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Conformational Changes of Transcobalamin Induced by Aquocobalamin Binding

MECHANISM OF SUBSTITUTION OF THE COBALT-COORDINATED GROUP IN THE BOUND LIGAND*

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Binding of aquo-, cyano-, or azidocobalamin (Cbl·OH_2, Cbl·CN, and Cbl·N_3, respectively) to the recombinant human transcobalamin (TC) and haptocorrin from human plasma was investigated via stopped-flow spectroscopy. Association of cobalamins with haptocorrin always proceeded in one step. TC, however, displayed a certain selectivity for the ligands: Cbl·CN or Cbl·N_3 bound in one step with $k_{+1} = 1 \times 10^8$ M^{-1} s^{-1} (20 °C), whereas binding of Cbl·OH_2 under the same conditions occurred in two steps with $k_{+1} = 3 \times 10^7$ M^{-1} s^{-1} (E_a = 30 kJ/mol) and k_{+2} = 0.02 s^{-1} (E_a = 120 kJ/mol). The second step of Cbl·OH_2 binding was interpreted as a transformation of the initial "open" intermediate TC·Cbl·OH_2 to the "closed" conformation TC(Cbl) with displaced water. The backward transition from the closed to the open conformation was the reason for the identical rate-limiting steps during substitution of H_2O in TC·Cbl·OH_2 for cyanide or azide according to the reaction TC(Cbl) → TC·Cbl·OH_2 + CN^-/N_3^-. The cyano and azido forms of holo-TC which were produced behaved as the open proteins. Different conformations of holo-TC, determined by the nature of the active group in the bound Cbl, may direct transportation of cobalamins in the organism.

Vitamin B_{12} (cobalamin, Cbl)[1] is synthesized by certain microorganisms, and humans are ultimately dependent on its external sources. Assimilation of the dietary B_{12} is a complex process with three successive Cbl transporters haptocorrin (HC), intrinsic factor (IF), and transcobalamin (TC) as well as several specific receptors involved (1–3). It makes the mechanism both efficient and fragile, *i.e.* any failure in the internalization process causes B_{12} deficiency. In fact, this is a commonplace problem for approximately 10% of the elderly human population despite a normal dietary supply of B_{12} (4).

Efficient uptake of the very minute amounts of Cbl by an organism attracted attention to the Cbl-binding proteins, and some of their properties are well established. All binders retain Cbl extremely strongly with the dissociation constants of the protein-Cbl complexes lower than 1 pM (2, 5–7). Binding of Cbl affects the structure of specific proteins. Both TC and IF shrink after attachment of Cbl (2, 8), and the compact protein-Cbl complex shows higher affinity to the corresponding receptor (2, 3). HC, however, does not change its size upon Cbl binding (2, 9).

Structural deformations during Cbl binding were observed not only for specific proteins but also for Cbl itself, as concluded from studies on both Cbl-dependent enzymes (10) and Cbl transporters (2). Distortion of enzyme-bound Cbl influences the properties of its catalytically essential upper axial group (called the β-group), which changes mobility by many orders of magnitude during the catalysis (10). However, the structural characteristics of cobalamins, associated with the transporting proteins, remain relatively unexplored. Changes in the absorbance spectrum of Cbl upon its binding to TC, IF, and HC (11–13) were interpreted as stabilization of Cbl in a more rigid state (12) with the restricted mobility of the upper axial ligand (14, 15). Thorough kinetic investigation of the reactions between a transporting protein and cobalamins with different β-groups may help to elucidate the mechanism of formation and the internal organization of the protein-Cbl complex.

In the present work we demonstrate that the interactions between TC and Cbl are influenced by the nature of the upper axial group of the vitamin. Human TC was expressed in yeast *Pichia pastoris* and purified. Cbl binding to TC was studied by stopped-flow spectroscopy. Our experiments showed that the direct attachment of aquo-Cbl (Cbl·OH_2) to TC was followed by a slow structural rearrangement of the TC·Cbl·OH_2 complex, in contrast to a simple one-step binding of two other cobalamins (Cbl·CN and Cbl·N_3). Exchange of the β-group in cobalamins bound to TC pointed to a peculiar conformation of TC·Cbl·OH_2 when compared with TC·Cbl·CN/N_3. Different conformations of holo-TC may potentially provide for specific targeting of different cobalamins in an organism.

EXPERIMENTAL PROCEDURES

Materials

All salts and media components were purchased from Merck, Roche Molecular Biochemicals, Sigma, and Difco and were of analytical grade. The enzymes and kits for DNA handling were obtained from New England Biolabs, Stratagene, and Roche Molecular Biochemicals. The kit for polymerase chain reaction was from HT Biotechnology Ltd. Human kidney Quick-Clone cDNA was purchased from Clonotech. Oligonucleotides were synthesized by DNA technology. The yeast expression system was purchased from Invitrogen. Fast Flow CM-Sepharose and Sephacryl S-200 were obtained from Amersham Pharmacia Biotech.

Methods

Preparation of the DNA Material—A TC-encoding fragment of DNA was amplified from a Quick-Clone cDNA preparation (human kidney)

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[1] The abbreviations used are: Cbl, cobalamin; B_{12}, vitamin B_{12}; Cbl·OH_2/·CN/·N_3, aquo/cyano/azidoCbl; Cbl*·CN, [^{57}Co]cyanocobalamin; Gnd, guanidine hydrochloride; HC, haptocorrin; IF, intrinsic factor; TC, transcobalamin; E_a, activation energy.

by polymerase chain reaction using TC-specific primers with adaptors for *Sal*I and *Spe*I endonuclease sites. The major 1.2-kilobase pair DNA band on agarose electrophoresis was purified by electroelution and ligated to *Xho*I and *Spe*I sites of the expression plasmid pPIC-Z. The designed sequence of the fusion protein contained the secretion α-signal, the site for yeast protease Kex2, and the mature human TC. This construction ensured cleavage of the NH_2-terminal peptides from the recombinant protein during its secretion: LDKR ↓ EMCEI (TC residues are underlined).

Expression and Purification of the Recombinant Human TC—The approach was analogous to the one described for bovine TC (15). In short, recombinant yeast cells SMD 1168 were grown to high density in glycerol-containing medium (4 liters). They were subsequently incubated in the same volume of the induction medium with methanol and Cbl·OH_2. Proteins in the cell-free supernatant were precipitated by ammonium sulfate and subjected to ion exchange chromatography. Batch adsorption on CM-Sepharose (12 ml) was performed in 5 mM P_i buffer (NaH_2PO_4/Na_2HPO_4), pH 6, and the matrix was washed sequentially with 0.02, 0.05, and 0.1 M P_i buffer, pH 6. Red-colored holo-TC was eluted from CM-Sepharose with 0.2 M P_i buffer, pH 7.5. The obtained preparation was treated with 2,500 units of peptide *N*-glycosydase F at 37 °C for 2 days to remove carbohydrates from the protein. Then, TC was subjected to gel filtration on Sephacryl S-200 (250 ml) equilibrated with 0.1 M Tris, 1 M NaCl, pH 7.5. The fractions with red-colored protein were pooled and repurified on a 2-ml CM-Sepharose column. The correct NH_2-terminal sequence of human TC was confirmed on a protein sequencer 477A (Perkin-Elmer). The final preparation contained 10–15 mg of the recombinant TC saturated with Cbl·OH_2. It was either stored at −20 °C or dialyzed against H_2O and lyophilized.

Preparation of Unsaturated TC—The isolated holo-TC was depleted of endogenous Cbl by dialysis against 5 M Gnd for 30–40 h at 30 °C. The denaturing agent was removed during extensive dialysis against 0.2 M P_i buffer, pH 7.5, for 24 h at 5 °C. The preparation was centrifuged briefly to remove a small amount of insoluble protein, and the supernatant with apo-TC was either stored at −20 °C or dialyzed against H_2O and lyophilized.

Isolation of Human HC—Purification procedure for human holo-HC has been described elsewhere (16). Apo-HC was prepared by dialysis of holo-HC against 8 M Gnd as described in the above paragraph.

Determination of Cbl Binding Capacity—We have employed a new spectrophotometric method suitable for the determination of apo-TC at concentrations 0.1–1 mg/ml (2–20 μM). Apo-TC in 0.2 M P_i buffer, pH 7.5, was titrated with Cbl·OH_2 (1–4 μM) at 37 °C. Absorbances at 352 nm and 363 nm were measured after every addition, and the ratio of A_{363} to A_{352} was plotted *versus* the concentration of the added Cbl·OH_2. The ratio of A_{363} to A_{352} remained approximately constant at Cbl·OH_2 < TC, but it decreased steeply when the concentration of Cbl·OH_2 exceeded the binding capacity of TC. The point of intercept between the two linear parts of the chart indicated the equivalence of the apo-TC and Cbl·OH_2 concentrations. Spectrophotometric determination of the binding capacity was confirmed by measurements of the binding capacity employing radioactive Cbl^*·CN according to Gottlieb *et al.* (17). The concentration of unsaturated HC was measured by the method of Gottlieb *et al.* (17).

Determination of the Extinction Coefficients for TC·Cbl—Cyano and azido forms of holo-TC were prepared by incubation of TC·Cbl·OH_2 with 10 mM KCN or 50 mM NaN_3 for 5 h at 37 °C. The spectra of TC·Cbl·OH_2/CN/N_3 (20–25 μM) were recorded. The amount of TC-associated Cbl was evaluated according to the concentration of the liberated Cbl as described earlier (12).

Binding of Cobalamins to Apo-TC and Apo-HC—The kinetics of apo-TC and apo-HC interaction with cobalamins was investigated on a DX.17MV stopped-flow spectrofluorometer from Applied Photophysics. The reaction was carried out in 0.2 M P_i buffer, pH 7.5, at either 20 °C or 37 °C. A 250-μl portion of the apoprotein (20–30 μM) in one of the drive syringes was mixed instantly with an equal volume of 10–100 μM Cbl from another drive syringe. The process of binding to apo-TC was monitored by the change in the absorbance of the γ1-peak at 363 nm for Cbl·OH_2/N_3 and 365 nm for Cbl·CN. The analogous reaction with apo-HC was followed at 355 nm for Cbl·OH_2 (γ1-peak), 363 nm for Cbl·CN (γ1-peak), and 342 nm for Cbl·N_3 (γ2-peak). In all cases the light pass was 1 cm, and the band pass was 18.6 nm. The dead time of the stopped-flow mixing was determined to be 1.6 ± 0.2 ms, and the measurements were assumed to be reliable after 2 ms of the record. Every transient was a mean of two or three consecutive measurements. Four independent preparations of TC and one preparation of HC were used in the experiments.

Exchange of the Cobalt-coordinated β-Group in Cbl and TC·Cbl—The reaction was performed in 0.2 M P_i buffer, pH 7.5, at either 20 °C or 37 °C. The concentration of the examined object (either Cbl·OH_2 or TC·Cbl·OH_2) was kept constant (20–30 μM) while the concentrations of added azide and cyanide varied in a broad range. The specific absorbance changes were followed either on an M350 double beam UV-visible spectrophotometer (Camspec) or on the stopped-flow equipment.

Mathematic Analysis—Fitting of the curves was performed by a program for nonlinear regression analysis[2] using the appropriate equations for the monomolecular and bimolecular reactions. Complex kinetics was simulated by the computer program Gepasi (18). The rate coefficients used in the simulations were obtained by trial and error, moving from an initial approximate estimate to the more appropriate set of parameters. Activation energies of the reactions were calculated from the rate constants at 20 °C and 37 °C according to the equation $E_a = R \cdot \ln(k_{37}/k_{20})/(1/310\ K - 1/293\ K)$, where $R = 8.315\ J\ mol^{-1}deg^{-1}$. Results of statistical analysis are presented as mean ± S.D.

RESULTS

Isolation and Characterization of Recombinant Human TC—The genetic material for the expression of human TC was produced from commercially available human kidney cDNA. The corresponding cDNA fragment was amplified by polymerase chain reaction using TC-specific primers, and the TC cDNA obtained was ligated to the expression plasmid. The sequence corroborated accuracy of the construction and was consistent with the known alleles of human TC (19, 20). In fact, it was identical to the sequence presented by Platica *et al.* (19) except for Ser^{376} (TCG codon) replaced in our preparation by Leu^{376} (TTG codon). The Leu residue at position 376 was also observed in other TC alleles (20).

Isolation of the recombinant human TC was in many aspects similar to the method described for the recombinant bovine TC in our recent publication (15). SDS-electrophoresis of the final preparation contained a major band of 46 kDa and a number of minor bands (46–48 kDa) all having the NH_2-terminal sequence of human TC (not shown). The bands with a molecular mass of above 46 kDa presumably represented human TC containing a small amount of carbohydrates remaining after deglycosylation (see "Methods").

The characteristics of Cbl binding and receptor recognition observed for the recombinant TC were similar to those for the natural TCs (11, 13, 21) and for the recombinant bovine TC (15). Thus, the protein-ligand complex (TC·Cbl·OH_2) was very stable, and the procedure of apo-TC preparation required prolonged incubation in Gnd. When apo-TC after Gnd treatment was resaturated with [^{57}Co]cyanocobalamin (Cbl^*·CN) and exposed to a high excess of the nonradioactive Cbl·CN, no considerable release of Cbl^*·CN was observed during 240 h of incubation at 20 °C. Liberation of Cbl^*·CN from holo-TC at a higher temperature (37 °C) was exponential with the rate constant $k_- = 0.006\ h^{-1}$ (not shown). For comparison, the recombinant bovine TC showed analogous behavior in our previous work (15), whereas holo-TC from bovine milk was characterized by $k_- = 0.007\ h^{-1}$ in the analogous experiment at 20 °C (7). The recombinant human holo-TC was capable of binding to the specific receptor in the membranes of human placenta in a way indistinguishable from the natural human TC (not shown). Unfolded human TC did not contain free SH groups (<0.05 SH/TC molecule) as demonstrated by treatment of the protein with 5,5′-dithiobis(2-nitrobensoic acid) in 5 M Gnd. This fact confirmed the presence of four S-S bridges in human TC proposed in our previous publication (15).

Treatment of Cbl·OH_2 (either free or bound to TC) with an excess of cyanide or azide converted the vitamin to its cyano or azido form, respectively, with the characteristic spectral changes (Fig. 1). The recorded absorbance spectra of human holo-TC in complex with different cobalamins (Fig. 1*B*) were

[2] S. N. Fedosov, unpublished data.

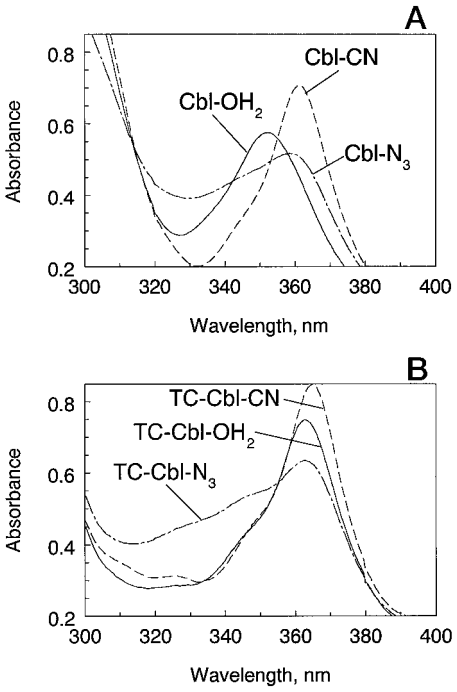

FIG. 1. **Absorbance spectra of different cobalamins in the solution (*panel A*) and in the protein-bound state (*panel B*).** The examined substance (Cbl·OH_2 or TC·Cbl·OH_2, 25 μM) was incubated with a saturating concentration of either CN^- or N_3^- in 0.2 M P_i buffer, pH 7.5, 37 °C. The determined extinction coefficients of the most intensive γ-peak in free Cbl were: ϵ_{352} = 23,000 ± 700 (Cbl·OH_2 at pH 7.5, n = 8), ϵ_{361} = 28,060 (Cbl·CN (22)), and ϵ_{352} = 22,300 ± 700 (Cbl·N_3, n = 6). Analogous coefficients for holo-TC were: ϵ_{363} = 30,000 ± 500 (TC·Cbl·OH_2, n = 6), ϵ_{365} = 33,300 ± 200 (TC·Cbl·CN, n = 6), and ϵ_{363} = 25,800 ± 600 (TC·Cbl·N_3, n = 6).

comparable to those of holo-IF/-HC/-TC from different sources (11–13). Comparison with the spectra of the free ligands (Fig. 1*A*) revealed the expected shift and intensification of the γ-peak in all protein-ligand complexes. It was particularly clear in the case of Cbl·OH_2 where the original γ-peak was 11 nm red-shifted, and its intensity increased by a factor of 1.3 upon binding to TC.

Binding of Cobalamins to Apo-TC—Binding of Cbl·CN and Cbl·N_3 to TC proceeded quickly and monophasically in accordance with an irreversible bimolecular mechanism (Fig. 2*A*). The high values obtained for the association rate constants at 20 °C (Table I) precluded reliable measurements at 37 °C.

The absorbance change induced by the binding of Cbl·OH_2 to TC was biphasic both at 20 °C and 37 °C (Fig. 2, *A* and *B*). The first phase was accomplished mainly in 20 ms and revealed a dependence on the Cbl concentration (Fig. 2*B*). The process corresponded to an irreversible bimolecular reaction with the rate constant shown in Table I and the activation energy E_a = 30 ± 8 kJ/mol. The second phase was much slower and continued for 200 s at 20 °C and 20 s at 37 °C (Fig. 2, *A* and *B*). The time course of that reaction was independent of Cbl concentration and could be fitted adequately to a monomolecular process (either reversible or irreversible) with the rate coefficient presented in Table I. The activation energy for the second phase E_a = 120 ± 10 kJ/mol exceeded the one for the first phase considerably.

Binding of cobalamins to TC can be represented by Scheme 1a for Cbl·CN and Cbl·N_3 or Scheme 1b for Cbl·OH_2 (Fig. 3) where one can expect that $k_{+1} \gg k_{-1}$ and $k_{+2} > k_{-2}$ (*i.e.* $k_1 \approx k_{+1}$ and $k_2 \approx k_{+2}$).

Binding of Cobalamins to Apo-HC—The observed reactions corresponded to an irreversible bimolecular process (see Fig. 2*C* and Table I). None of the cobalamins revealed a slow phase during the binding to HC.

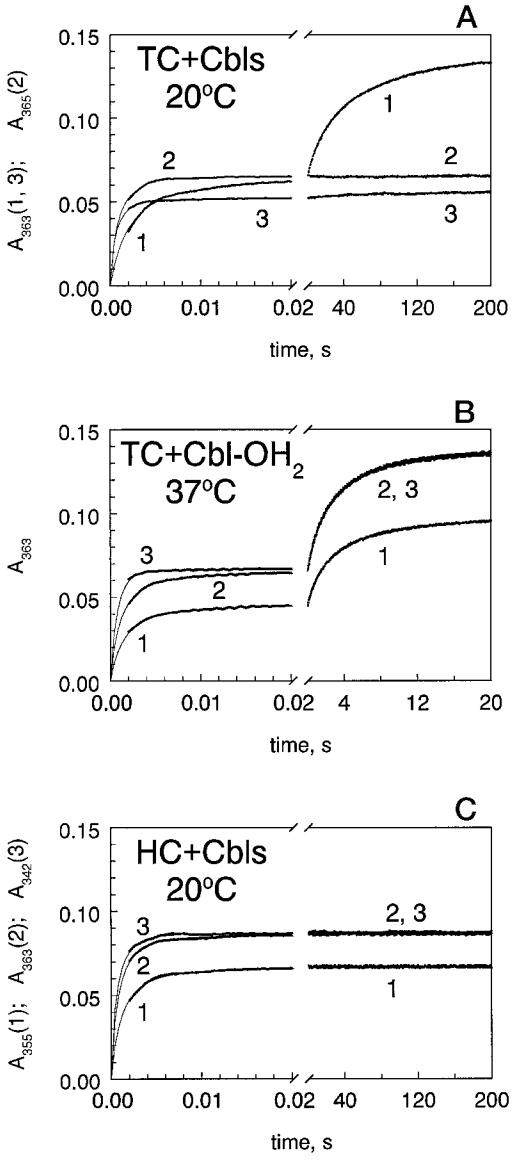

FIG. 2. **Change in absorbance of cobalamins during their binding to apo-TC (*panels A* and *B*) or apo-HC (*panel C*) measured by stopped-flow equipment.** The protein (12 μM) in 0.2 M P_i buffer, pH 7.5, was mixed with different concentrations of cobalamins, and the change in the specific absorbance was followed in time. *Thin lines* represent the best fit to a bimolecular or a monomolecular reaction. *Panel A*, transient changes in the absorbance of 16 μM cobalamins induced by mixing with apo-TC at 20 °C. In the first phase the calculated rate constants of the bimolecular reaction were 32 μM^{-1} s^{-1} for Cbl·OH_2, 88 μM^{-1} s^{-1} for Cbl·CN, and 126 μM^{-1} s^{-1} for Cbl·N_3 (*curves 1*, *2*, and *3*, respectively). In the second phase the calculated rate constant of the monomolecular reaction for Cbl·OH_2 was 0.020 s^{-1} (*curve 1*). No change in the absorbance was detected for Cbl·CN (*curve 2*) or Cbl·N_3 (*curve 3*). *Panel B*, transient changes in the absorbance of Cbl·OH_2 induced by mixing with apo-TC at 37 °C. The reaction was performed with 8, 16, and 32 μM Cbl·OH_2 (*curves 1*, *2*, and *3*, respectively). In the first phase the calculated rate constants of the bimolecular reaction were 59, 62, and 46 μM^{-1} s^{-1} (*curves 1*, *2*, and *3*, respectively). In the second phase the calculated rate constants of the monomolecular reaction were 0.26, 0.29, and 0.28 s^{-1} (*curves 1*, *2*, and *3*, respectively). *Panel C*, transient changes in the absorbance of 16 μM cobalamins induced by mixing with apo-HC at 20 °C. In the rapid phase the calculated rate constants of the bimolecular reaction were 62 μM^{-1} s^{-1} for Cbl·OH_2, 94 μM^{-1} s^{-1} for Cbl·CN, and 125 μM^{-1} s^{-1} for Cbl·N_3 (*curves 1*, *2*, and *3*, respectively). No following change of the specific absorbance was detected for these ligands.

Exchange of the Cobalt-coordinated β-Group in the Free

TABLE I

Rate coefficients measured for the Cbl binding to apo-TC or apo-HC and for the displacement of cobalt-coordinated H_2O in Cbl·OH_2 or TC·Cbl·OH_2

Reaction	Temperature	No. of experiments	Bimolecular reaction	Monomolecular reaction
	°C	*n*	*k*, M^{-1} s^{-1}	*k*, s^{-1}
			Phase 1	*Phase 2*
TC + Cbl·CN	20	3	100×10^6	
TC + Cbl·N_3	20	3	130×10^6	0.02 ± 0.001
TC + Cbl·OH_2	20	9	$(30 \pm 6) \times 10^6$	0.3 ± 0.08
TC + Cbl·OH_2	37	14	$(60 \pm 10) \times 10^6$	
			Phase 1	*Phase 2*
HC + Cbl·CN	20	3	90×10^6	
HC + Cbl·N_3	20	3	120×10^6	
HC + Cbl·OH_2	20	3	60×10^6	
			Forward	*Reverse*
Cbl·OH_2 + CN^-	20	10	40 ± 4	
Cbl·OH_2 + CN^-	37	15	230 ± 20	
Cbl·OH_2 + N_3^-	20	5	680 ± 6	ND[a]
Cbl·OH_2 + N_3^-	37	9	$2{,}500 \pm 150$	0.125 ± 0.025
				Forward
$CN^-/N_3^- \rightarrow \infty$				
TC·Cbl·OH_2 + CN^-	37	3		50×10^{-5}
TC·Cbl·OH_2 + CN^-	37	3		50×10^{-5}
TC·Cbl·OH_2 + N_3^-	20	3		8×10^{-5}
TC·Cbl·OH_2 + N_3^-	37	3		55×10^{-5}

[a] ND, not determined.

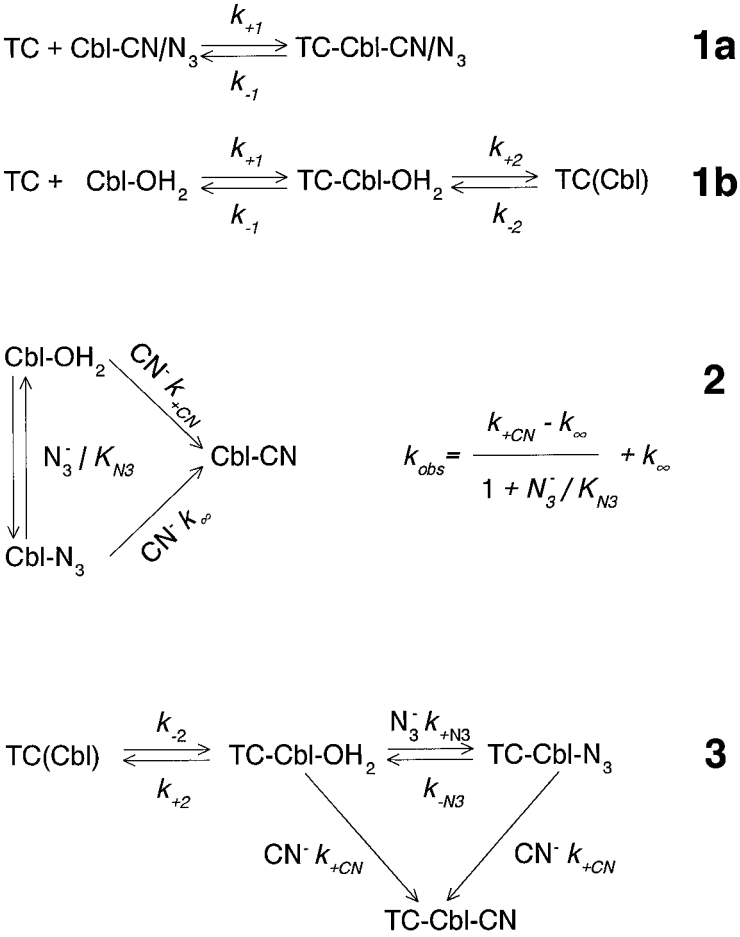

FIG. 3. **Kinetic schemes used for description of Cbl binding to TC and of the β-group exchange.**

Cbl—Displacement of H_2O in Cbl·OH_2 (20–30 μM) by either cyanide or azide was followed by a change in the specific absorbance of Cbl. The reaction with cyanide proceeded according to a simple bimolecular mechanism Cbl·OH_2 + CN^- → Cbl·CN and was irreversible under the conditions of the experiment (Fig. 4*A*). The rate constant of this reaction (k_{+CN}) was measured at two temperatures, and the obtained values are presented in Table I. They correspond to the activation energy E_a = 80 ± 5 kJ/mol. The binding of azide (Fig. 4*B*) was a reversible process Cbl·OH_2 + N^-_3 ⇔ Cbl·N_3 described by the parameters shown in Table I. The activation energy of the binding step (k_{+N3}) was E_a = 60 ± 4 kJ/mol. The calculated rate constants

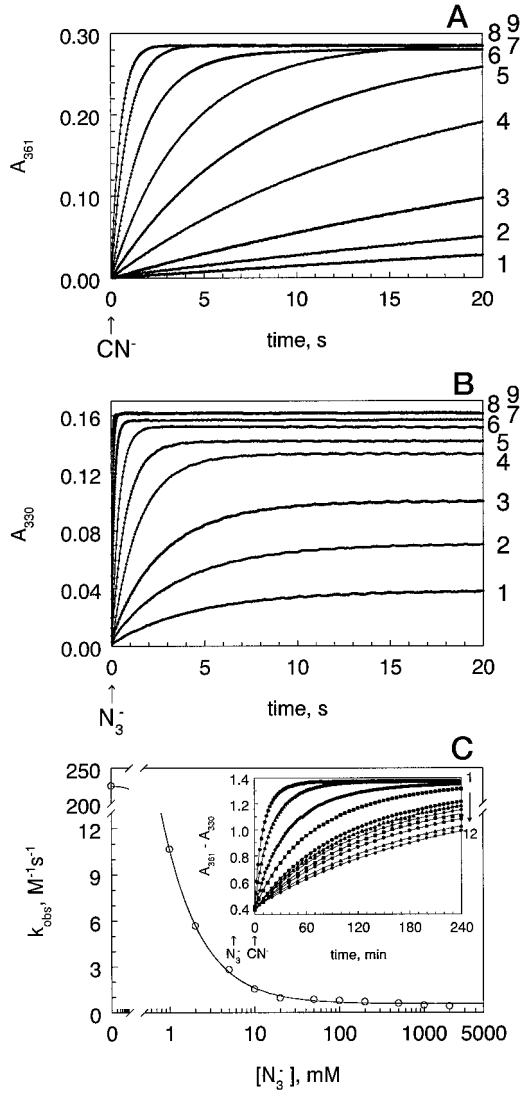

FIG. 4. **Exchange of the β-group in the protein-free Cbl, 0.2 M P_i buffer, pH 7.5, 37 °C.** *Panel A*, reaction of Cbl·OH_2 (32 μM) with cyanide (mM): 0.025, 0.05, 0.1, 0.25, 0.5, 1, 2.5, 5, and 10 (*curves 1–9*, respectively). The calculated rate constant of the irreversible bimolecular reaction was $k_{+CN} = 228 \pm 18$ M^{-1} s^{-1} ($n = 15$). *Panel B*, reaction of Cbl·OH_2 (32 μM) with azide (mM): 0.025, 0.05, 0.1, 0.25, 0.5, 1, 2.5, 5, 10 (*curves 1–9*, respectively). The calculated rate constants of the reversible bimolecular reaction were $k_{+N3} = 2{,}500 \pm 150$ M^{-1} s^{-1} ($n = 9$) and $k_{-N3} = 0.125 \pm 0.025$ s^{-1} ($n = 6$). *Panel C*, reaction of the mixture Cbl·OH_2/Cbl·N_3 with cyanide. *Inset*, time dependence. In *step1*, Cbl·OH_2 (65 μM) was incubated for 30 min with different concentrations of azide (mM): 0, 1, 2, 5, 10, 20, 50, 100, 200, 500, 1,000, and 2,000 (*curves 1–12*, respectively). In *step 2*, the sample was exposed to 172 μM cyanide, and the changes in absorbance were monitored. *Main figure*, the observed rate coefficients of the bimolecular reaction were calculated for each time curve and plotted *versus* the N_3^- concentration. The data were fitted by Scheme 2 and the equation $k_{obs} = 225/(1 + x/0.047) + 0.57$.

were consistent with the known values of K_d for the dissociation of cyanide or azide from their complexes with Cbl: $K_d < 1$ pM and $K_d = 40$ μM, respectively (22). Substitution of water in the β-position of Cbl was a monophasic reaction in both cases, *i.e.* the acid-base mixture Cbl·OH_2/OH observed at pH 7.5 (12) behaved as a homogeneous pool of molecules but not as the independently reacting species Cbl·OH_2 and Cbl·OH.

When Cbl·OH_2 was converted to Cbl·N_3 by 1–2,000 mM N_3^-, the following reaction with cyanide (172 μM) was essentially decelerated (Fig. 4*C*, *inset*). The rate coefficient of CN^- binding on the background of saturating azide decreased by a factor of 400 and stabilized on a constant level (Fig. 4*C*). This observation implied that cyanide was able to displace cobalt-coordinated azide in two ways: 1) by competition for the remaining Cbl·OH_2 and reversion of N_3^- binding and 2) by direct interaction with Cbl·N_3. The system is described by Scheme 2 (Fig. 3) and fitted to a second order reaction with the observed rate coefficient k_{obs}. The calculated kinetic parameters were: $k_{+CN} = 225$ M^{-1} s^{-1}, $K_{N3} = 47$ μM, $k_\infty = 0.57$ M^{-1} s^{-1}, 37 °C (Fig. 4*C*).

Exchange of Cobalt-coordinated β-Group in Holo-TC—Similar experiments on the group exchange were carried out with cobalamins bound to TC. Reaction of TC·Cbl·OH_2 with CN^- resembled the H_2O substitution in the protein-free Cbl·OH_2 but only at a low concentration of cyanide (Fig. 5*A*). Thus, the initial velocity of the reaction increased proportionally to the concentration of the added compound at $CN^- < 1$ mM. At higher concentrations of the reagent the initial velocity showed a clear tendency to stabilize at a constant level independently of the CN^- concentration. Extrapolation to an infinite CN^- concentration resulted in a rate coefficient shown in Table I where the activation energy of the process was equal to E_a = 70–80 kJ/mol.

The TC·Cbl·OH_2 interaction with azide (Fig. 5*B*) also deviated from the reversible bimolecular mechanism observed for the binding of azide to free Cbl·OH_2. Although the N_3^- concentration increased from 0.15 to 50 mM, the rate coefficient of the process decreased 10 times and stabilized at a constant level with E_a = 85 kJ/mol. The parameters of the reaction with holo-TC at saturating azide were almost identical to those at saturating cyanide (Table I).

In the next assay TC·Cbl·OH_2 was preequilibrated with 0–100 mM azide and exposed to a constant level of cyanide (Fig. 5*C*). The reaction revealed two distinct phases at subsaturating azide, where the amplitude of the rapid phase was more prominent at higher N_3^- (Fig. 5*C*). Velocity of the first phase declined with increasing N_3^- and stabilized at a constant level. Velocity of the second phase of cyanide binding was independent of N_3^- and corresponded to the reaction TC·Cbl·OH_2 + CN^-.

The general behavior of the system during exchange of the β-group in holo-TC (Fig. 5, *A–C*) was simulated according to Scheme 3 (Fig. 3) where $k_{+2} = 0.3$ s^{-1}, $k_{-2} = 5 \times 10^{-4}$ s^{-1}, $k_{+N3} = 2{,}500$ M^{-1} s^{-1}, $k_{-N3} = 7.5 \times 10^{-3}$ s^{-1}, $k_{+CN} = 250$ M^{-1} s^{-1}, $k_\infty = 7 \times 10^{-3}$ M^{-1} s^{-1} at 37 °C. The results of simulation are depicted in Fig. 5, *A–C*, as *solid lines*.

Effect of Different Cobalamins on the Solubility of Holo-TC—Irreversible precipitation of both TC·Cbl·CN and TC·Cbl·N_3 was induced by the temperature jump from −20 °C to 37 °C as well as by prolonged (20–30 h) storage at 20–37 °C. No effect of that kind was observed for TC·Cbl·OH_2 at concentrations as high as 7 mg/ml. Treatment of holo-TC with 10 mM dithiothreitol also revealed different solubilities of the complexes: the aquo complex precipitated after 1 h of incubation at 37 °C whereas the cyano and azido complexes remained soluble. Prolonged dialysis against SH-containing buffer resulted in liberation of the endogenous Cbl, as it was established earlier for the recombinant bovine TC (15).

DISCUSSION

The experimental data on cobalamins binding to the specific transporters are rather scattered and not always comparable. In addition, most of the available information was obtained in experiments under equilibrium conditions, thus precluding identification of the individual steps of the binding process. Expression of the recombinant human TC in a sufficient amount provided the opportunity to perform the stopped-flow spectroscopic measurements and analyze details of the binding process.

Those experiments demonstrated that the binding of the vitamin to TC was rapid and followed a one-step mechanism for

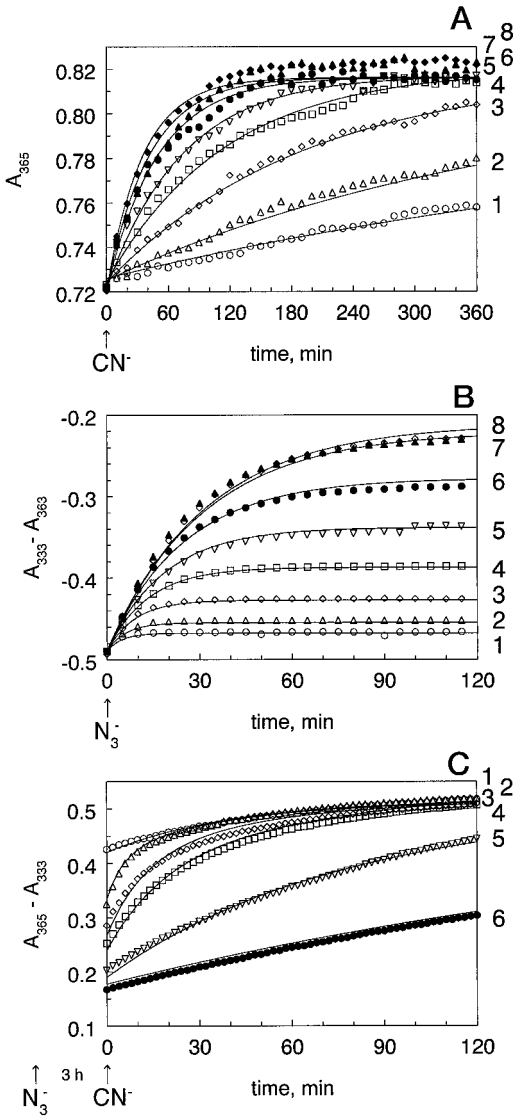

FIG. 5. **Exchange of the β-group in the TC-bound Cbl, 0.2 M P_i buffer, pH 7.5, 37 °C.** *Panel A*, reaction of TC·Cbl·OH_2 (24 μM) with cyanide (mM): 0.05, 0.01, 0.25, 0.5, 1, 2.5, 5, and 20 (*curves 1–8*, respectively). The process obeyed an irreversible mechanism with two steps: TC(Cbl) ⇔ TC·Cbl·OH_2 + CN^- → TC·Cbl·CN, where transition on the first step became rate-limiting at $CN^- \rightarrow \infty$ ($k \rightarrow 5 \times 10^{-4}$ s^{-1}). *Solid lines* represent simulations based on Scheme 3 considering that conversion of 1 μM Cbl·OH_2 into 1 μM Cbl·CN corresponds to an increase of A_{365} by 0.004 of the optical units. *Panel B*, reaction of TC·Cbl·OH_2 (24 μM) with azide (mM): 0.15, 0.25, 0.5, 1, 2, 5, 25, and 50 (*curves 1–8*, respectively). The process obeyed a reversible mechanism with two steps: TC(Cbl) ⇔ TC·Cbl·OH_2 + N_3^- ⇔ TC·Cbl·N_3 where transition on the first step was always rate-limiting ($k \rightarrow 5 \times 10^{-4}$ s^{-1} at $N_3^- \rightarrow \infty$). *Solid lines* represent simulations based on Scheme 3 considering that conversion of 1 μM Cbl·OH_2 into 1 μM Cbl·N_3 corresponds to an increase of A_{333} to A_{363} by 0.0115 of the optical units. *Panel C*, reaction of the mixture TC·Cbl·OH_2/TC·Cbl·N_3 with cyanide. In *step1* TC·Cbl·OH_2 was incubated for 3 h with different concentrations of azide (mM): 0, 1, 3, 5, 20, and 100 (*curves 1–6*, respectively). In *step2* the sample was exposed to 5 mM cyanide. The process obeyed a mechanism shown in Scheme 3 with two major branches of the reaction: rapid TC·Cbl·N_3 ⇔ TC·Cbl·OH_2 + CN^- and slow TC(Cbl) ⇔ TC·Cbl·OH_2 + CN^-. *Solid lines* represent simulations according to Scheme 3 where the values of A_{365} to A_{363}/1 μM of the corresponding complex were 0.0175 for TC(Cbl), 0.0215 for TC·Cbl·CN, and 0.0072 for TC·Cbl·N_3 (absorbance of TC·Cbl·OH_2 intermediate was of no practical importance because of its low concentration in comparison with other intermediates).

Cbl·CN and Cbl·N_3 (analogous to the interaction of Cbl·CN with bovine TC (7)), while the same process for Cbl·OH_2 comprised both rapid and slow phases (Fig. 2). Fast reactions observed for all cobalamins were dependent on the Cbl concentration, *i.e.* they apparently reflected direct attachment of the ligand to the protein. The binding rate constants of the ligands increased in the following order: Cbl·OH_2 ($1 \times k_{+1}$), Cbl·CN ($3 \times k_{+1}$), Cbl·N_3 ($4 \times k_{+1}$) (Table I). The similar array of values was obtained in experiments with another Cbl-specific protein, human HC, although the dispersion of k_{+1} for Cbl·OH_2/CN/N_3 binding was less pronounced (Table I). The observed difference in the binding rate constants probably has a structural basis: NMR analysis (23) as well as crystallographic studies (24) revealed variations in the three-dimensional structure of Cbl, depending on the compound coordinated at the β-position.

Although the concentration-dependent rapid phase was observed for all three ligands, the slow phase of the absorbance change was associated specifically with the Cbl·OH_2 binding to TC (Fig. 2, *A* and *B*). The biphasic nature of this reaction does not seem to be caused by the acid-base heterogeneity of Cbl·OH_2/OH at pH 7.5 (12) for the following reasons. First, the half-reaction time of the second phase was independent of the concentration of Cbl·OH_2. Second, the activation energy of the reaction was very high (E_a = 120 kJ/mol). In fact, the calculated E_a value was of the same order of magnitude as the activation energy observed for some essential rearrangements in the protein structure (25). Therefore, the slow phase of absorbance change can be attributed to a significant transformation in the ternary structure of TC·Cbl·OH_2 complex affecting properties of Cbl·OH_2 in the binding site. The slow phase was not observed when cobalamins reacted with another specific protein (HC) under identical conditions (Fig. 2, C). This points to lower sensitivity of HC to the ligand structure, which was also observed during binding of Cbl analogs (1, 2, 5).

The ability of TC to distinguish among cobalamins with different upper axial groups (in our case -OH_2, -CN, and -N_3) is apparently associated with the internal properties of these cobalt-coordinated compounds. It is known that Cbl (both free and in complex with a specific protein) can exchange its β-group in favor of an external compound capable of complex formation with corrins and present in the sufficient concentration (12, 15, 22, 26). These compounds can be classified according to their affinity to the Cbl ring as strong, intermediate, and weak substituents, where CN^-, N_3^- and H_2O are typical representatives from the above classes (22). Amino acid residues (histidine, lysine) are also potential candidates for the coordination at β-position of Cbl as imidazole and amino groups exhibit intermediate affinity to this site (12, 22, 26). The above classification may serve as a clue to the nature of the conformational transition inferred from the kinetic experiments. Upon binding of Cbl·OH_2 to TC, the weakly associated water of Cbl·OH_2 can be displaced by an amino acid residue of the protein, which is reflected by the second slow phase of the absorbance change. At the same time, the relatively strong substituents (CN^- and N_3^-) persist this displacement because of their high affinity to Cbl. The conformational rearrangement of the protein during the binding of Cbl·CN and Cbl·N_3 is therefore hampered, and the slow phase of the reaction is not observed.

The designed kinetic model of interaction between TC and Cbl, presented in Fig. 6, postulates the existence of at least two distinct conformations of holo-TC: "open" and "closed." The complexes of TC with Cbl·CN and Cbl·N_3 exist exclusively in the open conformation, whereas the prevailing form of the aquo complex is the closed one, where the upper surface of the bound Cbl is inaccessible for the external agents. We suggest here a substitution of the upper axial water in Cbl·OH_2 for an amino acid residue, but a simple shielding of the upper surface of Cbl·OH_2 by the protein is also a feasible explanation. If one

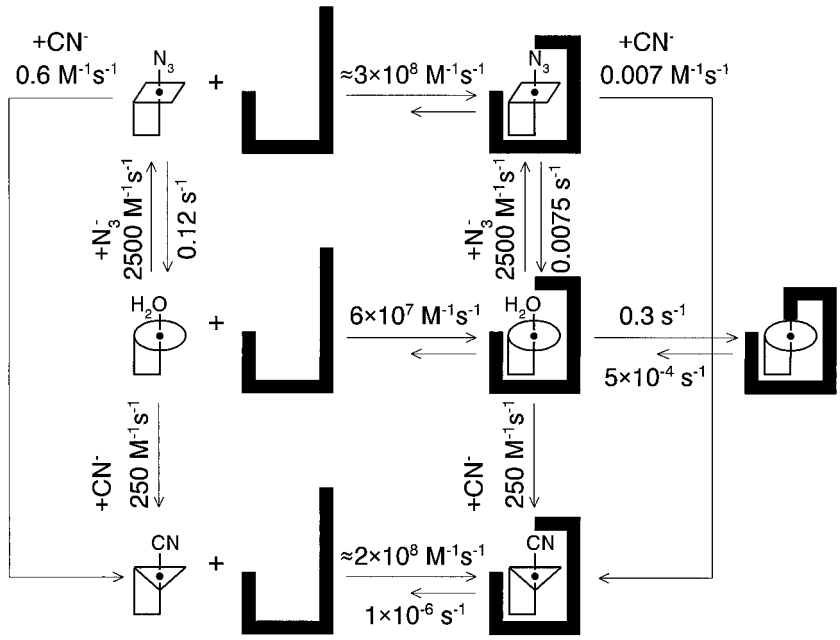

FIG. 6. **Mechanism of binding of different cobalamins to TC and exchange of the β-groups in the free and TC-bound vitamin.** The shown values of the rate constants correspond to the reactions at 37 °C. The rate constants for the binding of Cbl·CN and Cbl·N_3 to TC at 37 °C were calculated from the data at 20 °C assuming the same activation energy as for Cbl·OH_2.

exposes the aquo form of holo-TC to a high concentration of an external cobalt complexion, the added ligand would react rapidly only with the open conformation of TC·Cbl·OH_2. This would make the slow transition between the protein conformations rate-limiting for the whole process: TC(Cbl) → TC·Cbl·OH_2 + CN^-/N_3^- . . . Under these circumstances, one can expect the apparent mechanism of binding to be consistent with the monomolecular reaction (but not the bimolecular one) and characterized by the same parameters for any ligand. This effect was confirmed experimentally when the aquo form of holo-TC was exposed to either CN^- or N_3^- (known to bind to free Cbl·OH_2 with different velocities). At high concentrations of the reagents both reactions became monomolecular (Fig. 5, *A* and *B*) and were described by the comparable rate coefficients (Table I) and the similar activation energies E_a = 80 kJ/mol. This is a strong argument for the identity of the critical steps in both processes corresponding in our model to the backwards transition between the closed and open conformations.

Other evidence supporting the suggested model comes from the biphasic kinetics seen for the interactions of cyanide with the heterogeneous pool comprising both azido and aquo forms of holo-TC (Fig. 5*C*). The rapid phase reflects the reaction of cyanide with the available and fast equilibrating open pool TC·Cbl·N_3 ⇔ TC·Cbl·OH_2, whose absolute size increases at high concentrations of N_3^-. Cyanide preferentially interacts with the TC·Cbl·OH_2 component of the open pool as follows from the declining velocity of the rapid phase at increasing N_3^- concentrations. The second phase of the process, seen at subsaturating N_3^- concentrations, was associated with the slow transition from the remaining closed form to the open conformation TC(Cbl) ⇔ TC·Cbl·OH_2 followed by immediate equilibration of TC·Cbl·OH_2 with N_3^- and CN^-. Validity of the proposed model has been supported by the fact that the computer simulation of its kinetic behavior resulted in the appropriate fit of the experimental data (Fig. 5).

Stabilities of the open and closed conformations of holo-TC in solution seem to be different: whereas the aquo complex (the only species with the closed conformation) was soluble at a high protein concentration in a wide range of temperature, the cyano and azido complexes precipitated irreversibly during storage or because of temperature jumps. Treatment of holo-TC with dithiothreitol also caused different consequences: the aqua form precipitated within 1 h, whereas cyano- and azido-TC remained soluble during that time.

The presented interpretation of the data also has a physiological aspect. If different tissues exhibit some preferences toward certain forms of cobalamin, the ability of holo-TC to exist in different conformations (depending on the bound ligand) may be important for the trafficking of the vitamin and the receptor recognition. In fact, it was shown that the uptake of 5′-deoxyadenosyl-Cbl by reticulocytes was four times more efficient than that of Cbl·CN (27). The relatively high content of Cbl·OH_2 in the body (20–30% of all cobalamins (28)) may imply biological significance of the closed TC conformation during transportation of Cbl.

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