Comparative Analysis of Cobalamin Binding Kinetics and Ligand Protection for Intrinsic Factor, Transcobalamin, and Haptocorrin*

Sergey N. Fedosov‡§, Lars Berglund‡, Natalya U. Fedosova¶, Ebba Nexø, and Torben E. Petersen‡

From the ‡Protein Chemistry Laboratory, Department of Molecular and Structural Biology, University of Aarhus, Science Park, Gustav Wieds Vej 10, 8000 Aarhus C, Denmark, the §Department of Biophysics, University of Aarhus, Ole Worms Alle 185, 8000 Aarhus C, Denmark, and the ¶Department of Clinical Biochemistry, AKH Aarhus University Hospital, Nørrebrogade 44, 8000 Aarhus C, Denmark

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Changes in the absorbance spectrum of aquo-cobalamins (Cbl\(-\text{OH}_2\)) revealed that its binding to transcobalamin (TC) is followed by slow conformational reorganization of the protein-ligand complex (Fedosov, S. N., Fedosova, N. U., Nexø, E., and Petersen, T. E. (2000) J. Biol. Chem. 275, 11791–11798). Two phases were also observed for TC when interacting with a Cbl-analogue cobinamide (Cbi), but not with other cobalamins. The slow phase had no relation to the ligand recognition, since both Cbl and Cbi bound rapidly and in one step to intrinsic factor (IF) and haptocorrin (HC), namely the proteins with different Cbl specificity. Spectral transformations observed for TC in the slow phase were similar to those upon histidine complexation with Cbl\(-\text{OH}_2\) and Cbi. In contrast to a closed structure of TC-Cbl\(-\text{OH}_2\)\(\text{OH}_2\), the analogous IF and HC complexes revealed accessibility of Cbl's upper face to the external reagents. The binders decreased sensitivity of adenosyl-Cbl (Cbl\(\text{Ado}\)) to light in the range: free ligand, IF, HC, TC-Cbl\(\text{Ado}\). The spectrum of TC-Cbl\(\text{Ado}\) differed from those of IF and HC and mimicked Cbl\(\text{Ado}\) participating in catalysis. The above data suggest presence of a histidine-containing cap shielding the Cbl-binding site in TC. The cap coordinates to certain corrinoids and, possibly, produces an encapsulated Ado-radical when Cbl\(\text{Ado}\) is bound.

Intrinsic factor (IF), transcobalamin (TC) and haptocorrin (HC) are three proteins involved in assimilation and transport of cobalamin (Cbl) in an organism (1). They all have extraordinary affinity to the physiologically active forms of Cbl with \(K_d < 1\ \text{pm}\) \(\text{(1–4)}\) but exhibit different selectivity toward the non-functional Cbl analogues. IF and, to some extent, TC are sensitive to variations in the structure of the ligand, which helps these proteins to discriminate between the physiologically active and inactive corrinoids (2, 5). On the contrary, HC can successfully bind many defective corrinoids lacking even the whole nucleotide moiety (2, 5). Binding to the carriers shields the lower part of the Cbl molecule (also called \(\alpha\)-site), which contains the nucleotide. On the contrary, the upper surface of Cbl (\(\beta\)-site) with the active group is thought to be open, as judged from its reactivity with the external compounds in the case of holo-IF and holo-HC (6–8).

Low amounts of the Cbl-binding proteins available from the natural sources (1, 6, 9, 10) hampered their investigations until several binders were successfully expressed in the recombinant organisms (11–15). The sufficient amounts of both bovine and human transcobalamin were obtained from the recombinant yeast \(P\text{ichia pastoris}\). It allowed to establish the structure of the disulfide bridges in bovine TC (14) and investigate in detail Cbl\(\text{OH}_2\) binding to human TC by a stopped-flow technique (15). It was shown that the association between TC and Cbl\(\text{OH}_2\) occurred in two steps, when the initial attachment to an open conformation of the protein was followed by transition to a closed conformation with the shielded upper face of Cbl. As a result of this transition, cobalt-coordinated water in Cbl\(\text{OH}_2\) was thought to be displaced by a protein residue. The suggestion was supported by the fact that the external compounds coordinated to the \(\beta\)-position of TC-Cbl\(\text{OH}_2\) at exceedingly slow rates. The described features, however, appeared to be characteristic only for Cbl\(\text{OH}_2\) interacting with TC, whereas binding of Cbl\(\text{OH}_2\), for instance, to HC occurred in one step (15). Cobalamins with the tightly associated \(\beta\)-groups (Cbl-CN and Cbl\(\text{N}_2\)) bound both to TC and HC in one step as well (15).

The experiments on Cbl\(\text{OH}_2\) interaction with TC and HC suggested a correlation between high specificity of the carrier for Cbl and the biphasic nature of the binding reaction. In this paper we, therefore, investigated the rapid kinetics of Cbl\(\text{OH}_2\) binding to the most Cbl-specific protein IF. Interaction of two other ligands (Cbl\(\text{Ado}\) and an analogue Cbi) with IF, TC, and HC was also characterized. We found no correlation between the ligand specificity and the biphasic kinetics of binding. Slow spectral transformations were observed only for two ligands, Cbi (this paper) and Cbl\(\text{OH}_2\) (15), when interacting with TC. The character of these changes was identical to those induced by coordination of external histidine to Cbi or Cbl\(\text{OH}_2\). This fact supports the hypothesis of a cobalt-coordinated histidine residue within the complexes between TC and certain corrinoids. We also addressed the accessibility of bound Cbl to the external cobalt-specific reagents in different protein complexes. The results suggest that the \(\beta\)-surface of Cbl associated with IF or HC is moderately open, in contrast to practically closed complex with TC. Binding of Cbl\(\text{Ado}\) to the proteins protected to some extent this ligand from light-induced decomposition. In addition, the absorbance spectrum of TC-Cbl\(\text{Ado}\) alluded to homolytic cleavage of the carbon-cobalt bond in 10–20% of the associated ligand molecules.
EXPERIMENTAL PROCEDURES

Materials
All salts and media components were purchased from Merck, Roche Molecular Biochemicals, Sigma, and Beckton Dickinson. The enzymes and kits for DNA handling were obtained from New England Biolabs, Stratagene, and Roche Molecular Biochemicals, the kit for the PCR reaction was from HT Biotechnology Ltd. Oligonucleotides were synthesized by DNA technology. The employed yeast expression system was established by Invitrogen. The fermentor Biotest B from B. Braun Biotech International was employed during expression of the recombinant proteins. Sephacryl S-200 and CM Sepharose were obtained from Amersham Biosciences, Inc. The anti-IF serum was raised by DAKO.

Methods
Preparation of the DNA Material for Expression of Human IF—IF-encoding fragment of DNA was produced from a gastric cDNA by the reverse transcriptase and polymerase chain reactions employing IF-specific primers with adaptors for XhoI and NotI endonuclease sites. The obtained product was purified and ligated to the corresponding sites in the expression plasmid pGAPZa. The designated sequence of the fusion protein contained the following components counting from the N terminus: a yeast secretion signal (a-factor), the site for yeast protease Kex2, and the mature human IF. This construction ensured cleavage of the N-terminal peptides from the recombinant protein during its secretion: ...LEKR...STOT0... IF residues are underlined.

Expression and Purification of Human IF—The recombinant IF was expressed according to recommendations of the manufacturer (Invitrogen) in yeast P. pastoris (strain SME 1168). The constitutive promoter of glyceroldehyde-3-phosphate dehydrogenase induced the expression. The fermentation of the recombinant yeast was carried out at 30 °C for 2 days in 1 liter of YPD medium (containing 0.5 M CblOH2) with the constant supply of glucose. The level of oxygen and pH in the medium were maintained at 25% and 6.0, respectively. The cell-free supernatant was saturated with ammonium sulfate (520 g/liter) and centrifuged at 4,000 × g for 40 min. The pellet was dissolved in 50 ml of 0.05 M P, buffer, pH 7.5, whereupon centrifuged once more at 12,000 × g for 10 min. The solution was concentrated by ultrafiltration to the volume 10–15 ml and applied to a 250-ml Sephacryl S-200 column equilibrated with 0.1 M Tris, 1 M NaCl, pH 7.5. The fractions with red protein were pooled, concentrated to 5–8 ml, and subjected to repeated gel filtration under analogous conditions. The red fractions with IF were collected, concentrated, and stored frozen. SDS electrophoresis, staining of the gel by Coomassie, staining of the glycoproteins by PAS method, and Western blotting were performed according to the standard procedures.

Expression and Purification of Human TC—The recombinant TC was produced as described in our previous publication (15).

Isolation of Human HC—The protein was purified from human plasma as described elsewhere (17). Preparation of the Apo Forms of CM Binders—Holo forms of IF, TC, and HC were dialyzed against 5 M GdnHCl (IF and TC) or 8 M GdnHCl (HC) at 30 °C for 2 days with one change. Liberation of Cbl was monitored by overnight dialysis against 0.2 M P, buffer, pH 7.5, 5 °C.

Spectral Measurements—The spectra were recorded on M350 Double Beam UV Visible Spectrophotometer (Camspec) or on the stopped-flow equipment, see the next paragraph.

Stopped-flow Experiments on Cbl Binding—Binding of different corrinoids to the specific apo-proteins was followed on DX17MV stopped-flow spectrophotometer (Applied PhotoPhysics) using difference in the excitation wavelengths: A580/A500, A519/A580, and A560/A500 for IF, TC, and HC, respectively.

Exchange of the Cobalt-coordinated Groups in the Corrinoids—The displacement of the cobalt-coordinated groups in Cbi, Cbl-CN, IF-Cbl-OH2, and HC-Cbl-OH2 was induced by light when the sample in a quartz cuvette was placed in front of a 30 W daylight lamp at the distance of 20 cm. The changes in absorbance were measured at 352 nm (free Cbi) and 359 nm (protein-bound Cbi) with 1-min intervals.

Mathematical Analysis—Fitting of the curves was performed by a computer program for nonlinear regression analysis or a program Gepasi (18). The presented data were obtained from two to four parallel experiments and are shown as the mean values.

RESULTS

Purification of the Cbl-binding Proteins—Details of the isolation procedures for human HC and human recombinant TC were described elsewhere (15, 17). Human recombinant IF was expressed in yeast P. pastoris and purified as described under “Methods.” The gel filtration profile of the purified recombinant IF contained one protein peak of 70 kDa saturated with Cbl-OH2. SDS electrophoresis in the presence of a reducing agent revealed the major protein pool of 50–55 kDa (Fig. 1, lane 2), which was reactive toward IF-specific antibodies (Fig. 1, lane 4). The determined N-terminal sequence was identical to human gastric IF (STQTQTS...). The 50–55 kDa band was not sharp, probably because of variation in the composition of carbohydrates coupled to the protein core of IF. The presence of carbohydrates on recombinant IF was confirmed by PAS staining of the gel (Fig. 1, lane 3).

Changes of Cbl Absorbance Upon Its Binding to the Cbl-specific Proteins—Association of Cbls with IF, HC, and TC caused typical changes in the ligand spectrum (Fig. 2) (6, 8, 14, 15). The extinction coefficients of Cbl-OH2 in complex with the proteins investigated are shown in Table I. These data were obtained on the originally purified holo forms as well as on the GdnHCl-treated, renatured and resaturated proteins. GdnHCl treatment had certain effects on the extinction coefficients. It

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2 S. N. Fedosov, unpublished data.
was particularly evident for IF where all peaks increased by 15–20% (Table I) mainly due to intensified absorbance of the apo-protein (Fig. 2, B and C). The corresponding changes were insignificant for TC and practically absent for HC (Table I). The spectra of the free ligands are given for comparison in Fig. 2D.

The most significant shifts in the absorbance spectra of all ligands took place after their association with TC (Fig. 2, solid lines): (i) for Cbl-OH$_2$ one can see a noticeable red shift for all peaks (Fig. 2A); (ii) for Cbl-Ado this was a distortion of the shape at 350–380 and 480–550 nm (Fig. 2B); (iii) for Cbi there occurred an unusual redistribution of intensities from $A_{356}$/$A_{502}$ < 1 to > 1 (Fig. 2C). Curiously enough, the spectrum of TC-Cbl-Ado reminded very much of those for Cbl-OH$_2$ and its protein complexes at pH 7.5, 37 °C. On the contrary, the spectra of analogous complexes with IF and TC were similar to glutamate mutase-Cbl-Ado in rest (19).

**Development of Slow Spectral Distortions**—All spectral changes induced by the binding of Cbl-OH$_2$, Cbl-Ado, and Cbi to the specific proteins were accomplished in less than 1 s, except for the pairs TC + Cbl-OH$_2$ and TC + Cbi. Those cases attracted our special attention.

During the binding of Cbl-OH$_2$ to TC, the initial jump of the $\gamma$-peak was followed by continual spectral changes during the next 3 min (Fig. 3A). These slow perturbations significantly contributed to initially moderate red shift and amplification of the $\gamma$-peak. The process developed exponentially in time with the rate constant of $2.5 \times 10^{-2}$ s$^{-1}$, which did not differ from $k_{+2}$ obtained earlier in the stopped-flow experiments at a single wavelength (15). Increase of the temperature essentially accelerated the slow phase but had no affect on its amplitude (at least between 20 and 37 °C).

The slow decrease of absorbance for the $\gamma$-peak, induced by attachment of Cbi to TC, was not very noticeable due to the originally high absorbance of Cbi at 350–370 nm (not shown). The spectral transition was more evident for the smaller $\alpha$- and $\beta$-peaks (Fig. 4A), and the effect was expressed better at 37 °C than at 20 °C. The transition was exponential with the rate constants of $4.3 \times 10^{-3}$ s$^{-1}$ (20 °C) and $1.1 \times 10^{-2}$ s$^{-1}$ (37 °C).

**Imitation of the Slow Phases by Coordination of Histidine to Cbl-OH$_2$ and Cbi**—We have suggested earlier that the unusual spectral behavior of Cbl-OH$_2$ during binding to TC may have been caused by coordination of a protein residue to cobalt (15). The control experiment with several amino acids and free Cbl-OH$_2$ showed that only incubation with histidine caused noticeable spectral response (Fig. 5D, solid line), at least under the shown conditions. This is not surprising since imidazol is a known ligand with intermediate affinity to Cbl (21). The reaction between histidine and Cbl-OH$_2$ was reversible and characterized by the rate constants $k_{-\text{His}} = 0.92 \times 10^{-1}$ s$^{-1}$ and $k_{+\text{His}} = 2.2 \times 10^{-4}$ s$^{-1}$ ($K_{\text{His}} = 0.24$ mm) at pH 7.5 and 20 °C. At higher temperature (37 °C) the rate coefficients increased 2.0–2.2-fold without significant change in the equilibrium constant $K_{\text{His}}$. Addition of 5 mM histidine to either IF or HC complex with Cbl-OH$_2$ caused gradual shift of the $\gamma$-peak (Fig. 3, B and C, respectively) analogous to the reaction between histidine and free Cbl-OH$_2$ (Fig. 3D). All above processes were similar in

**Table I**

| Compound          | $\gamma$ | $\delta$ | $\beta$ | $\alpha$ |
|-------------------|----------|----------|---------|----------|
| Cbl-OH$_2$        | 352 nm, 23,000 | 412 nm, 3,900 | 505 nm, 8,400 | 527 nm, 9,000 |
| IF-Cbl-OH$_2$     | 356 nm, 28,200 | 412 nm, 5,100 | 505 nm, 8,800 | 531 nm, 9,300 |
| IF-Cbl-Ado        | 354 nm, 33,300 | 411 nm, 6,300 | 502 nm, 10,200 | 528 nm, 10,600 |
| TC-Cbl-OH$_2$     | 362 nm, 30,000 | 420 nm, 6,400 | 517 nm, 8,500 | 546 nm, 9,200 |
| TC-Cbl-Ado        | 362 nm, 31,400 | 421 nm, 5,900 | 516 nm, 9,000 | 545 nm, 9,700 |
| HC-Cbl-OH$_2$     | 356 nm, 28,600 | 421 nm, 5,400 | 507 nm, 10,300 | 528 nm, 10,400 |

$^a$The purified holo-protein.

$^b$The purified protein treated with GdnHCl, renaturated and saturated with Cbl to 90% of the potential binding capacity.
their manifestation to the second phase observed for TC + CblOH$_2$ interaction (compare Fig. 3, B and C), dashed lines, with A). The rate coefficients of the forward reaction determined for IF-CblOH$_2$ and HC-CblOH$_2$ were equal to 0.44 m$^{-1}$ s$^{-1}$ and 0.65 m$^{-1}$ s$^{-1}$, respectively. The complex TC/CblOH$_2$ did not react with histidine for at least 2 h (not shown).

Addition of histidine to Cbi also evoked spectral changes (Fig. 4B), which testified for coordination of the imidazol group to either $\alpha$ or $\beta$ surface of the corrinoid (21). The recorded spectra reversibly mirrored those during displacement of the dimethyl-benzimidazol base by cyanide: Cbl-CN$_2$ + CN$^-$ $\Leftrightarrow$ CN$_2$-Cbl-CN$_2$ (Fig. 4C). This may suggest attachment of histidine to the lower axial site of Cbi. The half-maximal optical response was reached at His = 20 mM (not shown). The apparent rate coefficient of the process $k_{\text{app}} = 0.021$ s$^{-1}$ (20 °C), $k_{\text{app}} = 0.077$ s$^{-1}$ (37 °C), was, however, practically independent on histidine concentrations at His = 5–100 mM. This means that the velocity of conversion CN-Cbi-CN $\rightarrow$ His-Cbi-CN is not limited by attachment of histidine to cobalt, although, the details of kinetics are not quite understood. Coordination of 15 mM histidine to Cbi (Fig. 4B) caused the same type of the spectral response as the binding TC + Cbi (Fig. 4A). Protection of the TC-associated Cbi was not as good as for CblOH$_2$, and addition of 15 mM histidine caused further spectral transitions with the velocity 14 times slower, than for free Cbi (not shown).

**Binding Kinetics of Different Corrinoids**—The change in the absorbance of Chls and Cbi upon their attachment to the proteins was used to monitor these processes on stopped-flow equipment. The data depicted in Fig. 5, A–C, represent the rapid phase of the binding. The reactions were fitted to a bimolecular mechanism A $+$ B $\rightarrow$ C with the rate constants shown in Table II. Ligand binding to IF was characterized by
the lowest rate constants. Association of Cbi (corrinoid with incomplete structure) with all proteins was almost as quick as for Cbl. No slow phase was found for IF and HC in the time region 1–100 s (not shown). The values of $k_{-\text{CblCN}}$ from the literature: 236 $\mu\text{M}^{-1}\text{s}^{-1}$ for chicken HC (3) and 10 $\mu\text{M}^{-1}\text{s}^{-1}$ for immobilized bovine TC (4), are in good agreement with our results (Table II).

As was already mentioned, the initial attachment of Cbi to TC was followed by a slow monomolecular reaction $C \rightarrow D$. The detected decrease of the $\gamma$-peak was difficult to follow at 12 $\mu\text{M}$ TC due to low response on the background of a relatively high absorbance. Therefore, the time course of this second phase was recorded at increased concentrations of TC and Cbi (both 40 $\mu\text{M}$) and at another wavelength corresponding to $\gamma$-peak (Fig. 5D). The rate coefficients, determined from continuous measurements, were $5.6 \times 10^{-2} \text{s}^{-1}$ (20 °C) and $1.4 \times 10^{-2} \text{s}^{-1}$ (37 °C). They did not differ from the data in Fig. 4A. Thorough investigation of Cbl-Ado binding to TC at different concentrations and wavelengths did not reveal any additional phase in this process besides the spectral changes during the first 10 ms induced by attachment of the ligand to the protein.

**Table II**
The rate constants ($k_1$) for binding of different corrinoids to Cbl-binding proteins at pH 7.5, 20 °C.

| Ligand     | Rate constant for the rapid phase ($\mu\text{M}^{-1}\text{s}^{-1}$) |
|------------|---------------------------------------------------------------|
|            | IF | TC | HC |
| Cbl-CN     | 8.5 | 100° | 90° |
| Cbl-OH$_2$ | 11.9 | 30° | 60° |
| Cbl-Ado    | 4.5 | 57 | 140 |
| Cbi        | 21.1 | 21 | 61 |

* Data from Ref. 15.

**Dissociation of the Ligand-Protein Complexes**—High velocity of association between Cbi and the recombinant IF or TC raised a question about their affinity to this analogue, since Cbi is known to be a poor substrate for IF and TC from the natural sources (1, 2, 5, 16). We have, therefore, characterized dissociation of the protein-Cbl-OH$_2$ or protein-Cbi complexes by gradual replacement of the original ligand with added Cbl-CN or Cbl-OH$_2$, respectively (Fig. 6, A and B). The process was followed in time by the spectral changes of the protein fraction after charcoal treatment.

The data in Fig. 6A show the reaction, where a 4-fold excess of Cbl-CN was added to the halo-proteins saturated with Cbl-OH$_2$. Computer simulation of the curve obtained for IF allowed to calculate the dissociation rate constants both for Cbl-OH$_2$ ($k_{-\text{CblOH}} = 4.2 \times 10^{-6} \text{s}^{-1}$) and Cbl-CN ($k_{-\text{CblCN}} = 9.2 \times 10^{-6} \text{s}^{-1}$), using the known values of $k_{+\text{Cbl}}$ from Table II. The values of $k_{-\text{CblOH}}$ for dissociation of the corresponding TC and HC complexes were estimated from the initial slopes ($v = k_{-\text{CblOH}} [\text{complex}]$) as $1 \times 10^{-7} \text{s}^{-1}$ and $6 \times 10^{-7} \text{s}^{-1}$, respectively. Our previous measurements of $k_{-\text{CblCN}}$ for bovine and human TCs at higher temperature (37 °C) were in the range of $1 \times 10^{-6}$ to $3 \times 10^{-6} \text{s}^{-1}$ (4, 15).

When the apo forms of recombinant IF and TC were saturated with Cbi and exposed to equal concentration of external Cbl-OH$_2$, the complete substitution occurred in less than 1 min (Fig. 6B, upper curves). No detectable dissociation of HC-Cbi was found under the same conditions (Fig. 6B, lower curve). The rate constants of Cbi liberation were estimated as $k_{-\text{Cbi}} > 5 \times 10^{-2} \text{s}^{-1}$ (IF, TC) and $k_{-\text{Cbi}} < 1 \times 10^{-5} \text{s}^{-1}$ (HC).

**Exchange of the $\beta$-Group in Cbl-OH$_2$ Associated with IF or HC**—It has already been shown that accessibility to the upper
face of the ligand in the TC-Cbl-OH$_2$ complex is hindered (15). In this assay we exposed IF (Fig. 7, A and B) and HC (Fig. 7, C and D), saturated with Cbl-OH$_2$, to different concentrations of CN$^-$ or N$_2$ and then followed replacement of the original $\beta$-group by changes in the absorbance. The observed reactions were practically irreversible in the case of CN$^-$ and reversible for N$_2$. The calculated rate coefficients are shown in Table III, where the previous results for TC-Cbl-OH$_2$ and free Cbl-OH$_2$ (15) are given for comparison. As one can see, neither IF nor HC rendered significant protection against CN$^-$. At the same time, coordination of N$_2$ to cobalt was somewhat decelerated in both directions when compared with free Cbl-OH$_2$.

### Specific Proteins Protect Cbl-Ado against Light-induced Decomposition—When Cbl binders saturated with Cbl-Ado were exposed to light, a gradual transformation of Cbl-Ado to Cbl-OH$_2$ was observed (Fig. 8). The time course of these photoactivated reactions was monitored spectroscopically and compared with decomposition of free Cbl-Ado under analogous conditions. The performed measurements showed a 7-, 15-, and 17-fold deceleration of Cbl-Ado decay when the ligand was bound to IF, HC, and TC, respectively.

### DISCUSSION

Binding of the Cbl molecule to the specific proteins affects its absorbance spectrum, which turns spectroscopy to an easy and convenient method for monitoring the protein-ligand interactions. The advantages of the method were used for the investigation of Cbl binding to three transporting proteins: IF, TC, and HC. Two first binders were expressed in recombinant yeast, and HC was purified from human plasma. All proteins were isolated as holo forms with bound Cbl-OH$_2$ and their absorbance spectra (Fig. 2A, Table I) were typical for the binders from other sources. GdnHCI treatment, necessary for production of the apo-proteins, had practically no effect on the spectra of HC and TC. At the same time, the treatment influenced IF, and the increased absorbance of the apo-protein (Fig. 2, B and C) resulted in artificially high extinction coefficients of the newly bound ligand (Table I). The earlier determined extinction parameters of gastric IF (8) were, nonetheless, closer to the observed absorbance of recombinant holo-IF after GdnHCI than to the coefficients of “fresh” recombinant holo-IF (Table I). This observation stresses importance of IF’s history for its spectral features.

Comparison of the data in Fig. 2, A–C, with Fig. 2D showed that the most pronounced alternations in the ligand spectra took place after binding to TC (solid lines). Thus, the record for TC-Cbl-OH$_2$ at pH 7.5 (Fig. 2A) demonstrated a remarkable red shift of the $\gamma$-peak (362 nm) and strong expression of the $\alpha$-peak (546 nm). This pattern mimicked better Cbl-CN or Cbl-imidazol than Cbl-OH$_2$ at neutral pH (21). The spectrum of another complex TC-Cbl-Ado (Fig. 2B) was characterized by increased optical density at 350–380 and 400–450 nm as well as by decreased absorbance at 500–550 nm accompanied by separation of the individual peaks $\alpha$ and $\beta$. Similar spectra were observed for enzyme-bound Cbl-Ado in catalysis (19, 20), which may suggest partial homolysis of the carbon-cobalt bond also in TC-Cbl-Ado, not trivial for a transporting protein like TC. The complex of TC with the third ligand Cbi (Fig. 2C) likewise revealed some redistribution of intensities between the peaks, i.e. decrease of the $\alpha$-peak (578 nm) and increase of the $\beta$-peak (544 nm). Analogous spectra can be observed, for instance, for Cbl during transition (base on) $\gamma$-Cbl-CN$_2$ $\leftrightarrow$ (base off), Cbl-Cbl-CN$_5$, when cyanide and the nucleotide base compete for the lower coordination position at cobalt (Fig. 4C).

The peculiar spectra of the above protein-ligand complexes prompted us to thorough investigation of the binding kinetics. Change in the absorbance of the $\gamma$-peak during the ligand binding was followed by a stopped-flow technique (Fig. 5, A–C). All ligands, including the analogue with the missing nucleotide moiety, attached rapidly and in one step to two proteins with widely different Cbl specificity, IF and HC (Fig. 5, A and B). There was no visible indication of any second phase during 100 s of the binding as well, unlike the interaction between TC and Cbl-OH$_2$ examined earlier (15). This fact implies that the slow phase is not an attribute of the selective recognition of Cbl but rather a specific characteristic of TC, when interacting with certain ligands. We doubt that presence of carbohydrates on IF and HC (1, 6) and their absence on TC (1, 6, 15) has anything to do with the described effects, because glycosylation does not seem to interfere with the binding of Cbl to IF (22).

There was an interesting observation, concerning the high velocity of association between Cbi and IF or TC. The incomplete ligand bound to these two proteins, known to be Cbl selective (1, 2, 4, 5), almost as quick as the ligand with the correct structure, i.e. Cbl. We compared the association rate constants from Table II with the collision rate constant $k_{coll} = 5 \times 10^{9} \text{ M}^{-1} \text{s}^{-1}$ at 20 °C estimated for a corrinoid and a binding site of appropriate geometry (23). The calculations showed that the number of the efficient impacts varied from 2 to 20 per 1000 collisions without particular correlation between $k_{coll}$ and the ligand structure. Similar rate constants found for Cbi and Cbl mean that the Cbl-specific site is not originally tuned to any particular ligand and can accommodate for a time being even some defective molecule. The sensitivity of IF and TC to the substrate’s geometry, and its absence in the case of HC, was
The rate constants of $\beta$-exchange in Cbl-Ado were subjected to thorough analysis. We have recorded it for TC (15). Two atypical ligands (Cbl and Cbl) revealed only in the dissociation experiments (Fig. 6, A and B).

Calculated values of the rate constants allowed us to make the following estimates of $K_d$ for Cbl-Ado: 1 pm (IF), 0.01 pm (HC), and 0.005 pm (TC). The values for Cbl were: $K_d < 0.1$ pm (HC), $K_d > 1$ nm (TC and IF). The earlier published $K_d$ for Cbl and the specific binders varied in the range $10^{-16}$ to $10^{-9}$ M (see, for review, Refs. 1-4, 8, 9, 15, and 22), which could hardly be explained by real fluctuations of the affinity. Such a broad dispersion was rather caused by inappropriate mathematical approach to the case when the total concentrations of the binding site $E_T$ and the ligand $L_T$ are close to each other (complicated by $K_d < E_T, L_T$). Under these conditions, half-saturation would be reached at $L_{T0.5} = K_d + 0.5 E_T$, which may represent rather concentration of the binding site than the dissociation constant. More accurate presentation of the results as $EL$ versus $L_{T0.5}$ may also lead to an erroneous evaluation of $K_d$ if the reaction is almost irreversible. Under these circumstances even a small but reproducible overestimate of $L_{T0.5}$ (e.g., $L_{T0.5} = 0.05 L_T E_T$) inevitably causes great overestimate of $K_d$ (e.g., half-saturation at $L_{T0.5} = 0.05 K_d + 0.025 E_T E_T$). In such a difficult case, determination of $K_d$ from the ratio of the rate constants $k_{-1} / k_{+1}$ may be advantageous. This statement can be illustrated by comparison of $K_d$ measured for chicken HC in an equilibrium assay ($10^{-11}$ M) and from $k_{-1} / k_{+1}$ ($10^{-16}$) by the same authors (3).

Investigation of the rapid kinetics contributed to our understanding of the substrate binding, although, it did not give us a clue to the anomalous appearance of the holo-TC spectra. Therefore, the stopped-flow experiments were repeated at different wavelength and higher concentration of TC. They did not exhibit any second phase for the reaction TC + Cbl-Ado but revealed it for TC + Cbi (Fig. 5D). This result demonstrated that Cbl-Ado was not the only corrinoid characterized by biphasic binding to TC (15). Two atypical ligands (Cbl and Cbl) were subjected to thorough analysis. We have recorded deformations of the $\gamma$-peak for Cbl-OH$_2$ (Fig. 3A) and $\alpha, \beta$-peaks for Cbi (Fig. 4A) at 20 and 37 °C in an attempt to get the best response for each corrinoid. In both cases the initial attachment of the ligands caused slight increase and red shift of the peaks without significant distortions of their shape (see the 1-s records in Figs. 3A and 4A). Continuation of the reactions was, although, accompanied by more pronounced changes in the

### TABLE III

| Complex                  | $+ \text{CN} \rightarrow \text{CblCN}, k_+$ | $+ \text{N}_3 \rightarrow \text{CblN}_3, k_+$ |
|-------------------------|------------------------------------------|------------------------------------------|
| Cbl-OH$_2$              | 250                                      | 16 M                                     |
| IF-Cbl-OH$_2$           | 77                                       | 200                                      |
| HCC-Cbl-OH$_2$          | 107                                      | 100                                      |
| TC-Cbl-OH$_2$ (+L = ∞)  | $5 \times 10^{-4}$ s$^{-1}$              | $5 \times 10^{-4}$ s$^{-1}$              |

$^a$ Data from Ref. 15.

$^b$ $K_{app}$ was calculated from dependence of the amplitude of the spectral response on $N_3$ concentration.

![Fig. 9](image-url)
sight. Thus, alignment of the pairs (IF, TC, or HC):methylmalonyl-CoA mutase or glutamate mutase) showed 15–19% of homology in all cases, although, at different regions. Anyway, the unusual spectral properties of the TC-Cbl-Ado complex require additional analysis.

The presented data throw some light upon the structure of the binding sites of Cbl transporting proteins. One can imagine that all three carriers are supplemented with a lid- or a cap-like structure at the entrance to the site cavity. At the same time, development of this structure in the Cbl transporters appears to be different. The cap in IF does not seem to cover any appreciable part of the upper face of Cbl and, therefore, holo-IF demonstrates quite rapid exchange of the β-groups as well as a relatively moderate protection of Cbl-Ado. The kindred protein HC is rigged somewhat better. Its cap shields to a certain extent the upper plane of Cbl and hinders the inwards-outwards movements of the β-coordinated groups, at least for bulky complexons. Analogous cap in TC renders much better protection against all substituents, and it might even produce and stabilize the Ado radical above the upper plain of Cbl. In addition, the protective shield of TC is, presumably, supplemented by an active His residue, which can coordinate to cobalt and dislodge β-water in CblOH2 or α(?)-cyanide in Cbl (the latter case requires, although, additional clarification). Coordination to cobalt at the β-position locks the lid above the binding site and Cbl (but not Cbi) becomes encapsulated inside holo-TC, with occasional and short-time openings to occur.

The performed investigation strengthens the view on TC as the best protector of the associated Cbl. It also raises a question about the role of the protective cap in stabilization and destabilization of the cobalt-coordinated groups in TC-bound corrinoids.

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