  K  R  F  L  E  D  E  K  R  A  I  G     125

CACAACCACCAGGGTCACCCCCGCACCAGCTACTACCAGTACAGCCTGGGCATCCTGGCCCTGTGTGTCCACCAG
H  N  H  Q  G  H  P  R  T  S  Y  Y* Q  Y  S  L  G  I  L* A  L* C* V  H  Q     150

AAGCGAGTCCACGACAGTGTGGTGGGCAAGCTCCTGTACGCCGTGGAACACAAGCCGCATCTCCTGCAGGACCAC
K  R  V  H  D  S  V  V  G  K  L  L  Y  A  V  E  H  K  P  H  L  L  Q  D  H     175

GTCTCTGTGGACACCATGGCCATGGCAGGCATGGCCTTCTCCTGTCTGGAGCTGTCCAACCTCAACCCCAAGCAG
V  S  V* D* T* M  A* M  A* G  M  A  F  S  C* L  E  L  S  N  L  N  P  K  Q     200

AGAAACCGGATCAACCTGGCCCTCAAGAGAGTGCAAGAGAAGATCCTGAAGGCCCAGACCCCAGAGGGCTACTTC
R  N  R  I  N  L  A  L  K  R  V  Q  E  K  I* L  K  A  Q  T  P  E  G  Y  F     225

GGGAATGTCTACAGCACCCCTCTGGCTTTGCAGTTGCTGATGGGCTCCCTCAGGCCCTCGGTGGAGCTGGGCACA
G  N  V  Y  S  T  P  L  A  L  Q  L  L* M  G  S  L  R  P  S  V  E  L  G  T     250

GCCTGCCTTAAAGCCAAGGCTGCTCTGCAGGCCAGCCTACAGCACAAGACCTTCCAGAACCCTCTCATGATCTCT
A  C  L  K  A  K  A  A  L  Q  A  S  L  Q  H  K  T  F* Q  N  P  L  M  I  S     275

CAGCTGCTGCCTGTCCTGAACCAGAAGAGCTATGTGGATCTCATCTCCCCAGACTGCCAGGCTCCAAGAGCCCTG
Q* L  L* P* V  L* N  K* S  Y  V  D* L  I  S  P  D  C  Q  A  P  R  A  Q  L     300

TTGGAACCGGCTCTGGAGACCCCGCCACAGGCCAAAGTCCCGAAGTTCATTGACGTCTTGCTGAAGGTCTCCGGC
L  E  P  A  L  E  T  P  P  Q  A  K  V  P  K  F  I  D  V  L  L  K  V  S  G     325

ATCTCCCCTTCATACAGACACTCTGTCTCTGTCCCTGCTGGCTCCTCCCTGGAAGACATCCTGAAGAACGCCCAG
I  S  P  S  Y  R  H  S  V  S  V  P  A  G  S  S  L  E  D  I  L  K  N  A* Q     350

GAGCATGGAAGATTCAGGTTTAGAACACAGGCCTCCCTGTCTGGCCCCTTCCTGACCTCCGTGCTGGGGAGAAAG
E  H  G  R  F  R  F  R  T  Q  A  S  L  S  G* P  F  L  T  S  V  L  G  R  K     375

GCTGGGGAACGTGAGTTCTGGCAGGTCCTCCGAGATCCTGACACCCCCTTGCAGCAAGGTATTGCTGACTACAGA
A  G  E  R  E  F  W* Q  V  L  R  D  P  D  T  P  L  Q  Q  G  I  A  D  Y  R     400

CCCAAGGATGGAGAGACCATCGAGCTGAGGCTGGTTGGCTGGTAG
P  K  D  G  E* T  I  E  L  R  L  V  G  W  STOP                                 414
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FIG. 1. **Nucleotide and amino acid sequences of bovine TC.** The conservative residues (*) identical in all Cbl-transporting proteins were established according to alignment of bovine TC with several Cbl binders: human TC (32, 33), human HC (34), porcine HC (35), human IF (36), rat IF (37), and mouse IF (38). Four distinct clusters of high similarity (30–50%) were detected: 85–98 (29%), 137–147 (40%), 178–190 (54%), and 268–288 (35%). Two regions of low similarity are present between residues 10–80 and 290–340. The potential glycosylation site (. . . N^{76} . . .) is shown as a symbol (‡). The sequence was submitted to the GenBank™ Database with accession number AF121289.

Preparation of Oligonucleotides and DNA Probes—TC-specific oligonucleotides (20-mers) were synthesized on the basis of the amino acid sequence of the bovine TC fragments: NI(C)EITE, (C)VHQKRV, GYFGNVY, KSYVDLI, and EDILKNA. Human TC-cDNA was kindly provided by Dr. E. V. Quadros. The sequence of oligonucleotides for β-actin (a control of RNA quantity on a Northern blot) was deduced from comparison of the conservative stretches in cDNAs of different species: CAACGGCTCCGGCATGTGCA, CCGGCTTCGCGGGCGACGAT, AAGATGACCCAGATCATGTT, and GATGATGATATCGCCGCGCTCGTCGTC.

Isolation of TC-encoding Clones and DNA Sequence—A mammary gland cDNA library cloned in vector λgt11 (CLONTECH) was plated and screened with a ^{32}P-labeled human TC cDNA probe according to the standard procedure (22). Positive clones were propagated and then rescreened with ^{32}P-labeled bovine TC oligonucleotides corresponding to different stretches in the sequence. It resulted in identification and

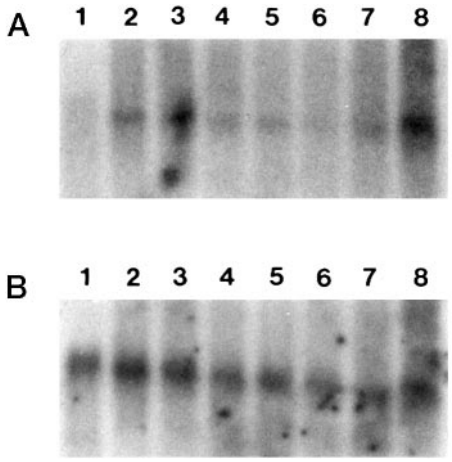

FIG. 2. **Northern blot analysis of the total RNA isolated from different bovine tissues.** *Lane 1*, mammary glands; *lanes 2,* and *3*, lymphatic nodes; *lane 4,* spleen; *lane 5,* intestine; *lane 6,* lung; *lane 7,* liver; and *lane 8*, kidney. *A,* the samples were hybridized to a ^{32}P-labeled probe for bovine TC-cDNA. *B,* the samples were hybridized to a ^{32}P-labeled probe for β-actin. Hybridization was carried out with the membrane from *A* stripped beforehand from the TC-specific probe.

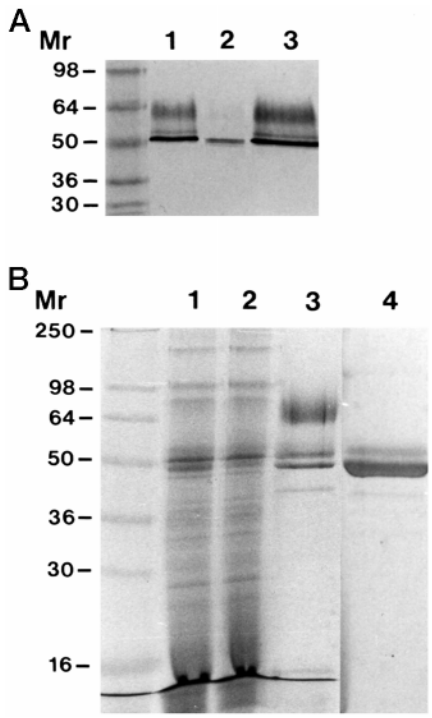

FIG. 3. **Electrophoretic analysis on different stages of purification of the recombinant bovine TC.** *A,* detection of the recombinant fusion protein mHX-TC on Western blot by anti-Myc antibody. *Lane 1,* 20 μl of the cell supernatant concentrated after ammonium sulfate precipitation; *lane 2,* 1 μg of the preparation obtained after His^6-specific purification; *lane 3,* 1 μg of the TC preparation purified on CM-Sepharose. *B,* Coomassie Blue-stained SDS-electrophoresis. *Lane 1,* 20 μl of the concentrated cell supernatant; *lane 2,* 20 μl of the same supernatant passed through CM-Sepharose; *lane 3,* 3 μg of the TC-containing fraction obtained after CM-Sepharose purification of the cell supernatant. The N-terminal sequences of the major 50 kDa and 46 kDa proteins corresponded to mHX-TC and TC, respectively. *Lane 4,* 6 μg of the final preparation of the recombinant bovine TC. The N-terminal sequence of the 46-kDa protein revealed an equimolar mixture of IEGR-TC and TC.

isolation of two cDNA clones with long overlapping TC fragments. They were subcloned into the *Eco*RI site of the Bluescript KS-II plasmid (Stratagene) and sequenced on ABI-Prism Genetic Analyzer (Perkin-Elmer).

Northern Blot of RNA from Bovine Tissues—Total RNA was isolated from different bovine tissues by ultracentrifugation in CsCl and blotted on a nitrocellulose membrane according to the standard recommendations (22). Hybridization was performed with a ^{32}P-labeled bovine TC cDNA probe and the probe for β-actin. The bands were visualized after exposure with intensifying screen for 2 weeks at −70 °C.

Construction of the Expression Vector—The cDNA encoding the mature TC was amplified by polymerase chain reaction from pBS-TC construction using the primers with *Not*I and *Spe*I adaptors. The polymerase chain reaction product was ligated at *Not*I and *Spe*I sites to the expression plasmid pPICZα-LB. It was derived in our laboratory from pPICαA (Invitrogen) by introduction of the Myc sequence, a His tag, and a factor X_a cleavage site before the protein sequence, for more details see "Results."

Expression of the Recombinant Bovine TC in Yeast P. pastoris—The expression scheme followed the general recommendations of the manufacturer (Invitrogen). The verified pPIC-TC construction was linearized inside the alcohol oxidase 1 promoter by treatment with endonuclease *Nsi*I. The methanol-metabolizing Mut$^+$ strain SMD 1168 of *P. pastoris* was transformed by electroporation and plated. The cell line with the highest level of TC synthesis was established during trial expressions.

The recombinant yeast was grown for 2 days in 1 liter of buffered glycerol complex medium supplemented with 1% casamino acids and 0.5 μM aqua Cbl. Then, the cells were pelleted and resuspended in 1 liter of induction medium (buffered methanol complex medium) containing the same additives and 1% methanol. The growth was continued for 24 h. Afterward, the suspension was centrifuged, and the cell-free supernatant was used for isolation of the recombinant bovine TC.

Purification of the Recombinant Bovine TC—Proteins in the obtained supernatant were precipitated by ammonium sulfate at 70% of saturation. The pellet was dissolved in 20 ml of 0.02 M P_i buffer, pH 7.5, and the protein solution was dialyzed against 3 liters of the same buffer overnight at 5 °C. Then, TC was adsorbed on 2 ml of CM-Sepharose at room temperature and washed in the column by increasing concentrations of P_i buffer: 0.02 M, 0.05 M, 0.1 M (pH 7.5). The protein was eluted with 2 ml of 0.5 M P_i buffer, pH 7.5, and the collected sample contained approximately 1 mg of the fusion protein mHX-TC. The sample was treated with 30 μg of factor X_a for 30 h at 37 °C to remove the service peptides from the N terminus. The preparation was applied to a 250-ml Sephacryl S-200 column equilibrated with 0.1 M Tris, 1 M NaCl, pH 8.0, at room temperature. The eluted fractions with maximal specific Cbl adsorption at 362 nm were pooled, dialyzed against 0.02 M P_i buffer, pH 7.5, and concentrated by adsorption on a small CM-Sepharose column (0.3 ml) as described above. The final preparation was stored frozen.

Spectral Measurements—The spectra of TC-Cbl were recorded on a M350 double beam spectrophotometer (Camspec). The TC concentration was measured at 205 nm according to the peptide bond adsorption (23) and used for estimation of an approximate extinction coefficient of TC-Cbl-OH_2 at 362 nm: E_{362}(mg/ml) ≈ 0.65 (ϵ_{362} ≈ 30,000 M^{-1} cm^{-1}).

Substitution of the Co^{2+}-coordinated β-group (H_2O) in Cbl-OH_2 and TC-Cbl-OH_2 by azide was followed according to the spectral changes developing at 37 °C in 0.5 M P_i buffer, pH 7.5, in the presence of different concentrations of sodium azide. The amplitudes ($A_{363} - A_{330}$) were plotted *versus* time to calculate the maximal values at $t \rightarrow \infty$.

Substitution of β-H_2O in TC-Cbl-OH_2 by cyanide was carried out for 5 h under analogous conditions in the presence of 10 mM KCN. The excess of KCN was removed by dialysis.

Determination of Endogenous Cbl and Cbl Binding Capacity—The amount of TC-bound Cbl was measured either by the extinction coefficient ϵ_{362} ≈ 30,000 M^{-1} cm^{-1} (see "Spectral Measurements") or by the isotope dilution method (24) employing human IF as the binding protein.

Concentration of apoTC was determined by incubation with an excess of [^{57}Co]Cbl followed by adsorption of unbound radioactive ligand on hemoglobin-coated charcoal (25). The assay was performed in an albumin solution containing 1 M NaCl to prevent artificial adsorption of bovine TC-[^{57}Co]Cbl on charcoal.

Determination of the Rate Constant for TC-Cbl Dissociation—Recombinant bovine TC was depleted of endogenous Cbl by dialysis against 8 M urea as described earlier (10, 13), and Cbl binding capacity in the obtained preparation was measured. The preparation of apoTC (1 nM) was exposed to 0.9 nM [^{57}Co]Cbl in the incubation solution (0.1 M P_i buffer, pH 8.0, 1 M NaCl, 1 mg/ml human serum albumin) for 1 h at room temperature. Afterward, the sample was diluted 1:1 with the same buffer (with or without 1 μM unlabeled cyano-Cbl) and incubated at 37 °C for 30 h. The remaining protein-associated [^{57}Co]Cbl was measured as a function of time.

Binding of Bovine TC-Cbl to the TC Receptor in Human Placenta—

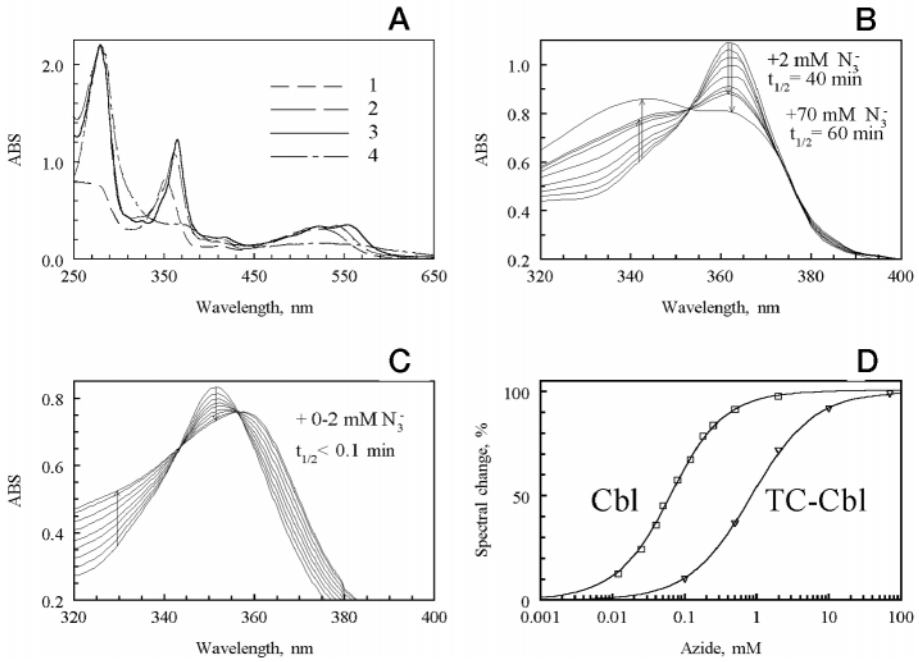

FIG. 4. **Spectral properties of the recombinant bovine TC (37 °C, 0.5 M P_i buffer, pH 7.5.).** *A,* absorbance spectra of Cbl-OH_2 (*spectrum 1*), TC-Cbl-OH_2 (*spectrum 2*), TC-Cbl-CN (*spectrum 3*), TC-Cbl-CN treated with dithiothreitol and dialyzed (*spectrum 4*), all ≈ 36 μM. *B,* spectral response of TC-Cbl-OH_2 (36 μM) to the added azide. Spectra at 2 mM azide were recorded after 5, 15, 30, 60, 120, 180, and 240 min of incubation. The presented spectrum at 70 mM azide was recorded after 360 min of incubation, when the reaction was practically accomplished. *C,* changes in the spectrum of Cbl-OH_2 (36 μM) in response to azide added at the following concentrations: 0, 12, 25, 40, 50, 80, 120, 180, 250, 500, and 2000 μM. There was no difference between the records after 1 and 60 min of incubation at the same N_3^-. *D,* relative spectral changes obtained for Cbl and TC-Cbl at different azide concentrations. The apparent K_d was 0.046 mM for Cbl-N_3^- and 0.84 mM for TC-Cbl-N_3^-.

The radioactive tracer, containing partially purified human TC-[^{57}Co]Cbl, was displaced from the receptor in the membranes of human placenta by different concentrations of bovine and rabbit TC-Cbl as described elsewhere (26).

Determination of S–S Bonds in the Recombinant Bovine TC—In the initial step, 0.5 mg of the protein in 1 ml was dialyzed against 100 ml of 8 M urea, 0.2 M Tris, pH 8, at room temperature for 2 days with one change to remove the endogenous Cbl. Radioactive iodoacetate was added for determination of possible free SH groups, and the mixture was incubated for 2 h. Urea and iodoacetate were removed by dialysis against 1 liter of 0.1 M ammonium bicarbonate, pH 8, for 48 h with one change. The final dialyzing buffer was 0.1 M acetate-pyridine, pH 6.5, whereupon the denatured TC (expected to retain the original pattern of S–S bridges) was subjected to digestion with 60 μg of thermolysin (50 °C, 4 h). The peptide mixture was separated by HPLC, and the fractions containing disulfide bonds were identified by amino acid analysis after performic acid oxidation. The detected fractions were additionally purified by HPLC, sequenced on Protein Sequencer 477A (Perkin-Elmer), and analyzed by matrix-assisted laser desorption ionization/time of flight mass spectrometry on Biflex™ (Bruker-Frauzer Analytik) (see Ref. 27 for more details).

Treatment of holo-TC with Dithiothreitol—TC-bound Cbl was converted to its cyano form (see "Spectral Measurements") to prevent coordination of dithiothrietol in the form of R–S^- to Co^{2+}. Then, the sample was treated with 10 mM dithiothreitol for 3 h at 37 °C and dialyzed for 24 h at room temperature against 0.5 M P_i buffer, pH 7.5, with 5 mM dithiothreitol. Analogous procedure was carried out with the control sample where the additive was H_2O. Absorbance spectra were recorded after dialysis in both preparations.

RESULTS

Cloning of Bovine TC and Analysis of Its Sequence—The cDNA of TC was cloned from a bovine mammary gland cDNA library and the deduced amino acid sequence of bovine TC is depicted in Fig. 1. The sequence showed 71% identity with human TC, whereas similarity with HC and IF from different sources varied in the range 21–25%. Bovine TC was characterized by a positive net charge +10 because of a high number of Arg and Lys residues. The net charge of human TC was, on the contrary, negative (−6). Peptides, obtained after trypsin cleavage of the native TC-Cbl from bovine milk, showed sequences identical to those predicted by cDNA. High recovery of the peptides was observed for the protein domains 16–41 and 285–347.

The Relative Level of TC in Different Bovine Tissues—Northern blot analysis was performed using both the TC-specific and β-actin radioactive probes (Fig. 2). The highest expression of TC-mRNA (≈1.9 kilobases) was associated with bovine kidney followed by lymphatic nodes and the liver, whereas bovine mammary gland contained a relatively low amount of TC-mRNA.

Expression and Purification of Bovine TC—Bovine TC was expressed as a fusion protein mHX-TC and was detected by Western blot employing an anti-Myc antibody (Fig. 3*A*). No TC expression was found when the incubation media did not contain Cbl (data not shown). Coomassie Blue staining of the crude cell-free supernatant after SDS-electrophoresis (Fig. 3*B*, *lane 1*) revealed two major bands of 50 and 46 kDa, which were present in the partially purified CM-Sepharose fractions as well (Fig. 3*B*, *lane 3*). N-terminal sequences showed that the 50-kDa protein corresponded to the immunoreactive form mHX-TC as detected by anti-Myc antibody and the 46-kDa band corresponded to the mature TC. The 50-kDa protein was still visible in the cell-free supernatant passed through CM-Sepharose (Fig. 3*B*, *lane 2*).

The final product was obtained after cleavage of mHX fragment by factor X_a, gel filtration on Sephacryl S-200, and concentration on CM-Sepharose. The preparation consisted mainly of a 46-kDa TC (Fig. 3*B*, *lane 4*). Still, the N-terminal sequence revealed the presence of two subforms: NI-(C)EITEVD . . . and IEGRNI(C)EI . . . (the TC residues are underlined). The yield of the recombinant TC corresponded to 0.5–1 mg from 1 liter of the incubation medium.

Verification of Structural and Functional Properties of the Recombinant TC—The absorbance spectra showed that the γ-peak in the free aqua Cbl (352 nm) shifted to longer wavelength (362 nm) and intensified after binding to TC (Fig. 4*A*). Conversion of TC-bound Cbl to its cyano form caused additional amplification of the γ-peak. The same effects were observed earlier for human TC-Cbl (18) and rabbit TC-Cbl (21). The preparation contained 1 mol of the endogenous Cbl/mol of the protein as judged from the absorbance ratio, $A_{280}/A_{362} \approx 2$, attributed to the saturated Cbl binders (17, 18, 21, 23). The stability of the TC-Cbl complex was analyzed according to the rate constant of its decomposition. A slow exponential decay of TC-Cbl* (Fig. 5*A*) was observed with the rate constant $k \approx 1.6 \times 10^{-4}$ min^{-1} at 37 °C. The recombinant bovine TC, saturated with Cbl, was able to bind to the specific TC receptor in membranes of human placenta (2, 4, 24) with the same effi-

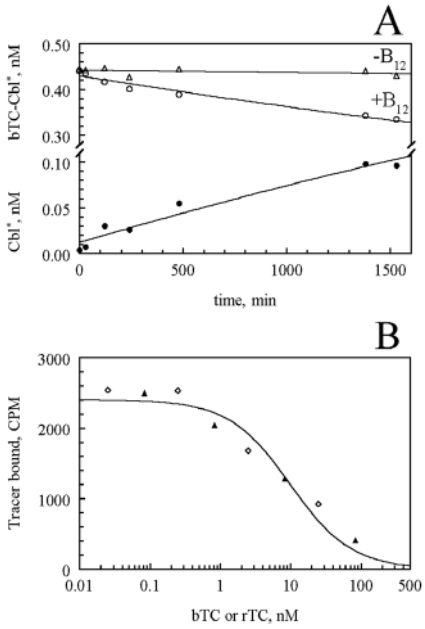

FIG. 5. **Ligand and receptor binding properties of the recombinant bovine TC.** *A,* liberation of the radioactive ligand (Cbl*) from TC-Cbl* at 37 °C. Remaining concentration of TC-Cbl* was followed in time in the presence of a 100-fold excess of the unlabeled vitamin B_{12} (○) or without additives (△). Subtraction of the first chart from the second reveals appearance of free Cbl* in the medium (●). The dependence of TC-Cbl* and Cbl* on time was fitted by equations: $0.43 \times \exp(-0.00017t)$ and $0.12 + 0.44 \times (1 - \exp(-0.000155t))$, respectively. The rate constant of Cbl* liberation can be estimated as $k \approx 1.6 \times 10^{-4}$ min^{-1}. *B,* binding of the recombinant bovine TC-Cbl (▲) and rabbit TC-Cbl (◇) to the TC-specific receptor in the membranes of human placenta. The assayed proteins displaced the radioactive tracer from the receptor at increasing concentrations of bovine TC-Cbl (*bTC*) and rabbit TC-Cbl (*rTC*).

ciency as TC-Cbl purified from rabbit plasma (Fig. 5*B*).

Determination of S–S Bridges—No free SH groups were detected in the denaturated TC as judged from the lack of an incorporated $HOOCCH_2$ group after treatment with iodoacetate. Three pairs of the peptides connected with S–S bonds were obtained after separation of thermolysin-digested TC by HPLC. Analysis by mass spectrometry and amino acid sequence identified these peptides as: 1) NI(C) and LGTA(C), 2) FS(C) and L(C), and 3) LRAN(C)EF and ISPD(C)QA. The location of the S–S bridges along the TC sequence is shown in Fig. 6.

A number of peptides were purified after treatment of the native TC isolated from bovine milk with trypsin. The following Cys-containing fragments co-eluted during HPLC: 1–15 and 218–254, 96–104 and 285–298, and 134–160 and 161–199. It indirectly testifies that they are bound together, and the pattern of S–S bridges in the native TC is the same as in the recombinant TC.

Intact S–S bonds are required for retention of Cbl in the TC-Cbl complex. Dialysis of TC-Cbl-CN against dithiothreitol-containing buffer was accompanied by the removal of the endogenous Cbl, Fig. 4*A*.

Spectral Studies of the β-group Exchange in the Free and TC-bound Cbl—We investigated the exchange of the original Co^{2+}-coordinated β-group (H_2O) with an external ligand in TC-Cbl. Sodium azide was chosen as a possible probe for the "base-on" and "base-off" structure of Cbl (29). Significant perturbations in the spectrum of the protein-bound Cbl upon azide coordination, accompanied by the appearance of a γ_2-peak, are believed to be an evidence in favor of the base-off structure of Cbl (28, 30).

Conversion of the TC-Cbl spectrum during substitution of

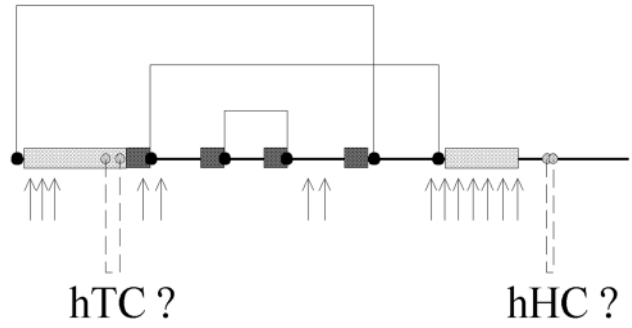

FIG. 6. **S–S bridges (⊓) between Cys residues (*closed circle*) in the molecule of the recombinant bovine TC.** The same motif of the conservative Cys residues was found in human, mouse, and rat IFs, which testifies for analogous organization of their S–S bridges. Human TC and HC have two additional Cys residues (*shaded circle*), which provide a condition for an additional S–S bridge in each protein. Conservative regions are indicated as *dark boxes*. Nonconservative domains are shown as *light boxes*. Stretches particularly susceptible to trypsin are indicated with *arrows* according to the number of cuts.

endogenous β-H_2O by external N_3^- developed exponentially in time (Fig. 4*B*). The amplitude and $t_{1/2}$ of the reaction increased at high concentration of azide: $t_{1/2}$ = 15 min at N_3^- = 0.5 mM, $t_{1/2}$ = 60 min at N_3^- = 10–70 mM (37 °C). Strong expression of the γ_2-peak at 343 nm was achieved at saturating concentrations of azide. The observed process was consistent with the two step scheme where the first stage is shifted to the left ($k_{-1}/k_{+1} > 50$) and is rate-limiting.

$$\text{TC-Cbl-OH}_2 \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} \text{TC-Cbl}' \underset{k_{-2}}{\overset{k_{+2} \times N_3^-}{\rightleftharpoons}} \text{TC-Cbl-N}_3$$

SCHEME 1

The form TC-Cbl′ corresponds to an intermediate capable of azide binding. Increasing concentrations of N_3^- reverse the balance in favor of TC-Cbl-N_3, and it takes a longer time to accomplish equilibration at high N_3^-. The scheme simplifies to TC-Cbl-OH_2 → TC-Cbl-N_3 at saturating concentration of azide when the measured rate coefficient k_{obs} = 0.012 min^{-1} should be close to k_{+1}. The amplitude of the spectral changes (calculated for $t \to \infty$) depended hyperbolically on azide concentration with the apparent dissociation constant K_d = 0.84 mM (Fig. 4*D*).

The analogous experiment was performed with protein-free Cbl-OH_2, which has a predominant base-on structure in water solution (29). The response to the added azide occurred immediately after mixing of the reagents ($t_{1/2} < 0.1$ min) for all concentrations used (Fig. 4*C*). The amplitude changes were much less than those for TC-Cbl, and the half-effect occurred already at $N_3^- \approx 0.06$ mM. It was comparable to concentration of the binding sites, Cbl = 0.036 mM, and required a fit according to the "square root" equation (31) with K_d = 0.046 mM.

DISCUSSION

A TC-cDNA was obtained from a bovine mammary gland cDNA library and used for expression of the recombinant TC in yeast. The choice of the cDNA library was based on our previous observation of a high TC concentration in bovine milk (3 nM) when compared with bovine plasma (0.2 nM) (10). Nevertheless, synthesis of the TC-mRNA in bovine mammary glands was lower than in other tissues, especially kidney, lymphatic nodes, and liver (Fig. 2*A*). This may suggest that TC in bovine milk does not originate from mammary glands but is transported from blood to the milk through the mammary gland epithelium.

Alignment of seven Cbl-binders bovine and human TCs

(present work and Refs. 32 and 33); human and porcine HCs (34, 35); human, mouse, and rat IFs (36–38) revealed positions of the conservative residues identical in all proteins (Fig. 1). The earlier analysis of such a kind (32, 33) resulted in identification of 7 and 6 regions of high similarity according to the alignment of three and four Cbl-binding proteins, respectively. Our data sustain conserved structure of four regions, II, III, IV, and V, according to the nomenclature used by Li *et al.* (33). All these segments are situated in the middle part of the sequence (Fig. 6) and may be responsible for Cbl binding. It is interesting that the nonconservative domains in bovine TC were particularly susceptible to trypsin (Fig. 6), suggesting that these regions are exposed to the external medium.

Recombinant bovine TC was successfully expressed in yeast and the pattern of the disulfide bridges was established (Fig. 6). The organization of S–S bonds in the recombinant bovine TC was identical to that deduced for the natural TC from bovine milk. The six Cys residues, found in bovine TC and involved in S–S bonding, were present in three known IFs (36–38), as well as in human TC (32, 33) and human HC (34). Positions of these residues along the sequences varied insignificantly, and one can assume the pattern of S–S bonds between six of the Cys-residues (Cys^1-Cys^5, Cys^2-Cys^6, Cys^3-Cys^4) to be general for all Cbl binders. The absence of free SH groups in human IF (39) supports the above suggestion. Three S–S bridges between the distant Cys residues embrace the possible sites of Cbl binding and may ensure extraordinary stability of the Cbl-protein complexes observed for TC, IF, and HC (1–4, 11–13). Liberation of the endogenous Cbl from bovine TC in the presence of an S–S reducing compound demonstrates functional importance of the disulfide bonds.

The absorbance spectra of TC-Cbl treated with azide gave some evidence for a significant deformation in the Cbl molecule when bound to bovine TC. Remarkable perturbations in the typical absorbance spectrum of Cbl, evoked by coordination of azide to Co^{2+} in the upper axial position (β), are believed to be associated with substitution of dimethylbenzimidazol in the lower axial position (α) by another ligand (28, 30). A His residue coordinates to Co^{2+} and substitutes dimethylbenzimidazol in many enzymes (29), which is supposed to be important for stabilization and destabilization of the functional β-group during catalysis. Disruption or weakening of the dimethylbenzimidazol–Co^{2+} bond may also take place in bovine TC-Cbl.

The binding of Cbl to bovine TC decreased the mobility of the β-group in the Cbl molecule according to velocities of $H_2O \leftrightarrow$ azide exchange. A similar effect had been observed for another protein HC-Cbl (40, 41). The mechanism of protein-enforced partial immobilization of the β-group is not clear. The Cbl-binding proteins can, for example, poise some "β-stable" conformation of Cbl and restrict its transition to another "β-mobile" structure. One cannot exclude complete encapsulation of Cbl inside the protein molecule when the "open" conformation of TC-Cbl is unfavorable. Both suggestions can be described by Scheme 1, and clarification of this question requires some additional work. Anyway, the physiological role of such a protection is consistent with safe transportation of the active cofactor when circulating in blood bound to TC and HC. Another observation concerns the affinity of different β-groups to the TC-Cbl complex. Thus, the ability of azide to compete with water significantly decreased after the binding of Cbl to TC (Fig. 4*C*), which may suggest Co^{2+}-coordinated H_2O to be more preferable than Co^{2+}-coordinated N_3^-.

In conclusion, we have expressed functional bovine TC in yeast *P. pastoris* and performed structural and functional analysis of the isolated recombinant protein. The established pattern of S–S bridges in bovine TC seems to be general for other known Cbl carriers. Protective influence of TC, imposed on Co^{2+}-coordinated upper group of Cbl, was revealed during $H_2O \leftrightarrow$ azide exchange. The process was accompanied by essential spectral perturbations presumably caused by significant deformations of Cbl bound to bovine TC.

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