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Transcobalamin from cow milk: isolation and physico-chemical properties

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Abstract

The concentration of endogenous cobalamin (Cbl) in cow milk was 3.3 nM while the Cbl-binding capacity was 0.05 nM. Both endogenous and newly added Cbl showed similar quantitative distribution between a 280 kDa protein complex (45%) and a 43 kDa Cbl-binder (55%). Long time incubation, as well as urea treatment, was accompanied by a slow release of the 43 kDa Cbl-binder from the 280 kDa fraction. No other Cbl-binding proteins appeared after these procedures. The 43 kDa binder from cow milk, depleted of the ligand by urea treatment, reacted with Cbl even in the presence of a B_{12}-analogue cobinamide (Cbi) at the ratio Cbl:Cbi = 1:40. The Stokes radius of the binder changed from 2.7 nm for the Cbl-free protein to 2.5 nm for the Cbl-saturated form and the Cbl-saturated binder was able to displace human transcobalamin (TC) from the TC-receptor. The interaction between the protein and Cbl was significantly suppressed at pH 2.0. The N-terminal sequence of the purified 43 kDa Cbl-binder revealed homology with TC from human and rabbit plasma. In conclusion we have shown that TC is the main Cbl-binding protein in cow milk. This is surprising, since previous studies on human and rat milk have shown another Cbl-binder, apo-haptocorrin, to be the dominating Cbl-binding protein.

Keywords: Cobalamin; Cow milk; Isolation; Sequence; Transcobalamin

1. Introduction

Vitamin B_{12} or cobalamin (Cbl) is important as a coenzyme for methylmalonyl-CoA mutase and methionine synthase [1]. The vitamin is synthesized only by microorganisms in different chemical forms classified as 'true' Cbl-forms and analogues [2,3]. The first group includes the forms of Cbl (e.g., cyano-Cbl, hydroxo-Cbl) which can be converted to the coenzymes after their incorporation into animal tissues. The other group includes cobinamide (Cbi) and pseudo-B_{12}, which have no catalytic function, at least for animals. Nevertheless, some of the analogues compete with the coenzymes and suppress Cbl-dependent biochemical reactions [4,5].

The uptake of Cbl from food and its dissemination among animal tissues require the expression of three Cbl-binding proteins: intrinsic factor (IF), transcobalamin (TC) and haptocorrin (HC) [6,7]. Released from the food Cbl binds to gastric IF and the complex IF-Cbl enters the ileal mucosal cells by a receptor mediated mechanism [6–9]. Cbl is transferred inside the enterocytes from IF to TC and TC-Cbl, as well as unsaturated TC, is released into portal plasma [6,7,9–12]. Plasma TC-Cbl binds to specific receptors in the tissue [6,7,13]. The internalized Cbl is metabolized to the coenzymes: methyl-Cbl and 5′-deoxyadenosyl-Cbl [1,6,7].

The role of the third Cbl-binding protein HC is not completely clear. Its probable function in plasma is withdrawal of Cbl-analogues from the circulation, since HC has a high affinity to many Cbl-forms in contrast to TC and IF [2,3,6,7]. HC is a pH-resistant protein that binds Cbl even at pH 1.5–2.0 while TC loses its Cbl-binding capacity at acidic pH [14].

Cbl-binding proteins are also present in excreted body fluids. Human and rat saliva and milk [15–17] contain a relatively high concentration of generally unsaturated HC (30–100 nM) [15–18]. The physiological role of HC in

Abbreviations: B_{12}, vitamin B_{12}; Cbl, cobalamin; Cbl*, [^{57}Co]cyanocobalamin; Cbi, dicyanocobinamide; HC, haptocorrin; IF, intrinsic factor; TC, transcobalamin; P_i-buffer NaH_2PO_4/Na_2HPO_4 buffer.

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excreted body fluids is unknown. An antibacterial function was proposed because apo-HC 'traps' Cbl in a form unaccessible for microorganisms [2,6,7].

The Cbl-binders in cow milk have not been characterized so far. In the present paper we show that the main Cbl-binding protein in cow milk is transcobalamin and not haptocorrin as expected.

2. Materials and methods

2.1. Cow milk and plasma gel filtration

An aliquot of milk, collected from several thousand cows, was received from a dairy. The sample was centrifuged at $3000 \times g$, 5°C for 10 min and the lipid fraction on the top was removed. Skim milk (3 ml) or cow plasma (1 ml of plasma + 2 ml 0.1 M NaCl) was incubated either with 0.75 pmol of radioactive Cbl* (Amersham, 3.9 kBq per pmol) or with the mixture of 0.25 nM Cbl* and 10 nM Cbi (Sigma) for 5 h at room temperature. The sample was subjected to gel filtration on a 2×83 cm Sephacryl S-200 column (Pharmacia) equilibrated with 0.1 M Tris, 1 M NaCl, 0.2% sodium azide (pH 8.0) with an elution flow of 10–20 ml/h at room temperature. The protein profile was recorded as $A_{280}(E_{280}^{mg/ml} \approx 1)$ or $A_{293}(E_{293}^{mg/ml} \approx 0.5)$. The value of $A_{280} - A_{330}$ or $A_{293} - A_{330}$ was used for the turbid milk sample. Cbl*-containing radioactive fractions (2.5–3 ml) were measured in a γ-counter.

2.2. Determination of Stokes radius

Stokes radius was determined by gel filtration on a 2.5×100 cm Sephadex G-200 column (Pharmacia) according to Ackers [19]. All conditions were the same as described above for gel filtration on Sephacryl S-200.

2.3. Determination of the Cbl-binding capacity

The total amount of unsaturated Cbl-binders was determined by the method of Gottlieb et al. [20]. The solution with 0.05–0.2 nM of unsaturated Cbl-binders was incubated with 0.25 nM Cbl* for 1 h at room temperature. Afterwards, the free ligand was adsorbed on haemoglobin-coated charcoal, removed by centrifugation and the protein-associated radioactivity was measured in the supernatant. The determination of the unsaturated binding capacity in partially purified protein from cow milk was performed in the presence of 1 M NaCl in order to suppress adsorption of TC on haemoglobin-coated charcoal at low ionic strength [21].

The alternative way for the determination of Cbl-binding capacity was based on gel filtration. The assayed sample was diluted with 0.1 M Tris, 1 M NaCl, 0.2% sodium azide (pH 8.0) to 0.05–0.2 nM of unsaturated Cbl-binders. Then, 0.25 nM Cbl* was added and after incubation for 1 h at room temperature the sample was applied to a Sephacryl S-200 column, see above.

2.4. Determination of the endogenous Cbl

Quantification of Cbl was performed by an isotope dilution method [22]. The protein-bound Cbl (100–500 pM) was released by boiling at pH 4.8 in the presence of 75 μM KCN and then subjected to competition with radioactive Cbl* for IF or HC. The IF-method measures the concentration of cobalamins in the assayed solution, while the HC-method measures the sum of cobalamins and analogues [23].

2.5. pH Stability of Cbl-binders

Acid-resistant binding capacity in cow plasma was determined as follows. The plasma sample (1 ml of plasma + 2 ml 0.1 M NaCl) was loaded with radioactive Cbl* as described above. Then, the pH of the sample was adjusted to 2.0 by 1 N HCl and the mixture was incubated for 30 min at room temperature. The distribution of Cbl* in protein fractions of the acidified plasma was traced by gel filtration on a Sephacryl S-200 column, see above.

The pH stability of the partially purified Cbl-binder loaded with Cbl* was analyzed identically to plasma.

2.6. Displacement of human TC from the human TC-receptor by Cbl-binder from cow milk

The procedure of displacement was performed under the conditions described earlier [24]. The binding of 0.2 nM human TC saturated with Cbl* to the TC-receptor from human placenta was carried out with or without 5 nM Cbl-protein from cow milk. The Ca^{2+}-sensitive specific binding was analyzed.

2.7. Isolation of transcobalamin from cow milk

Fresh skimmed milk (10 l) was mixed with 20 pmol of radioactive Cbl* and left for 2–3 days at 2–5°C.

The milk proteins were fractionated by $(NH_4)_2SO_4$ at 2–5°C. The salt (0.226 kg/l) was added to 10 l of stirred milk in increments until 40% of saturation (1.6 M) was reached. This procedure took about 1 h. The mixture was left for another 0.5 h for equilibration and then centrifuged at $5000 \times g$, 2°C for 10 min. The supernatant was collected. The bulky precipitate was suspended in 2 l of 40%-saturated $(NH_4)_2SO_4$-solution for additional extraction. The obtained suspension was centrifuged and the two supernatants ($\approx$ 10 l) were pooled. Then 0.187 kg/l of $(NH_4)_2SO_4$ was added for 70%-saturation. After 1.5 h, the mixture was centrifuged at $5000 \times g$, 2°C for 20 min. The precipitate was collected and suspended in 250 ml of 0.04 M P_i-buffer (pH 7.5). The volume after dissolving was $\approx$ 500 ml. The main amount of the Cbl-binder in this

preparation had a molecular mass of 43 kDa as judged from gel filtration.

The obtained solution was prepared for CM ion-exchange chromatography by dialysing against 5 l of 0.04 M P_i-buffer, pH 7.5 (24 h at 5°C with one change of the buffer). Neutral pH of the adsorption medium was preferable to pH 5.2 (conventionally used for human TC purification [25,26]). The dialysed sample (≈ 700 ml) was applied to 2 × 16 cm CM-Sepharose column (Pharmacia) at room temperature with the flow of 40 ml/h. The radioactive band drifted behind a coloured brownish protein during adsorption of the protein sample. We observed no drift during the following wash with 25 ml of 0.04 M P_i-buffer (pH 7.5). The next solution (70–150 ml of 0.12 M P_i-buffer, pH 7.5) was applied until the brownish protein was completely removed from the matrix and the protein concentration in eluate became 0.3–0.5 mg/ml. Afterwards, the Cbl-binder was desorbed in 0.2 M P_i-buffer, pH 7.5 (flow rate 10 ml/h).

The fractions with maximal Cbl*-radioactivity were pooled (final volume ≈ 15 ml). The sample was dialysed for 2 days at room temperature against 150 ml of 8 M urea dissolved in 0.2 M P_i-buffer (pH 7.5) with one change of the medium. 95–98% of endogenous Cbl was released into the external medium according to Cbl*-liberation. Protein renaturation was reached in 2 days during dialysis against 1 l of 0.04 M P_i-buffer (pH 7.5 at 2–5°C) with one change of the medium. The recovery of Cbl-binder after renaturation was 30–50% as judged from the Cbl-binding capacity related to the amount of released Cbl.

The Cbl-free protein was applied to a 2 ml affinity column, prepared as previously described [27]. Adsorption of the sample was carried out at 2–5°C at a flow of 10–20 ml/h. The matrix, containing adsorbed Cbl-binder, was washed in steps with: 10 ml of 0.04 M P_i-buffer (pH 7.5); 10 ml of 0.2 M P_i-buffer (pH 7.5) and 20 ml of 0.1 M Tris, 1 M NaCl (pH 8.0). The washed column was incubated at 37°C overnight for disruption of the thermolabile bond between the matrix and Cbl-protein. Desorbed Cbl-binder, together with the free hydroxo-Cbl, was eluted at 37°C in Tris + NaCl buffer. The coloured fractions were collected and pooled, final volume ≈ 3 ml.

The eluate from the affinity column was dialysed at 5°C against 1 l of 10 mM P_i-buffer (pH 7.5) in order to remove free hydroxo-Cbl. The external solution was changed every 24 h until no spectral difference between the dialysing medium and the pure buffer was obtained. The procedure usually took 4 days. At this step, the protein concentration was measured according to the peptide bond absorbance coefficient $E_{205}^{mg/ml} = 31 \pm 2$ [28]. If the assayed solution had a light turbidity, measurement of $A_{205} - A_{250}$ was performed. The final dialysing medium was 100 ml of 0.1 M Tris, 1 M NaCl (pH 8). Then, the protein was applied to a Sephacryl S-200 column (2 × 83 cm) equilibrated with the same buffer at room temperature. Protein elution was recorded at 230 nM ($E_{230}^{mg/ml} \approx 6$) and the main peak of 43 kDa protein was collected for SDS-electrophoresis and N-terminal sequence determination according to standard procedures [29].

3. Results

3.1. Distribution of Cbl-binders in cow milk

The Cbl-binding capacity in cow milk was 0.05 ± 0.02 nM ($\bar{X} \pm$ S.D., $n = 8$) and the concentration of endogenous Cbl was 3.3 ± 0.3 nM ($n = 11$). The determined Cbl-concentration was the same when measured employing IF and HC as the binding proteins. This indicates the absence of detectable analogues in cow milk [23].

The allocation of endogenous and radioactive Cbl was assayed during fractionation of Cbl*-treated milk. The same distribution of both Cbl's was observed employing the following methods of fractionation: gel filtration, acidic precipitation of milk proteins at pH 4.6, $(NH_4)_2SO_4$-fractionation and ion-exchange chromatography. Therefore, protein-bound Cbl* can be used as a measure of Cbl-saturated proteins during milk fractionation.

Gel filtration of cow milk revealed two peaks of Cbl-binders, see Fig. 1A. One of them was associated with a bulky protein peak ($M_r \approx 280$ kDa) and did not fit according to its molecular mass to any known Cbl-binder. The other peak had the same elution volume as TC ($M_r \approx 43$ kDa). The 280 kDa and the 43 kDa peaks comprised 45 ± 6% and 55 ± 6% ($n = 8$) of the milk Cbl-binders, respectively.

Incubation of a milk sample with a Cbl* + Cbi mixture showed similar low suppression of Cbl*-binding in both peaks, see Fig. 1A. It points to the absence of a considerable amount of the Cbi-sensitive apo-HC in cow milk.

The 280 kDa Cbl-peak precipitated together with the caseins when milk was subjected to chymosin-treatment, acidification of the medium (pH 4.6) and 40% saturation with $(NH_4)_2SO_4$ (1.6 M). We failed to release a Cbl-binder from the isolated 280 kDa complex by treatment with 20 mM EDTA, 1 M NaCl or 0.1 mM KCN for several hours. However, incubation at 5°C was accompanied by a slow liberation ($\tau_{1/2} = 17$ days) of a 43 kDa Cbl-binding protein, as judged by gel filtration.

Dialysis of a milk sample against 8 M urea for 2 days increased the Cbl-binding capacity up to 0.4 nM and decreased the concentration of endogenous Cbl down to 1 nM. The urea treatment was also accompanied by a redistribution of Cbl-binders: 80% of the endogenous Cbl and 85% of the binding capacity were associated with the 43 kDa fraction. No additional Cbl-binding protein appeared during the procedure. These observations led to the conclusion that the main Cbl-binder in cow milk had a molecular mass of 43 kDa but approximately half was associated with other proteins in cow milk.

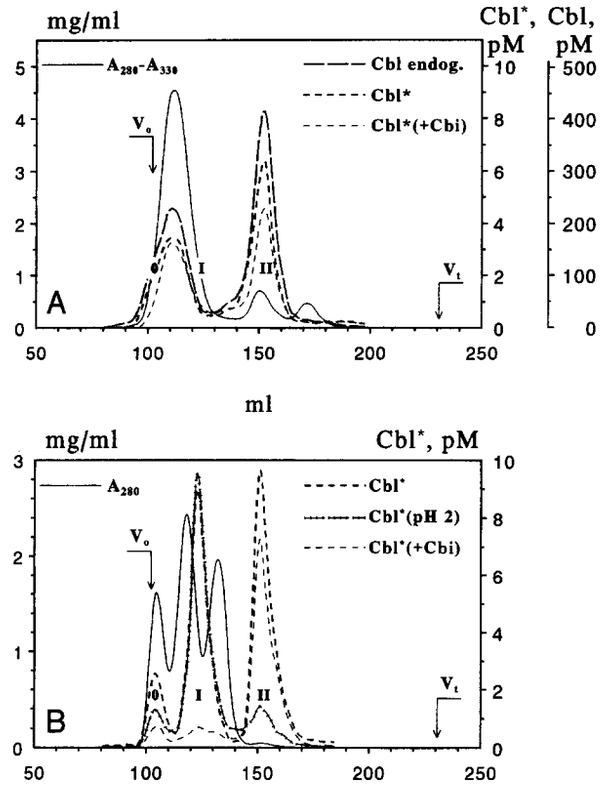

Fig. 1. Gel filtration on Sephacryl S-200 column (2×83 cm). (A) Cow milk (3 ml) and (B) cow plasma (1 ml plasma+2 ml 0.1 M NaCl) were treated either with 0.25 nM Cbl* (---) or with 0.25 nM Cbl* + 10 nM Cbi (—) for 5 h at room temperature. The Cbl*-profile (+ + +) on (B) corresponds to a plasma sample treated with Cbl* and then exposed to pH 2.0 for 30 min prior to gel filtration. The profiles of the protein (—) and endogenous Cbl (– –) are indicated. Numbers O, I, II mark positions of cow plasma Cbl-binders both in (A) and in (B).

3.2. Distribution of Cbl-binders in cow plasma

The Cbl-binding capacity varied in cow plasma from 0.1 nM to 0.3 nM and the gel filtration profile of Cbl*-treated plasma (Fig. 1B) was similar to those obtained for other species [10,11,30]. Three peaks comprised the following amounts of the incorporated Cbl*: 10% in peak 0 with $M_r > 500$ kDa, 40% in peak-I with $M_r \approx 120$ kDa and 50% in peak-II with $M_r \approx 43$ kDa. The exposure of Cbl*-treated plasma to pH 2.0 reduced the amount of Cbl* eluted with peak II, Fig. 1B.

Quantification of endogenous Cbl in cow plasma was hampered because of an extreme stability of the 120 kDa Cbl-protein complex. Thus, Cbl*-loaded 120 kDa binder retained 50–70% of Cbl* even after boiling in the presence of KCN. The concentration of endogenous Cbl attached to the 43 kDa Cbl-binding protein was estimated as 0.1 nM.

The plasma sample was incubated with a mixture of 0.25 nM Cbl* and 10 nM Cbi in order to characterize ligand specificity of the Cbl-binding proteins. Only partial suppression of Cbl*-binding by the analogue was observed

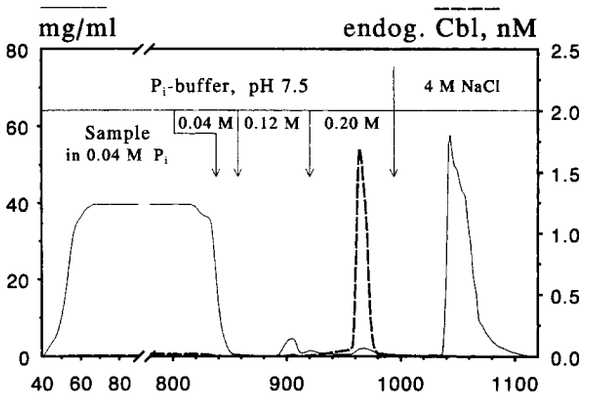

Fig. 2. CM-Sepharose chromatography profile of a Cbl-binding protein partially purified from cow milk. 700 ml of the preparation, precipitated from cow milk between 40% and 70% of $(NH_4)_2SO_4$-saturation, was dialysed against 0.04 M P_i-buffer (pH 7.5) and applied to a 50 ml CM-Sepharose column. The elution profile was as indicated in the figure: (—) protein profile, (– –) profile of endogenous Cbl calculated from the direct measurements as well as from associated radioactivity.

in peaks 0 and II while radioactivity in peak I almost disappeared, Fig. 1B.

Molecular masses, pH-stability and substrate specificity point to the following allocation of Cbl-binders in cow plasma: peak 0, high molecular aggregate containing both TC and HC; peak I, HC; and peak II, TC.

3.3. Purification of a Cbl-binding protein from cow milk

We chose the 43 kDa Cbl-binder from cow milk as the aim of isolation. The 280 kDa aggregate, containing associated 43 kDa Cbl-carrier, was ignored as a protein complex unaccessible for the preparative purposes. The procedure of isolation was complicated by the almost complete ligand saturation of the Cbl-binder, which precluded the direct application of affinity chromatography [25–27]. Therefore, the following purification strategy was attained. The 43 kDa Cbl-binding protein underwent partial purification by $(NH_4)_2SO_4$ precipitation and CM-Sepharose ion-exchange chromatography, Fig. 2. Then, the sample was depleted of endogenous Cbl by dialysis against 8 M

Table 1
Purification of a Cbl-binding protein from cow milk

Step of isolation	Total volume (ml)	Total protein (mg)	Total 43 kDa TC *) (nmol)	Purific. factor
Skimmed milk	10000	280000	18	1
$(NH_4)_2SO_4$ precipitate	750	30000	13	9
CM ion-exchange	15	25	9	$1.2 \cdot 10^4$
Urea treatment	15	23	3.5	$1.3 \cdot 10^4$
Affinity column	3	0.12	3	$2.6 \cdot 10^6$
S-200 gel filtration	15	0.10	2.5	$3.0 \cdot 10^6$

*) The value represents the sum of protein-bound Cbl and Cbl-binding capacity in the 43 kDa protein fraction.

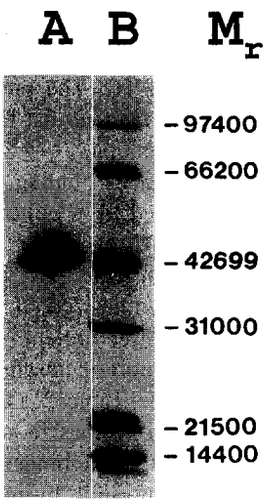

Fig. 3. SDS-polyacrylamide gel electrophoresis of (A) the Cbl-binding protein isolated from cow milk, (B) standard proteins. Molecular mass of the purified Cbl-binding protein is 43 kDa, as compared to the standards. The gel gradient is 10–20%.

urea. Afterwards, the protein was subjected to affinity chromatography. The final gel filtration on Sephacryl S-200 had more analytical than preparative character.

The process of purification is described in Section 2 and summarized in Table 1.

The partially purified protein, depleted of endogenous Cbl by urea treatment, was examined concerning its ligand binding properties. The Cbl-binding protein decreased in size after Cbl-attachment and the Stokes' radii of its apo- and holoforms were 2.7 nm and 2.5 nm, respectively. The holoform was able to displace about 50% of human TC from the human TC-receptor under the experimental conditions. The unsaturated protein had a low affinity to Cbi (a B_{12}-analogue) which is a feature of IF or TC. Thus, incubation with a mixture of 0.25 nM Cbl* and 10 nM Cbi was accompanied only by 10–20% suppression of Cbl*-binding. The Cbl*-saturated 43 kDa binder easily released the ligand at pH 2.0. No protein associated radioactivity was found at pH 2.0 as judged by charcoal-treatment and only 5–10% as judged by gel filtration.

The Cbl-binding protein obtained after affinity chromatography and gel filtration on Sephacryl S-200 was pure according to SDS-electrophoresis (Fig. 3). It had the molecular mass of 43 kDa and the amount of bound Cbl was estimated as one mol per mol of the protein. The absorbance spectrum of the preparation showed peaks at 280 nm, 363 nm, 415 nm and 535 nm. The ratio A_{280}/A_{363} was estimated as 1.9. Unfortunately, the low protein concentration precluded more careful spectral analysis.

The N-terminal sequence of the 43 kDa Cbl-binder isolated from cow milk revealed its homology with human and rabbit TC [31–33] while similarity to HC from human plasma [34] was much less (Fig. 4). There was no ambiguity in the obtained N-terminal sequence.

4. Discussion

We have purified and characterized the Cbl-binding protein in cow milk. It was classified as TC rather than HC because of the following reasons. N-terminal sequence revealed a homology between the isolated milk protein and TC from man and rabbit [31–33]. The protein decreased in size after Cbl-binding which is considered to be a characteristic feature [7]. The affinity to the Cbl-analogue (Cbi) was relatively low and could be estimated as at least 500-times less than that to Cbl. Low affinity to Cbi was earlier established for human and rabbit TC [2,3], while HC from these species was able to bind Cbi relatively well [2,3]. The complex between the protein and Cbl was unstable at pH 2.0 which is also a feature of TC and not of

```
                1   2   3   4   5   6   7   8   9   10  11  12  13
TC,  cow    : Asn-Ile(Cys)Glu-Ile-Thr-Glu-Val-Asp-Ser-Thr-Leu-Val-
TC,  man    : Glu-Met-Cys-Glu-Ile-Pro-Glu-Met-Asp-Ser-His-Leu-Val-
TC, rabbit: Glu-Ile-Cys-Gly-Val-Pro-Lys-Val-Asp-Ser-Glu-Leu-Val-
HC,  man    : Glu-Ile-Cys-Glu-Val-Ser-Glu-Glu-Asn-Tyr-Ile-Arg-Leu-

                14  15  16  17  18  19  20  21  22  23  24  25  26
TC,  cow    : Glu-Arg-Leu-Gly-Gln-Arg-Leu-Leu-Pro-Xaa-Met-Asp(Arg)...
TC,  man    : Glu-Lys-Leu-Gly-Gln-His-Leu-Leu-Pro-Trp-Met-Asp-Arg...
TC, rabbit: Glu-Lys-Leu-Gly-Gln-Arg-Leu-Leu-Pro(Trp)Met(Thr)...
HC,  man    : Lys-Pro-Leu-Leu-Asn-Thr-Met-Ile-Gln-Ser-Asn-Tyr-Asn...
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Fig. 4. N-terminal sequence of different Cbl-binders: TC from cow milk (present work), TC from human plasma [31,32], TC from rabbit plasma [33], HC from human plasma [34]. Amino-acid residues, equivalent to those in cow TC, are marked in bold letters.

Table 2
The concentrations of apo- and holo-TC and HC in plasma and milk from different species

Protein	Cow (nM)	Man (nM)	rat (nM)
Plasma	present work	[6,7,10,11,30,36]	
HC	0.08	0.2	– [a]
TD	0.1	0.7	1.5
HC-Cbl	– [a]	0.2	– [a]
TC-Cbl	0.1	0.04	– [a]
Milk	present work	[15–18,35,36]	
HC	≈ 0 [b]	90	20
TC	0.05	0.06	– [a]
HC-Cbl	– [a]	0.3	7
TC-Cbl	3.2	≈ 0 [b]	– [a]

[a] No data available.
[b] ≈ 0 – not detected.

HC [14]. All the data provide an unambiguous proof that the Cbl-binder isolated from cow milk is TC and not HC.

The endogenous Cbl in cow milk showed an equal distribution between a large protein complex (280 kDa) and TC (43 kDa). The high molecular mass complex liberated a 43 kDa Cbl-containing protein during long time incubation and after urea treatment. It testifies that the Cbl-binding protein incorporated into 280 kDa aggregate is, probably, the same 43 kDa TC. Unsaturated HC was not detected, as concluded from the low affinity to Cbi in all milk fractions. Still, we cannot exclude the presence of HC-Cbl in cow milk. Extreme stability of this complex, revealed in plasma preparations, provides a condition for its masking during Cbl-determination according to the standard procedure [22].

The total Cbl-binding capacity in cow milk was unusually low, when compared to other species, while the concentration of endogenous Cbl was intermediate between man and rat, Table 2. Cbl-analogues were not revealed in cow milk and the amount of TC-Cbl (3 nmol per liter) was quite sufficient to satisfy the demands of daily intake for man (1 nmol) [35]. The instability of TC-Cbl at pH 2 guaranties the liberation of Cbl in acidic medium of human stomach and its binding to human IF and HC [37,38]. It means that cow milk is an appropriate source of B_{12} for man.

While rat and human milk contain mainly HC, cow milk contains mainly TC. The different nature of Cbl-binders in milk from these species might be caused by their digestion distinctions. Thus, grass-eating animals have a strong dependence on microorganisms in their stomachs. A high concentration of unsaturated HC in milk might suppress the normal growth of the microorganisms due to 'trapping' of Cbl into the stable HC-Cbl complex. As a result of an evolutionary adaptation, grass-eating animals may have developed another way of Cbl-delivery by a less stable carrier, TC. Unfortunately, there is no available information in the literature about Cbl-binders in milk of other ruminants.

The gel filtration pattern of cow plasma was quite similar to human plasma. The main peaks of Cbl-binding capacity eluted as HC and TC. The presence of the two proteins was confirmed by the fact that HC bound Cbi and TC was sensitive to acidification.

The transport of Cbl and its binding protein from cow plasma to milk is unclarified. It is quite probable that the mechanism of HC and TC excretion into milk of the investigated species differs. Thus, HC in human and rat milk is supposed to be synthesized in mammary glands and excreted as a substrate-free protein [16,17]. It explains a relatively high concentration of unsaturated HC in milk as compared to plasma, see Table 2. On the other hand, there is no obvious need to synthesize new TC-Cbl in the mammary glands. The saturated binder might be transported from cow plasma into milk by the receptor-mediated mechanism [6,7,13], provided that the TC-Cbl complex does not degrade inside the cells.

In conclusion, we have purified TC from cow milk and shown that TC rather than HC is the dominating Cbl-binding protein in cow milk. Further, our results indicate that cow milk is an excellent source of Cbl for human beings due to a relatively high concentration of Cbl and due to the absence of B_{12}-analogues.

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