Binding of Cobalamin and Cobinamide to Transcobalamin from Bovine Milk†

Sergey N. Fedosov,‡,§ Torben E. Petersen,§ and Ebba Nexø*,‡

Protein Chemistry Laboratory, Department of Molecular Biology, University of Aarhus, Science Park, Gustav Wieds Vej 10, 8000 Aarhus C, Denmark, and Department of Clinical Biochemistry, KH University Hospital of Aarhus, Nørrebrogade 44, 8000 Aarhus C, Denmark

Received August 15, 1995; Revised Manuscript Received October 9, 1995⊗

ABSTRACT: We have studied the interaction between transcobalamin (TC) and the ligands cobalamin (Cbl) and cobinamide (Cbi). Partially purified TC from bovine milk was depleted of endogenous Cbl by 8 M urea treatment. Unsaturated TC was adsorbed on CM-Sepharose in order to ensure fast separation of the matrix-bound protein from the reaction medium. The forward reaction TC + Cbl → TC−Cbl (rate constant k_+^{Cbl}) and the backward reaction TC−Cbl → TC + Cbl (k_-^{Cbl}) were followed in time. A single-step binding model (with no intermediate protein−ligand complex) was sufficient to fit the data. The calculated rate constants were $k_+^{Cbl} = 0.6$ nM^{-1} min^{-1} and $k_-^{Cbl} = 1.3 \times 10^{-4}$ min^{-1}, which corresponded to the TC−Cbl dissociation constant $K_D^{Cbl} = 0.2$ pM. Reaction between TC and Cbl developed against electrostatic forces, and the effective charges of the interacting species were estimated as both +1 or both −1. The competition between Cbl and Cbi for TC was studied, which resulted in determination of the relevant rate constants for Cbi: $k_+^{Cbi} = 0.03$ nM^{-1} min^{-1}, $k_-^{Cbi} = 0.03$ min^{-1}, and $K_D^{Cbi} = 1$ nM. Slow dissociation of TC−Cbl guarantees its stability in plasma for 5−10 h, while Cbi bound to TC would be transferred to haptocorrin in less than 1 h.

The uptake of the vitamin B_{12}, i.e., cobalamin (Cbl),[1] in mammals proceeds in several steps and requires three specific proteins: intrinsic factor (IF), transcobalamin (TC), and haptocorrin (HC) (Allen, 1975; Nexø & Olsen, 1982). The vitamin is released during digestion as a free molecule that binds to gastric IF (Allen et al., 1977, 1978). A receptor on ileal mucosal cells with high affinity to IF−Cbl mediates entrance of the protein−ligand complex into the cell where Cbl is transferred to another Cbl carrier, TC (Donaldson et al., 1967; Hooper et al., 1973; Ramanujam et al., 1991). The TC−Cbl complex and the unsaturated TC circulate in plasma together with the third Cbl-binding protein HC (Hom, 1967: Hall & Finkler, 1971; Allen, 1976; England et al., 1976; Nexø & Andersen, 1977; Wickramasinghe & Fida, 1993). Target tissues recognize TC−Cbl due to a specific receptor on the cell surface (Kolhouse & Allen, 1977; Quadros et al., 1994).

IF and TC bind cobalamins relatively well, but they have a low affinity to Cbl analogues (e.g., cobinamide) (Kolhouse & Allen, 1977; Stupperich & Nexø, 1991). This feature prevents to some extent incorporation of the analogues into animal tissues where they may inhibit B_{12}-dependent biochemical reactions (Coates et al., 1960). Nevertheless, both Cbl and its analogues are able to enter the body just by passive diffusion through the cell membrane at high concentration of the ligand (Kolhouse & Allen, 1977; Ramanujam et al., 1991). A significant share of Cbl analogues has been found in human plasma (Muir & Chanarin, 1983) and in plasma of some animal species (Halpin et al., 1984). The analogues were attached mainly to HC (Muir & Chanarin, 1983). The HC-bound forms of Cbl are unaccessible for most tissues and are removed from the circulation by the liver (Kolhouse & Allen, 1977). Possibly, this mechanism prevents an organism from analogue poisoning.

The knowledge concerning Cbl binding and transfer of Cbl from one carrier to another is rather incomplete. No rate constants have been calculated for the reaction between ligand and binder, and the obtained values of the equilibrium dissociation constants vary considerably (Hippe & Olsen, 1971; Allen, 1973; Kolhouse & Allen, 1977, Allen et al., 1977, 1978).

The purpose of the present work was to describe the reaction between ligands and Cbl-depleted TC from bovine milk. Cyano-Cbl and Cbi were chosen as ligands, representing a biologically active form of B_{12} and a B_{12}-analogue, respectively.

MATERIALS AND METHODS

1. Partial Purification of the TC Preparation from Bovine Milk. The purification included two initial steps of the previously described isolation procedure (Fedosov et al., 1995). In short, precipitate of milk proteins was collected at 40%−70% of ammonium sulfate saturation and subjected to ion exchange chromatography on CM-Sepharose. Dialysis against 8 M urea for 2 days at room temperature was accompanied by 95%−98% release of endogenous Cbl. About 40% of the potential binding capacity was then recovered after 2 days of renaturating dialysis against 0.04 M P_i buffer, pH 7.5, 5 °C. The final amount of endogenous Cbl in the TC preparation, determined by the isotope dilution method (Nexø & Gimsing, 1981), did not exceed 2−5% of the binding capacity.

† This work is a part of the FØTEK program supported by the Danish Government and the Danish Dairy Board.

* To whom correspondence should be addressed. Tel.: (+45) 89 49 30 83. Fax: (+45) 89 49 30 60.

‡ KH University Hospital of Aarhus.

§ University of Aarhus.

⊗ Abstract published in *Advance ACS Abstracts,* November 15, 1995.

[1] 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 buffer.

0006-2960/95/0434-16082$09.00/0 © 1995 American Chemical Society

2. Adsorption of TC to CM-Sepharose. CM-Sepharose (Pharmacia) was washed on a filter with several volumes of 0.04 M P_i buffer, pH 7.5, and suspended in equal volume of the buffer.

A 9 mL amount of the TC solution (2.5 nM of unsaturated TC in 0.04 M P_i buffer, pH 7.5) was mixed with 1 mL of the CM-Sepharose suspension and incubated for 30 min with mild stirring at room temperature. The effective adsorption of TC on CM-Sepharose at pH 7.5 and $I = 0.1$ M was established earlier (Fedosov et al., 1995). CM-bound TC was pelleted by centrifugation (3000*g*, 5 °C, 10 min) and resuspended in 10 mL of 0.04 M P_i buffer pH 7.5. The total concentration of TC adsorbed on the matrix was checked in every new preparation by incubation with an excess of radioactive [^{57}Co]Cbl (Cbl*), see below. The final concentration of unsaturated TC in the suspension was adjusted to 2 nM.

3. Reaction between CM-Adsorbed TC and Cbl.* A 1 mL amount of matrix-bound TC (2 nM) was mixed with 9 mL of 0.04 M P_i buffer, pH 7.5 at room temperature. Afterward, 0.1−1 nM Cbl* was added (Amersham, 3.9 kBq per pmol of Cbl*). The reaction medium was stirred mildly at room temperature, and aliquots (1 mL) were removed at timed intervals. The removed samples were filtered under vacuum through a coffee filter, and the retained beads of CM-Sepharose were washed with 20 mL of 0.04 M P_i buffer, pH 7.5, for 1 min. The loss of protein-associated Cbl* was nonsignificant. The amount of TC−Cbl* on the filter was measured in a γ-counter. The control experiments with pure CM-Sepharose showed that the washing procedure ensured 99.5% removal of the unbound Cbl*.

The reaction between TC and Cbl* at different ionic strength was carried out as described above but in 1−40 mM P_i buffer, pH 7.5 ($I = 2.5-100$ mM).

4. Release of Cbl from CM-Adsorbed and Soluble TC−Cbl*.* CM-adsorbed TC preparation (10 mL, 0.2 nM) was incubated with 0.2 nM Cbl* for 5 h, as described above. The suspension was centrifuged, and the TC−Cbl* -containing pellet was resuspended in 10 mL of 0.04 M P_i buffer, pH 7.5, with or without 10 μM of nonradioactive Cbl. The mixture was incubated for 250 h at room temperature under mild stirring. Matrix-associated radioactivity was measured in 1 mL samples as a function of time.

In the alternative experiments, 2 mL of TC (0.2 nM, dissolved in 1 M NaCl) was incubated with Cbl* (0.2 nM) for 5 h. Afterward, the mixture was dialyzed against 20 mL of 0.04 M P_i buffer, pH 7.5 (with or without 10 μM Cbl), for 250 h at room temperature. The external solution was changed every 10−20 h and assayed for the radioactive Cbl*.

5. Release of Cbi from CM-Adsorbed TC−Cbi. A 10 mL amount of matrix-bound TC (0.2 nM) was incubated with 50−2000 nM Cbi (Sigma) for 2 h at room temperature with mild stirring. The excess Cbi was quickly removed by washing with 50 mL of P_i buffer on the filter. The matrix-bound TC−Cbi was suspended in 10 mL of the medium: 1 nM Cbl*, 0.04 M P_i buffer, pH 7.5. The substitution of TC-associated Cbi by Cbl* was followed in time by analysis of 1 mL aliquots as described above.

6. Competition between Cbl and Cbi: Time Dependence and Equilibrium Assay.* A set of 10 mL samples was prepared with the following concentrations of the following reagents: 0.2 nM CM-adsorbed TC, 0.25 nM Cbl*, and 0−5000 nM Cbi (Cbl and Cbi were added together). The samples were incubated in 0.04 M P_i buffer, pH 7.5, at room temperature with mild stirring. The 1 mL aliquots of CM-adsorbed TC−Cbl* were separated from the reaction medium either by the filtration procedure or by centrifugation (3000*g*, 5 °C, 10 min). The pellet was washed and counted. The reaction was followed in time until no change in TC−Cbl* occurred during the last 10 h.

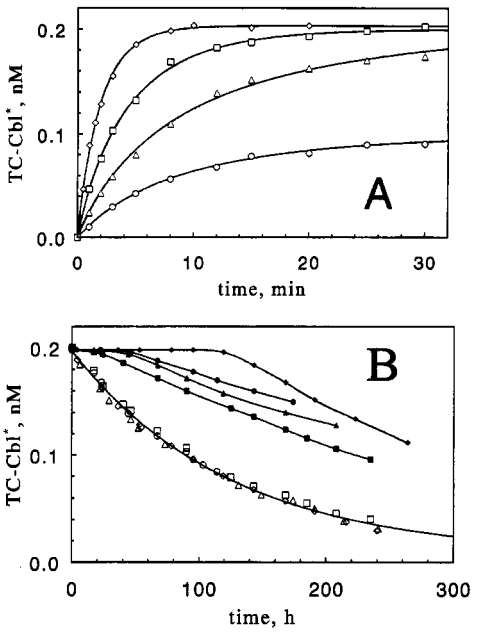

FIGURE 1: Reaction between bovine milk TC and Cbl*. (A) Association of TC (0.2 nM) and Cbl*: (○) 0.1 nM, (△) 0.25 nM, (□) 0.5 nM, (◇) 1.0 nM. The data were fitted by eq 1, see the Appendix. The mean values of the floating parameters for four curves were calculated: $TC_{t=0} = 0.20 \pm 0.01$ nM, $k_+^{Cbl} = 0.55 \pm 0.04$ nM^{-1} min^{-1} ($n = 4$). (B) Dissociation of the TC−Cbl* complex in four different TC preparations: (●, ▲, ■, ◆) nonspecific liberation of Cbl* in the absence of any additives; (○, △, □, △) specific liberation of Cbl* in the presence of nonradioactive Cbl (10 μM). Experiments were performed with CM-adsorbed TC except (□, ■) when a soluble preparation of TC was employed. The process of specific liberation, given as a function $y = f(x)$, was fitted by eq 2 in the Appendix: $y = 0.008 + 0.19 \exp(-0.00803x)$. The calculated rate constant is $k_-^{Cbl} = (1.34 \pm 0.06) \times 10^{-4}$ min^{-1} ($n = 4$).

7. Binding of Cbl to TC in Skim Milk: Time Dependence.* A sample of skim milk was incubated with 0.5 nM Cbl* at room temperature with mild stirring. The mixture was assayed in portions for protein-associated Cbl* by the method of Gilbert (1977), employing haemoglobin-coated charcoal for adsorption of the free Cbl*. The reaction time was calculated as the interval between addition of Cbl* to the milk and the addition of the charcoal to the mixture.

8. Computation and Statistics. The data fitting was performed by the computer program for regression analysis and simulation of metabolic chain reactions (Fedosov, 1995). The statistical data are presented as mean ±SD.

RESULTS

1. Cbl Binding to CM-Adsorbed TC.* The interaction between TC and Cbl* was almost irreversible under the conditions of the experiment, Figure 1A. Two possible mechanisms of the reaction were considered: (i) the single-step binding, TC + Cbl* → TC−Cbl*, and (ii) the binding with the formation of an unstable protein−ligand intermediate, TC + Cbl* ⇄ TC···Cbl* → TC−Cbl*.

The data fit in Figure 1A was performed on the basis of the single-step model, eq 1 in the Appendix. It revealed no

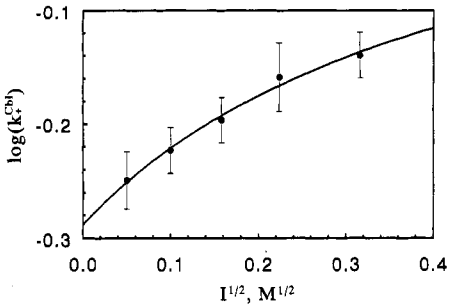

FIGURE 2: The influence of ionic strength on bovine milk TC and Cbl* association rate constant. The experimental points are presented as $y_{mean} \pm SD$ ($n = 4$). The dependence $y = f(x)$ was fitted by eq 2 in the Appendix in order to calculate the effective charges of the interacting species. The best fit gave the following equation: $y = -0.288 + 0.819x/(1 + 2.25x)$. The calculated value of Z_1Z_2 corresponds to $Z_1 = Z_2 = \pm 0.90 \pm 0.35$ ($n = 4$), see the text.

systematic error in the curve approximation, which testifies for the validity of the model. The analysis of four different TC preparations gave the rate constant of Cbl association: $k_+^{Cbl} = 0.6 \pm 0.2$ nM^{-1} min^{-1} ($n = 10$).

An attempt to fit the data according to the more complex scheme with an intermediate protein−ligand complex revealed no advantages when compared to the simple model fit (not presented). Therefore, the complex mechanism was rejected as groundless.

2. Release of Cbl from the TC−Cbl* Complex.* Both CM-adsorbed TC−Cbl* and soluble TC−Cbl* were examined for a nonspecific loss of Cbl* during incubation without additives. The protein−ligand complex was quite stable for 2−5 days of incubation (Figure 1B, filled symbols). The amount of the liberated radioactivity was ≈1% of the total value. Then, a release of Cbl* from TC−Cbl* was observed with a significant variation of the process among different TC preparations. The nonspecific loss of Cbl* developed faster in the soluble TC preparation (Figure 1B, filled squares) when compared to the CM-adsorbed preparations. The liberated ligand was not protein associated as judged from adsorption on haemoglobin-coated charcoal (Gilbert, 1977). The nonspecific loss of Cbl* was probably caused by protein denaturation.

The specific liberation of Cbl* was induced by an excess of external nonradioactive Cbl which provided irreversible substitution of Cbl* by Cbl on TC. The dissociation of TC−Cbl* was supposed to be the rate-limiting step. The process was identical in all examined TC preparations, (open symbols in Figure 1B. An attempt to correct the data for the nonspecific loss of Cbl* gave quite unreasonable shapes of the obtained curves. Therefore, the nonspecific loss was finally ignored and TC−Cbl* was believed to be denaturation resistant in the presence of the external Cbl (even after 100 h of incubation). If it is true, the exponential fit of the data according to eq 2 in the Appendix could be applied. The rate constant of TC−Cbl* dissociation was calculated as $k_-^{Cbl} = (1.34 \pm 0.06) \times 10^{-4}$ min^{-1} ($n = 4$).

The ratio between the rate constants k_-^{Cbl}/k_+^{Cbl} is equal to the equilibrium dissociation constant of TC−Cbl, $K_D^{Cbl} = 0.22 \pm 0.06$ pM.

3. Influence of Ionic Strength on the Reaction between Transcobalamin and Cbl.* The interaction between TC and Cbl* was accelerated at higher ionic strength (Figure 2), i.e., the charges of interacting species were both plus or both

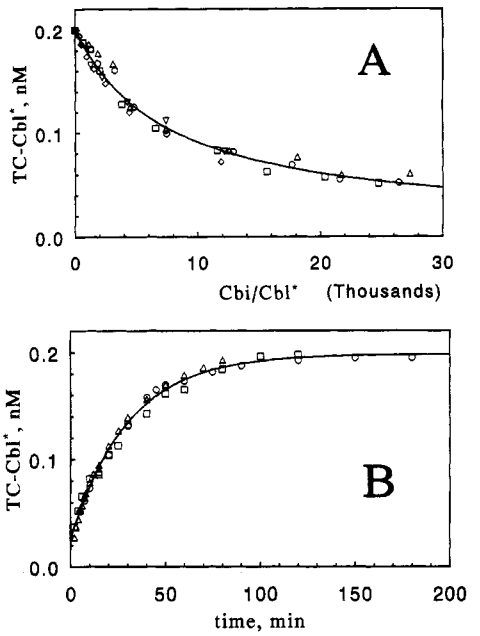

FIGURE 3: Reaction between bovine milk TC and Cbi followed by Cbl* displacement. (A) Competition between Cbl* (0.25 nM) and Cbi (0−5000 nM) for TC (0.2 nM) at equilibrium. The concentration of TC−Cbl*, reached after 50 h of incubation at room temperature, was plotted against the concentration ratio (Cbi/Cbl*) of the free ligands. Four different TC preparations (○, △, □, ◇) were analyzed. The dependence $y = f(x)$ was fitted by eq 4 in the Appendix in order to reveal the relative affinities of Cbi and Cbl* to TC. The fit resulted in the following equation: $y = 0.193/(1 + x/7376) + 0.009$, i.e., the ratio of the dissociation constants is $K_D^{Cbi}/K_D^{Cbl} = 7376 \pm 781$ ($n = 4$). (B) Dissociation of the TC−Cbi complex in the presence of 1 nM Cbl* followed by TC−Cbl* formation. The complex TC−Cbi was prepared using three different TC preparations (○, △, □) by treatment with Cbi: (○) 50 nM, (△) 1000 nM, (□) 2000 nM. The excess of Cbi was removed after 2 h of incubation at room temperature, and TC−Cbi was mixed with Cbl*. The dependence of TC−Cbl* on time $y = f(x)$ was fitted by eq 2 in the Appendix: $y = 0.198 - 0.173 \exp(-0.0332x)$. The calculated rate constant corresponds to the rate-limiting step of TC−Cbi dissociation: $k_-^{Cbi} = 0.033 \pm 0.001$ min^{-1} ($n = 3$). Extrapolation of the curve to t_0 of the reaction gave a positive value of TC−Cbl* in all three preparations. This effect could not be explained by an experimental error in time or in TC−Cbl* determination.

minus according to eq 3 in the Appendix. The increase of k_+^{Cbl} at higher ionic strength was not pronounced due to the low effective charges Z_1 and Z_2 on the interacting molecules. Regression analysis of the experimental data was performed on the basis of eq 2 in the Appendix and gave the following parameters: the charges of interacting species were estimated as $Z_1 = Z_2 = \pm 0.90 \pm 0.35$; the logarithm of the reaction rate constant at $I = 0$ was determined from the intercept with the Y-axis as 0.29 ± 0.03; one-half of the distance between bound ions was 0.7 ± 1.0 nm ($n = 4$).

4. Equilibrium Assay of the Cbl and Cbi Competition.* The amount of TC−Cbl*, obtained at equilibrium (50 h of incubation), was plotted against the ratio Cbi/Cbl*, where Cbi was the total concentration of the ligand (Cbi-total ≈ Cbi-free) and Cbl* was concentration of the free ligand. The data obtained (Figure 3A) were fitted according to the simple competitive model, eq 4 in the Appendix. The fit resulted in the following ratio of the equilibrium dissociation constants: $K_D^{Cbi}/K_D^{Cbl} = 7376 \pm 781$ ($n = 4$). The absolute value of the TC-Cbi dissociation constant, $K_D^{Cbi} = 1.6 \pm 0.5$ nM, was calculated on the basis of K_D^{Cbl} determined above.

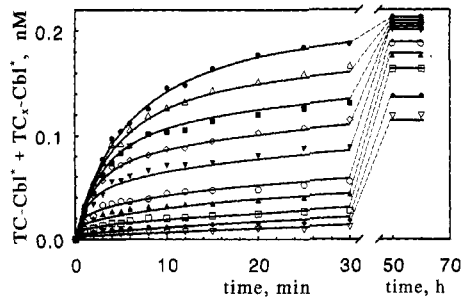

FIGURE 4: Binding of Cbl* to bovine milk TC followed in time during Cbl* and Cbi competition. The reagent concentrations at t_0 were $[TC]_0 = 0.21$ nM, $[Cbl^*]_0 = 0.25$ nM, $[Cbi]_0 =$ (●) 0 nM, (△) 2 nM, (■) 5 nM, (◇) 10 nM, (▼) 20 nM, (○) 50 nM, (▲) 100 nM, (□) 200 nM, (◆) 500 nM, and (▽) 1000 nM. The experimental points were fitted by the reaction modeling on the computer. The curves were simulated using a set of differential equations and the proposed Schemes 1 and 2 (see eq 5 in the Appendix). The optimal set of the rate constants is present in the main text.

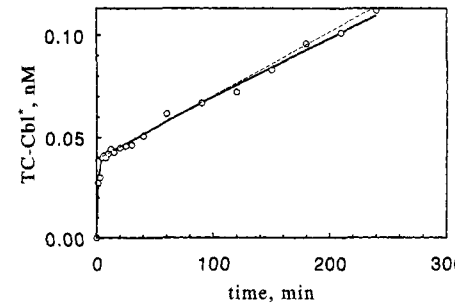

FIGURE 5: Treatment of a skim milk preparation with 0.5 nM Cbl*. Formation of TC−Cbl* occurred biphasic which corresponded to the reaction between Cbl* and unsaturated TC ("fast" phase) and to the substitution of endogenous Cbl with Cbl* ("slow" phase). The slope in the beginning of the "slow" phase (see the dashed line) is equal to $v = k_-^{Cbl}[TC-Cbl]$. The rate constant of TC−Cbl dissociation was calculated from the slope and [TC−Cbl] = 3.06 nM as $k_-^{Cbl} = 0.0001$ min^{-1}.

5. Cbi Release from TC−Cbi. The irreversible liberation of Cbi from TC−Cbi was induced by an excess of external Cbl* which provided substitution of Cbi by Cbl* on TC. The rate-limiting step was supposed to be TC−Cbi dissociation. The proposed scheme of Cbi liberation (eq 3 in the Appendix) did not completely satisfy the experimental data, see Figure 3B. Thus, the exponential fit gave a positive value of TC−Cbl* extrapolating the curve to t_0 of the reaction. It points to a "jump" in TC−Cbl* formation as if a small share of the protein (TC_x) was able to liberate bound Cbi more quickly than the main part of the TC preparation. The rest of the TC-preparation followed the proposed reaction model with the rate constant $k_-^{Cbi} = 0.033 \pm 0.001$ min^{-1} ($n = 3$), which was calculated on the basis of eq 3 in the Appendix.

It was easy to evaluate the missing Cbi binding rate constant (k_+^{Cbi}) from the equation $K_D^{Cbi} = k_-^{Cbi}/k_+^{Cbi}$. This value was estimated as $k_+^{Cbi} = 0.021 \pm 0.005$ nM^{-1} min^{-1}.

6. Competition between Cbl and Cbi for TC Followed in Time.* Several sets of experimental points with varying Cbi concentration were obtained, see Figure 4, open and filled symbols. The experimental data in Figure 4 were fitted using computer modeling on the basis of eq 5 in the Appendix. First, a minimal model including one protein (TC) and two competing ligands (Cbl* and Cbi) was analyzed (Scheme 1 in the Appendix). Surprisingly, it was impossible to fit all curves with the same set of the rate constants using this model. A systematic deviation from the experimental points was obtained at high concentration of Cbi, not presented.

As mentioned in section 5, 10%−15% of TC seemed to have a relatively high velocity of Cbi liberation from the protein−ligand complex. Because of this fact, an alternative model was used (Schemes 1 and 2 and eq 5 in the Appendix). It included the minor TC_x form with a decreased affinity to Cbi characterized by the rate constants k_{x-}^{Cbi} and k_{x+}^{Cbi} ($k_{x-}^{Cbi}/k_{x+}^{Cbi} \gg k_-^{Cbi}/k_+^{Cbi}$). The calculations performed according to this model gave the following parameters: [TC] = 0.178 nM, $[TC_x] = 0.032$ nM, $k_+^{Cbl} = 0.7$ nM^{-1} min^{-1}, $k_-^{Cbl} = 0.00014$ min^{-1} ($K_D^{Cbl} = 0.2$ pM), $k_+^{Cbi} = 0.03$ nM^{-1} min^{-1}, $k_-^{Cbi} = 0.03$ min^{-1}, ($K_D^{Cbi} = 1$ nM, $K_D^{Cbi}/K_D^{Cbl} = 5000$), $k_{x+}^{Cbi} = 0.003$ nM^{-1} min^{-1}, $k_{x-}^{Cbi} = 0.3$ min^{-1} ($K_{xD}^{Cbi} = 100$ nM). The theoretical curves for the acquired constants were simulated on the computer and gave an appropriate fit of the experimental points as is shown in Figure 4.

7. Treatment of Skim Milk with Cbl: Time Dependence of TC−Cbl* Formation.* The assay was undertaken in order to corroborate the validity of the rate constants calculated for the urea-treated TC preparation. The incubation of a milk sample with 0.5 nM Cbl* was accompanied by TC−Cbl* formation. The process developed biphasically, see Figure 5. The first phase showed the interaction between unsaturated TC and Cbl*. The process was too fast ($\tau_{1/2} > 2$ min) to follow by the method of Gilbert (1977), which includes a 10 min treatment with charcoal.

The substitution of endogenous Cbl by Cbl* occurred in the second phase. It was almost linear during 4 h, but then the reaction stopped and after 6−7 h of incubation the amount of TC−Cbl* steadily decreased toward zero, presumably due to protein denaturation. Therefore, the reaction was analyzed in the time interval 0−4 h. The rate constant k_-^{Cbl} was calculated from the slope of the curve in the initial part of the second phase. The slope was equal to the reaction velocity $v = k_-^{Cbl}[TC-Cbl]$, where [TC−Cbl] = 3.06 nM. The determined value of $k_-^{Cbl} = 0.0001$ min^{-1} showed a reasonable agreement with the rate constant obtained for the urea-treated TC preparation.

DISCUSSION

The partially purified TC from bovine milk was employed to study the binding characteristics of Cbl and Cbi. Adsorption on CM-Sepharose made it easy to separate TC from the reaction medium and to follow TC−Cbl* formation in time.

There was no difference between CM-adsorbed and soluble TC-preparations concerning the specific liberation of Cbl* from TC−Cbl*. It points to the absence of a matrix-induced effect on the reaction kinetics. Moreover, CM-Sepharose stabilized the protein against denaturation. The urea-treated TC and TC in a crude milk sample also showed the same value of k_-^{Cbl}. Thus, the validity of the data obtained with the urea-treated preparation was confirmed.

We have determined the individual rate constants both for TC + Cbl association ($k_+^{Cbl} = 0.6$ nM^{-1} min^{-1}) and for TC−Cbl dissociation ($k_-^{Cbl} = 1.3 \times 10^{-4}$ min^{-1}). The binding of an analogue (Cbi) to TC was 20 times slower and its release from the complex TC−Cbi occurred 230 times faster, when compared to Cbl. An interesting point is, that the affinity of Cbl and Cbi to TC differs particularly at the ligand liberation process.

The equilibrium dissociation constant of TC−Cbl* was derived from the ratio k_-^{Cbl}/k_+^{Cbl} and gave the value K_D^{Cbl} = 0.2 pM. It was comparable to data obtained for rabbit TC (K_D^{Cbl} = 0.4 pM) but differed from values obtained for human TC: K_D^{Cbl} = 2 pM (Kolhouse & Allen, 1977) and K_D^{Cbl} = 3 pM (Hippe & Olsen, 1971).

The process of interaction between TC and Cbl showed low sensitivity to the ionic strength of the medium. Moreover, it went against electrostatic forces. The effective charges on TC and Cbl were estimated as both +1 or both −1. The most probable values are −1 since the positive charge on the Cbl molecule is well shielded while its negatively charged phosphate group is exposed. The evaluation of the charge inside the Cbl-binding site of TC could be of assistance for its localisation.

The analogue-binding experiments showed the low affinity of TC to Cbi when compared to Cbl. The ratio of the equilibrium dissociation constants for these ligands was K_D^{Cbi}/K_D^{Cbl} = 7000 according to one set of the experiments and 5000 according to another. These values are relatively high when compared to K_D^{Cbi}/K_D^{Cbl} = 1500 (Kolhouse & Allen, 1977) and 2000 (Stupperich & Nexø, 1991) obtained for human TC. The ratio K_D^{Cbi}/K_D^{Cbl} < 2000 seems to be an underestimate caused by an incubation time insufficient for equilibration of the reagents. This point requires some explanations.

As shown in the present paper, the formation of TC−Cbl* developed biphasic after mixing of TC, Cbl*, and Cbi (Figure 4), still there is no surprise in this observation. The relatively fast and two-directional binding occurs on the first step of the reaction: TC−Cbi $\overset{+Cbi}{\leftarrow}$ TC $\overset{+Cbl^*}{\rightarrow}$ TC−Cbl*. On the second step, the redistribution of the ligands in favor of TC−Cbl* takes place: TC−Cbi $\overset{-Cbi}{\rightarrow}$ TC $\overset{+Cbl^*}{\rightarrow}$ TC−Cbl*. This process is quite slow at high Cbi concentrations, and the final equilibrium was reached only after 50 h of incubation. It means, that one must call attention to the careful equilibration of the mixture (binder + Cbi + analogue) because the error in K_D^{Cbi}/K_D^{Cbl} determination might be quite dramatic under preequilibrium conditions.

The rate constants obtained for the interaction between TC and Cbl/Cbi permit speculation about the relationships between different Cbl carriers in plasma. A fresh portion of TC−Cbl released into plasma becomes a subject of an equilibrium with HC which has a stronger affinity to Cbl than TC (Kolhouse & Allen, 1977; Stupperich & Nexø, 1991). Nevertheless, the main amount of Cbl would still be bound to TC even after 5−10 h, as estimated from the measured rate constants. This time is quite sufficient for internalization of TC−Cbl by different tissues (Hom & Olsen, 1969; Kolhouse & Allen, 1977). It explains why the usual way of Cbl transportation in plasma is TC−Cbl → (cell) → Cbl → HC−Cbl. On the other hand, liberation of the analogue from TC−Cbi occurs relatively quickly. Hence, HC would effectively remove Cbi (and, perhaps, other analogues) from TC if for some reason TC−Cbi appeared in plasma. Therefore, the analogue transport in plasma follows the scheme TC−Cbi → HC−Cbi → (hepatocyte).

The obtained heterogeneity of the TC-preparation from bovine milk according to its affinity to Cbi may reflect either the existence of different TC isoforms or the difference between individual cows. The minor high-molecular form of TC (Hom, 1967; Quadros & Rothenberg, 1986) may also add to the nonequivalence in Cbi-binding properties. In any case, the amount of TC_x with the reduced affinity to Cbi was relatively low and it could have been discarded from a keen scrutiny.

In conclusion, we have determined the rate constants for the process of association−dissociation between TC and Cbl/Cbi. They provided data for calculation of the ligand transfer from one Cbl carrier to another.

APPENDIX

The notation used throughout the Appendix is as follows: E is TC; S is Cbl*; L is Cbl; I is Cbi; and ES, EL, and EI are the corresponding protein−ligand complexes.

Equation 1. The bimolecular reaction E + S → ES (Figure 1A) followed in time (Mahler & Cordes, 1971) is expressed as

$$ES = \frac{1 - e^{-(S_0 - E_0)k_+ t}}{1 - \frac{E_0}{S_0} e^{-(S_0 - E_0)k_+ t}}$$

where ES is TC−Cbl* concentration at the corresponding t; t is time of the reaction; E_0 is TC concentration at $t = 0$; S_0 is Cbl* concentration at $t = 0$; k_+ is the rate constant of the forward reaction between TC and Cbl (k_+^{Cbl}), see Figure 1A.

Equation 2. The dependence of ES on time in the processes ES $\overset{-S+L}{\rightarrow}$ EL (Figure 1B) and EI $\overset{-I+S}{\rightarrow}$ ES (Figure 3B) was described by the exponential equation (Mahler & Cordes, 1971) below

$$ES = ES_\infty + ES_\Delta e^{-k_- t}$$

where ES is TC−Cbl* concentration at the corresponding t; t is time of the reaction; $ES_\Delta + ES_\infty$ is the TC−Cbl* concentration at $t = 0$; ES_∞ is the TC−Cbl* concentration at $t \rightarrow \infty$; and k_- is the rate constant of the process. Dissociation of the original complex was believed to be the rate-limiting step. Therefore, $k_- = k_-^{Cbl}$ for the data in Figure 1B and $k_- = k_-^{Cbi}$ for the data in Figure 3B.

Equation 3 shows the influence of ionic strength I on the rate constant k_+^{Cbl} of the bimolecular reaction $E^{Z_1} + S^{Z_2} \rightarrow ES^{Z_1+Z_2}$ where Z_1 and Z_2 are charges of the interacting species. The following form of the Debye−Hückel equation was applied to fit the data in Figure 2 (Guggenheim & Prue, 1955; Tanford,1961; Snyder et al., 1981; Fedosov et al., 1993):

$$\log(k_+) = \log(k_0) + \frac{2Z_1 Z_2 \alpha \sqrt{I}}{1 + \beta r \sqrt{I}}$$

where k_+ is the rate constant k_+^{Cbl} at corresponding ionic strength I; k_0 is the value of k_+^{Cbl} at $I = 0$; $\alpha = 0.509\ M^{-1/2}$ and $\beta = 3.29\ M^{-1/2}\ nm^{-1}$ at 20 °C (Guggenheim & Prue, 1955; Tanford, 1961); Z_1 and Z_2 are the effective charges of the interacting species; r is the mean radius of the interacting ions (usually, r = 0.5−1 nm for protein−ligand interaction (Snyder et al., 1981).

Equation 4. The equilibrium competition between S and I for E (Figure 2A) in the simple competitive mechanism EI $\overset{I/K_i}{\leftrightarrow}$ E $\overset{S/K_s}{\leftrightarrow}$ ES at $I \gg K_i$ (Mahler & Cordes, 1971) is expressed below

$$ES = \frac{ES_\Delta}{1 + \frac{I/S}{K_i/K_s}} + ES_\infty$$

where ES is TC−Cbl* concentration at the corresponding Cbi/Cbl* ratio; I/S is the ratio Cbi/Cbl* of the free ligand concentrations; K_i/K_s is the ratio of the dissociation constants for TC-Cbi (K_D^{Cbi}) and TC−Cbl* (K_D^{Cbl}), respectively; $ES_\Delta + ES_\infty$ is the TC−Cbl* concentration at Cbi = 0; and ES_∞ is the TC−Cbl* concentration at $I \rightarrow \infty$.

Equation 5. The competition between S and I for E and E_x followed in time is shown in Schemes 1 and 2. The

Scheme 1

$$EI \underset{k_{-i}}{\overset{k_{+i}I}{\rightleftharpoons}} E \underset{k_{-s}}{\overset{k_{+s}S}{\rightleftharpoons}} ES$$

Scheme 2

$$E_xI \underset{k_{-xi}}{\overset{k_{+xi}I}{\rightleftharpoons}} E_x \underset{k_{-s}}{\overset{k_{+s}S}{\rightleftharpoons}} E_xS$$

minimal and E-homogenous model implies $E_x = 0$ and could be described by Scheme 1. The schemes used were described by the following equations: $S = S_0 - ES - E_xS$; $I = I_0 - EI - E_xI$; $E = E_0 - ES - EI$; $E_x = E_{x0} - E_xS - E_xI$; $dES/dt = k_{+s}SE - k_{-s}ES$; $dE_xS/dt = k_{+s}SE_x - k_{-s}E_xS$; $dEI/dt = k_{+i}IE - k_{-i}EI$ and $dE_xI/dt = k_{+xi}IE_x - k_{-xi}E_xI$, where E_0, E_{x0}, S_0, and I_0 are the reagent concentrations at t_0; E, ES, EI, E_x, E_xS, E_xI, S, and I are the reagent concentrations at the time of the reaction t. The calculations were started from $t = 0$, $E_0 + E_{x0} = 0.21$ nM, $S_0 = 0.25$, $I_0 = 0-1000$ nM using the estimated values of the rate constants and E_0/E_{x0}. The reagent concentrations were simulated in time. The computation was performed by a differential equations analyzer for simulation of metabolic chain reactions (Fedosov, 1995). The calculated theoretical values of ES + E_xS were compared to the experimental points in order to reach the best fit. The optimal rate constants and E_0/E_{x0} were found by trials and errors using the separate analysis of the "fast" and "slow" phases in the preliminary fitting. The combination of all k_+-constants was important in the first phase, while k_- constants were crucial for the second phase. The influence of k_{x+} and k_{x-} on time course was negligible at [Cbi] < 50 nM and critical at [Cbi] > 200 nM. The value of E_0/E_{x0} influenced the relative amplitudes of the "fast" and "slow" phases. The calculated optimal parameters are given in the main text.

ACKNOWLEDGMENT

The authors appreciate greatly the perfect technical assistance of A. L. Christensen. We are grateful to Andreas Madsen, MD Foods Research and Development Centre, Brabrand, Denmark, for the supply of bovine milk samples.

REFERENCES

Allen, R. H., & Mehlman,C. S. (1973) *J. Biol. Chem. 248,* 3660−3669.

Allen, R. H. (1975) *Prog. Hematol. 9,* 57−84.

Allen, R. H. (1976) *Br. J. Haematol. 33,* 161−171.

Allen, R. H., Seetharam, B., Podell, E. R., & Alpers, D. H. (1977) *J. Clin. Invest. 61,* 47−54.

Allen, R. H., Seetharam, B., Allen, N. C., Podell, E. R., & Alpers, D. H. (1978) *J. Clin. Invest. 61,* 1628−1634.

Coates, M. E., Davies, M. K., & Harrison, G. F. (1960) *Arch. Biochem. Biophys. 87,* 93−99.

Donaldson, R. M., Jr., Mackenzie, I. L., & Trier, J. S. (1967) *J. Clin. Invest. 46,* 1215−1228.

England, J. M., Down, M. C., Wise, I. J., & Linnell, J. C. (1976) *Clin. Sci. Mol. Med. 51,* 47−52.

Fedosov, S. N., Belousova, L. V., & Plesner, I. W. (1993) *Biochim. Biophys. Acta 1153,* 322−330.

Fedosov, S. N. (1995) *Biochim. Biophys. Acta 1208,* 238−246.

Fedosov, S. N., Petersen, T. E., & Nexø, E. (1995) *Biochim. Biophys. Acta* (in press).

Gilbert, H. S. (1977) *J. Lab. Clin. Med. 89,* 13−24.

Guggenheim, E. A., & Prue, J. E. (1955) in *Physico-Chemical Calculations* (De Boer, J., Brinkman, H., Casimir, H. B. G., Eds.) pp 202−256, Interscience, New York.

Hall, Ch. A., & Finkler, A. E. (1971) in *Methods in Enzymology* (McCormick, D. B., & Wright, L. D., Eds.) Vol. 18, Part C, pp 108−129, Academic Press, New York.

Halpin, C. G., Harris, D. J., & Caple, I. W. (1984) *Res. Vet. Sci. 37,* 249−251.

Hippe, E., & Olsen, H. A. (1971) *Biochim. Biophys. Acta. 243,* 83−89.

Hom, B. L. (1967) *Clin. Chim. Acta 18,* 315−317.

Hom, B. L., & Olsen, H. A. (1969) *Scand. J. Clin. Lab. Invest. 23,* 201−211.

Hooper, D. C., Alpers, D. H., Burger, R. L., Mehlman, C. S., & Allen, R. H. (1973) *J. Clin. Invest. 52,* 3074−3083.

Kolhouse, J. F., & Allen, R. H. (1977) *J. Clin. Invest. 60,* 1381−1392.

Li, N., Seetharam, Sh., Lindemans, J., Alpers, D., Arwert, F., & Seetharam, B. (1993) *Biochim. Biophys. Acta 1172,* 21−30.

Mahler, H. R., & Cordes, E. H. (1971) in *Biological Chemistry* pp 267−324, Harper & Row Publishers, New York.

Muir, M., & Chanarin, J. (1983) *Br. J. Haematol. 54,* 613−621.

Nexø, E., & Andersen, J. (1977) *Scand. J. Clin. Lab. Invest. 37,* 723−728.

Nexø, E., Olsen, H., Bucher, D., & Thomsen, J. (1977) *Biochim. Biophys. Acta 494,* 395−402.

Nexø, E., & Gimsing, P. (1981) *Scand. J. Clin. Lab. Invest. 41,* 465−468.

Nexø, E., & Olesen, H. (1982) in B_{12} (Dolphin, D., Ed.) Vol. 2, pp 57−85, John Wiley & Sons Inc., New York.

Quadros, E. V., & Rothenberg, Sh.P. (1986) *J. Biol. Chem. 261,* 15455−15460.

Quadros, E. V., Sai, P., & Rothenberg, Sh. (1994) *Arch. Biochem. Biophys. 308,* 192−199.

Ramanujam, K. S., Seetharam, Sh., Ramasamy, M., & Seetharam, B. (1991) *Am. J. Physiol. 260,* G416-G422.

Snyder, G. H., Cennerazzo, M. G., Karalis, A. J., & Field, D. (1981) *Biochemistry 20,* 6509−6581.

Stupperich, E., & Nexø, E. (1991) *Eur. J. Biochem. 199,* 299−303.

Tanford, Ch. (1961) in *Physical Chemistry of Macromolecules* pp 458−489, John Wiley & Sons Inc., New York.

Wickramasinghe, S. N., & Fida, S. (1993) *J. Clin. Pathol. 46,* 537−539.

BI9519213