Kinetics and Thermodynamics of the Interaction of Cyanocobalamin (Vitamin B_{12}) with Haptocorrin: Measurement of the Highest Protein−Ligand Binding Constant Yet Reported

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Abstract: Kinetic studies of the binding of ^{57}Co-labeled cyanocobalamin to purified chicken serum haptocorrin resulted in an apparently diffusion-controlled second-order rate constant, k_b, of 2.36×10^8 M^{-1} s^{-1} at 25.0 °C, pH independent in the range 7.4−9.6, and $\Delta S^{\ddagger}_b = -4.48 \pm 0.34$ cal mol^{-1} K^{-1} and $\Delta H^{\ddagger}_b = 4.71 \pm 0.10$ kcal mol^{-1} (5−58 °C). Cyanide trapping of the slowly released cyanocobalamin at 85.0 °C and pH 7.4 resulted in a concentration-independent rate constant, k_d, of $(5.58 \pm 0.17) \times 10^{-5}$ s^{-1} for dissociation of the cyanocobalamin−haptocorrin complex, with $\Delta S^{\ddagger}_d = 13.4 \pm 1.6$ cal mol^{-1} K^{-1} and $\Delta H^{\ddagger}_d = 32.9 \pm 0.6$ kcal mol^{-1} (70−95 °C). This leads to a binding constant, K_b, of 5×10^{16} M^{-1} or log $K_b = 16.7 \pm 0.6$ at 25.0 °C, which is the largest protein−ligand binding constant yet reported and at least an order of magnitude higher than that for the biotin−avidin system.

Introduction

Cyanocobalamin (CNCbl,[1] vitamin B_{12}) and its derivatives are well-known to bind tightly to a number of vitamin B_{12} binding proteins including the intrinsic factors found in the gastric juices of numerous mammals, the serum transcobalamins, and a number of haptocorrins found in saliva, some gastric juices, and most sera.[2−9] The unifying property of such proteins is their high affinity for CNCbl, with reported values for binding constants as high as ca. 10^{12} M^{-1}.[10]

We have long been interested in such binding proteins as models for the cobalamin binding sites of B_{12}-dependent enzymes,[11−14] focusing on the haptocorrin from chicken serum, which is readily available in relatively large quantities. In attempts to understand how proteins can modulate carbon−cobalt bond reactivity, notably in the 10^9−10^{12}-fold catalysis of Co−C bond homolysis of 5′-deoxyadenosylcobalamin (AdoCbl,[1] coenzyme B_{12}) by AdoCbl-dependent enzymes,[15−18] we have previously studied the influence of complexation to haptocorrin on the carbon−cobalt bond homolysis of thermally labile alkylcobalamins.[11,14] These studies have suggested that binding of cobalamins to haptocorrin may involve substantial hydrogen bonding of cobalamin side chain amides to hydrogen bond acceptors in the protein binding pocket. Since a single hydrogen bond between a neutral hydrogen bond donor and acceptor in an enzyme active site is known to provide 0.5 to 1.5 kcal of binding free energy,[19] hydrogen bonding to the 13 amide side chain N−H's in cobalamins could well provide a substantial amount of binding energy. Studies of the coenzymic activity of side chain-altered AdoCbl analogs with the AdoCbl-dependent enzymes diol dehydrase,[20] glycerol dehydratase,[21] and ribonucleotide reductase[22] provide support for the idea that such hydrogen bonding is important in the interaction of cobalamins with these proteins. Thus, the *b*-, *d*-, and *e*-monocarboxylate derivatives of AdoCbl, their methyl esters, and their *N*-methylamides are active coenzymes with 7 to 86% of the activity

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(1) The abbreviations used are: AdoCbl, 5′-deoxyadenosylcobalamin (coenzyme B_{12}); Bicine, *N,N*-bis(2-hydroxyethyl)glycine; BSA, bovine serum albumin; Cbl, cobalamin; CNCbl, cyanocobalamin (vitamin B_{12}); $(CN)_2Cbl^-$, dicyanocobalamin; cpm, count per minute; Hc, purified apoprotein of the haptocorrin from chicken serum; MWCO, molecular weight cut-off; UCBC, unbound Cbl-binding capacity.

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of AdoCbl itself. Using the method of Wilkinson et al.,[23] the monocarboxylates and methyl esters can be shown to suffer a loss of 2.3−2.8 kcal of binding energy relative to AdoCbl itself, while the *N*-methylamides suffer a loss of about 1.5 kcal of binding energy. This suggests that each of the amide hydrogens of these side chains may function as hydrogen bond donors at the active sites of these enzymes.

As a result, we are interested in determining the importance of side chain amide hydrogen bonding in the association of cobalamins with haptocorrins *via* studies of the thermodynamics of the binding reactions of various Cbl analogs with altered hydrogen bonding capabilities. Such a study first requires the establishment of sufficiently accurate methods for measuring large binding constants in this system, and a reliable value for the binding constant of CNCbl itself. Literature values of the binding constant, K_b, for CNCbl to various vitamin B_{12} binding proteins vary in the range of ca. 10^9-10^{12} M^{-1},[2,3,8,10,24] although most such studies employed unpurified or partially purified proteins. These K_b values correspond to a single cobalamin binding site as determined for all three types of binding proteins (intrinsic factors, haptocorrins, and transcobalamins).[25] The analytical method of choice for these protein−ligand systems is radioassay using γ-emitting [^{57}Co]-CNCbl. This commercially available radiolabeled compound is used routinely for assays of cobalamin-binding proteins (so-called UCBC methods[1,12,26,27]) and for isotope dilution assays of cobalamins,[28−30] with adsorption of free cobalamin on protein-coated charcoal as the most common method of separation of bound and free cobalamin. The high specific activity of commercial [^{57}Co]-CNCbl (>2 × 10^5 Ci mol^{-1}, essentially carrier free) makes quantitation feasible at the low concentrations required for direct measurements of K_b in the reported range of values. However, to our great surprise, the careful equilibrium and kinetic studies reported below reveal that the binding constant for CNCbl to chicken serum haptocorrin is several orders of magnitude larger than the upper limit of the range of values reported in the literature, making it, to our knowledge, the largest protein−ligand binding constant yet measured.

Experimental Section

Materials. The apoprotein of chicken serum haptocorrin was obtained, purified, and analyzed as described previously[14,26] and stored at −20 °C. Cyanocobalamin was from Roussel. [^{57}Co]-Cyanocobalamin was obtained from Amersham International, with radioactivity at the reference date of 220 Ci g^{-1} or 10.5 mCi L^{-1} of its aqueous solution. Our tests confirmed its high radionuclidic purity. Bovine serum albumin, fraction V, charcoal, activated and neutralized, and dextran, molecular weight 79 100, were from Sigma. Glass-distilled water was used throughout.

General Methods. The cobalamin-binding capacity (UCBC) of haptocorrin was determined by the method of Gottlieb *et al.*[27,30] using charcoal coated with dextran instead of albumin, according to the following procedure. Charcoal suspension, containing 50 g L^{-1} of activated charcoal and 5 g L^{-1} of dextran in 0.1 M phosphate buffer at pH 7.4, was stirred on ice for 1 h and then stirred gently during its use. Aqueous solutions of [^{57}Co]-CNCbl and unlabeled CNCbl were mixed in the phosphate buffer to give a total concentration, $[CNCbl]_T$, of 1 × 10^{-8} M and radioactivity of ~5 × 10^7 cpm L^{-1}. BSA[1] solution, 1 g L^{-1} in the phosphate buffer, was prepared and stored on ice until use. Haptocorrin was diluted with the BSA solution to obtain a molar concentration of binding sites lower than $[CNCbl]_T$. To 1.5-mL microcentrifuge tubes were added sequentially 200 μL of the BSA solution, 100 μL of the haptocorrin solution, and 100 μL of the CNCbl solution. The tubes were then closed and *gently* vortexed. After a few min, 400 μL of the charcoal suspension was added, the tubes were vortexed for 2 min and centrifuged for 10 min in a microfuge, and 700 μL of supernatant was taken for radioactivity measurement. Besides samples processed as above, "blanks" were prepared by replacing the haptocorrin solution with BSA solution and "controls" were prepared by replacing the haptocorrin solution with BSA solution *and* replacing the charcoal suspension with water. The exact molar concentration of binding sites in the haptocorrin solution was calculated as follows: $[Hc] = [CNCbl]_T(S - B)/C$, where *S*, *B*, and *C* are sample, blank and control radioactivities, respectively. Throughout this report the haptocorrin concentration is expressed as molar concentration of its single cobalamin-binding sites. Frozen 0.1-mL aliquots of analyzed haptocorrin were thawed to prepare its stock solutions, then kept on ice or stored at 3 °C for up to several days. Except for pH-dependence experiments, the typical buffer solution contained 0.1 M phosphates at pH 7.4. All dilute (less than ~10^{-6} M) haptocorrin solutions contained freshly dissolved 1 g L^{-1} BSA as a protective protein. The exact concentrations of final dilutions were determined as required.

A Gamma 5500B gamma counter from Beckman Instruments was used to measure radioactivity of [^{57}Co]-CNCbl. Operating parameters were optimized and calibration of the instrument was performed as required. The standard curve for [^{57}Co]-CNCbl radioactivity measurement was linear up to at least 2 × 10^4 cpm with a slope of (5.62 ± 0.09) × 10^{17} cpm mol^{-1} (the actual error is ±8% as reported by Amersham for their radioactivity measurements), corresponding to 85% counting efficiency. Samples were counted for 10 min each in a series that included a number of water-containing background vials, and the entire series was counted repeatedly several times for acceptable statistics. A Spectrum 20-cell equilibrium dialyzer was used with micro, semimicro, and custom-made dialyzing cells. Spectra/Por membranes No. 6 (MWCO 25000) and No. 4 and 2 (both MWCO 12000−14000) were used with required pretreatment. Additional cleaning of the membranes did not affect the results of the binding experiments. For testing purposes, the dialyzer was used for the haptocorrin assay and gave the same (within <2%) result as that from the UCBC (charcoal) method.

CNCbl solutions were quantitated as described previously[11,31] with the use of a Cary 219 spectrophotometer and 1-cm path length quartz cuvettes. Measurements of pH were made using a Radiometer PHM 84 pH meter and Radiometer type GK533901 (at ambient temperature) or type GK2402B (at elevated temperatures) combination glass electrodes, with electrodes, standards, samples, and rinse water equilibrated at the appropriate temperature. Dynamic viscosities were obtained from viscosimetric and density measurements using the absolute viscosity of degassed water at 20 °C, 1.002 mN s m^{-1}, as a reference value.[32] Total cyanide concentrations were determined argentometrically,[33] while the actual concentrations of CN^- at the experimental conditions were calculated using the results of temperature and pH measurements and the known temperature dependence of pK_a of HCN.[13] Temperature was controlled in all experiments by means of a Lauda K4R Electronic or a Neslab RTE 220 circulating water bath. Measurements of temperature and of time required for thermal equilibration of samples were made using a calibrated YSI 702A thermistor probe or NBS calibrated thermometers.

Equilibrium Measurements. Direct binding equilibrium constant (K_b) determinations were performed by equilibrium dialysis. The time required to reach the diffusional equilibrium was determined without

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haptocorrin and with [^{57}Co]-CNCbl present initially in only one of the two dialysis half cells, under the same conditions as those used later for binding experiments. Micro cells, up to 15 at a time, and No. 2 membranes were used in most experiments.

In a typical experiment, for an anticipated K_b value, 200 μL of buffer solution containing about $2K_b^{-1}$ of haptocorrin was placed on one side of the membrane in all cells, whereas the opposite sides received the same volumes of buffer with concentrations of [^{57}Co]-CNCbl varied around the value of $3K_b^{-1}$. The initial concentrations were specifically related to the assumed K_b value in order to minimize the relative error in experimentally determined K_b.[34] The solutions were then dialyzed until equilibrated, usually for 20 h at 25.0 °C and 20 rpm, and drained into weighed scintillation vials with caps. The vials were then weighed again and their radioactivity was measured. The draining and weighing procedure was adapted because the design of the microcells does not permit efficient removal of the dialyzed solutions with a syringe and needle, as well as to maximize the sample volume available for radioactivity measurement. The weight and radioactivity data permitted calculation of the sum of equilibrium concentrations, [Hc-CNCbl] + [CNCbl], on one side of the membrane and of [CNCbl] alone on the other side. The concentrations were plotted as [Hc-CNCbl] *versus* [CNCbl] and fitted by a nonlinear least-squares method to the binding curve equation, $[\text{Hc-CNCbl}] = K_b[\text{Hc}]_0[\text{CNCbl}]/(1 + K_b[\text{CNCbl}])$. Values of K_b and initial haptocorrin concentration, $[\text{Hc}]_0$, were obtained as parameters of the fit.

Kinetic Measurements. The kinetics of formation and dissociation of the Hc-CNCbl complex were followed by taking 20 to 30 samples during the course of each reaction and separating free from haptocorrin-bound [^{57}Co]-CNCbl with dextran-coated charcoal in a manner similar to that used in UCBC assays. Radioactivity measurements of the haptocorrin-bound [^{57}Co]-CNCbl allowed determination of the complex concentration and its buildup or decay curves were analyzed by a nonlinear least-squares method to yield the rate constants.

The binding kinetics were studied under pseudo-first-order conditions using 2×10^{-12} to 2×10^{-11} M radiolabeled CNCbl with initial concentrations of haptocorrin being ca. 10 times lower. In a typical experiment, [^{57}Co]-CNCbl and Hc solutions, 7.5 mL each, in identical buffers were equilibrated in a water bath at the appropriate temperature and mixed at the start of a stopwatch into a 15-mL test tube in a water bath. At various times, 600-μL duplicate samples were transferred to 1.5-mL microcentrifuge tubes in a water bath and 600 μL of charcoal suspension was added as the elapsed time was measured. The mixtures were gently vortexed for 2 min and centrifuged for 10 min, and 1000 μL of supernatant was taken for radioactivity measurement. At the above concentrations the reaction rate was low enough, compared to the time required for mixing and adsorption, to make the charcoal method well suited to efficiently stop the binding, at least in the absence of viscogens. At the usual temperature of ~15 °C (resulting from the addition of ice-cold charcoal suspension to the reaction mixture), the half-time of the CNCbl adsorption was 10 s and the residual CNCbl concentration was <0.5% of its initial concentration (in the range 1×10^{-13} to 1×10^{-4} M), while the adsorption of the complex was immeasurably small. In viscogenic buffers a large excess of unlabeled cyanocobalamin in the same buffer was used to stop the binding of [^{57}Co]-CNCbl to haptocorrin, followed by separation with charcoal.

The dissociation rate constant of the ^{57}Co-labeled Hc−CNCbl complex in the 70−95 °C range was measured using cyanide to trap the released CNCbl as $(CN)_2Cbl^-$.[12] Here, KCN constituted a part of the buffer system as well, replacing Na_2HPO_4 at concentrations sufficient to achieve pH 7.4 at given temperature, and the starting concentration of the complex was kept low (usually 2×10^{-13} M), in order to assure efficient trapping, based on the known properties of the CNCbl/$(CN)_2Cbl^-$ system.[35] The radiolabeled complex was prepared and diluted as needed with cyanide-containing buffer, and the resulting mixture was distributed into microcentrifuge tubes which were then closed and completely immersed in a water bath at the appropriate temperature. In addition, some control tubes contained no cyanide. Over a time period ranging from 2 to 60 h, the tubes were subsequently transferred to a stirred ice−water bath to stop the dissociation, the protein-bound and free [^{57}Co]-CNCbl were separated with charcoal, and the supernatant was counted. A few measurements above 90 °C were carried out directly, without cyanide trapping, since the released haptocorrin denaturated sufficiently rapidly at these temperatures to allow determination of the dissociation rate constant without the need to trap the released CNCbl with CN^-.

Results

Binding Equilibrium. The middle of the range of K_b values reported in the literature was taken as a first approximation to plan preliminary equilibrium dialysis experiments. Results of these experiments were used to refine the conditions for subsequent experiments. Although plots of [CNCbl−Hc] *versus* [CNCbl] appeared to adequately fit binding curves in most cases, the reproducibility of the results was not satisfactory. As the details of the experimental conditions and data treatment were optimized to allow for lower concentrations to be used, the resulting K_b values gradually increased over the range of three orders of magnitude, reaching almost 1×10^{13} M^{-1}. Unfortunately, this occurred without improvement in precision and the K_b^{-1} values were generally much lower than $0.5[\text{Hc}]_0$, indicating the need to decrease the concentrations further. With that in mind, an improved set of equilibrium dialysis cells was designed and precisely manufactured that would allow measurement of equilibrium constants at least an order of magnitude higher without increasing the diffusional equilibration time. While this improvement was in progress, preliminary kinetic experiments started to point toward a significantly higher binding constant. Kinetic studies of binding and dissociation of the CNCbl−Hc complex subsequently revealed that K_b was substantially greater than 10^{13} M^{-1}, and thus exceeded the maximum binding constant that could reliably be measured by equilibrium dialysis using carrier-free [^{57}Co]-CNCbl.

Binding Kinetics. Second-order rate constants for the binding of cyanocobalamin to haptocorrin were expected to approach the diffusion-controlled limit and the pseudo-first-order conditions for their measurement were chosen accordingly. Formation of the CNCbl-Hc complex in a sufficient excess of cyanocobalamin followed first-order kinetics (Figure 1) for at least 4 half-times and had randomly scattered residuals. A plot of k_{obs} *vs* the average concentration of free CNCbl for each kinetic run was linear (Figure 1 inset) with an intercept value of 0 within the error limits. A weighted least-squares fit of this dependence yielded the second-order binding rate constant, $k_b = (2.36 \pm 0.04) \times 10^8$ M^{-1} s^{-1} at 25.0 °C, although the actual error in k_b may be higher than that resulting from the fit statistics if the uncertainty of the specific activity of the [^{57}Co]-CNCbl reagent is included. Similar kinetic experiments at temperatures between 5.4 and 57.7 °C allowed estimation of the thermodynamic parameters for binding kinetics from an Eyring plot (Figure 2). A weighted least-squares fit yielded $\Delta S^{\ddagger}_b = -4.48 \pm 0.34$ cal mol^{-1} K^{-1} and $\Delta H^{\ddagger}_b = 4.71 \pm 0.10$ kcal mol^{-1}. Attempts to study the binding kinetics at higher temperatures were prevented by the observed loss of ability of the free haptocorrin to bind cyanocobalamin.

Viscosity dependencies of the binding rate constant were determined at 25.0 and 2.0 °C at 4−8 different concentrations of glycerol or sucrose, while concentrations of other solution components remained unchanged. The data were plotted as $k_b°/k_b$ *vs* $\eta/\eta°$, where $k_b°$ and $\eta°$ refer to "standard" values, *i.e.* obtained in solutions lacking added viscogens, characterized by dynamic viscosity $\eta° = 0.950(4)$ mN s m^{-2}. When the microviscosity is the only property of the system that changes upon addition of a viscogen, such plots are expected to form straight lines going through the (1,1) coordinate with slopes

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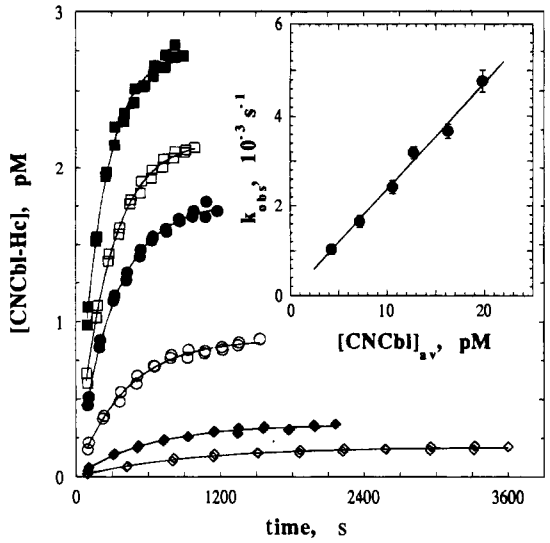

Figure 1. Kinetics of the formation of the cyanocobalamin–haptocorrin complex under pseudo-first-order conditions in excess CNCbl, at 25.0 °C and pH = 7.4 (0.1 M phosphate), and various concentrations of CNCbl. The curves represent least-squares fits of the data (collected to 5 half-lives) to an exponential growth equation, from which the pseudo-first-order rate constants, k_{obs}, were obtained. *Inset:* Plot of k_{obs} *vs* the average concentration of free CNCbl calculated for each kinetic run. The line is a weighted least-squares fit ($r^2 = 0.992$), from which $k_b = (2.36 \pm 0.04) \times 10^8$ M^{-1} s^{-1}.

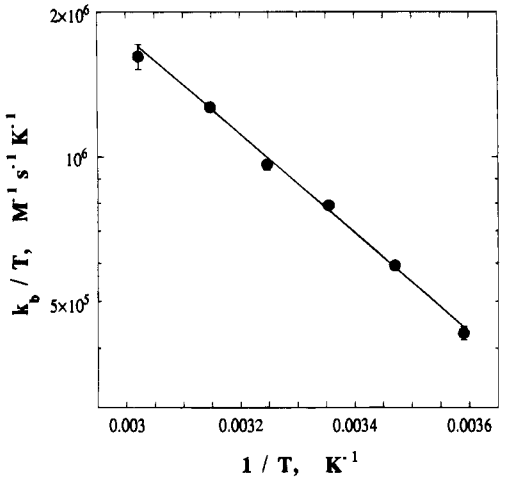

Figure 2. Plot of k_b/T *vs* $1/T$ for the formation of the CNCbl–Hc complex in the temperature range 5.4–57.7 °C. The solid line is a weighted nonlinear least-squares fit to the Eyring equation, from which $\Delta S^{\ddagger}_b = -4.48 \pm 0.34$ cal mol^{-1} K^{-1} and $\Delta H^{\ddagger}_b = 4.71 \pm 0.10$ kcal mol^{-1}. For some data points the error bar falls within the symbol.

within the limiting values of 1.0 for a fully diffusion-controlled reaction and 0.0 for a diffusion-independent one.[36,37] The observed effects on the rate constant for binding cyanocobalamin to haptocorrin at 25 °C are shown in Figure 3.

Dissociation Kinetics. The exceedingly high thermal stability of the CNCbl–Hc complex[11] permitted determination of complex dissociation kinetics, using cyanide to trap released CNCbl as the nonbinding base-off $(CN)_2Cbl^-$,[11] at temperatures as high as 95 °C. The use of cyanide as a trapping agent for the CNCbl-Hc dissociation rate constant determination required some preliminary experiments to assure that the trapping method was effective and that valid kinetic data could be obtained. The binding of CN^- to CNCbl is only moderately strong and fast,[35]

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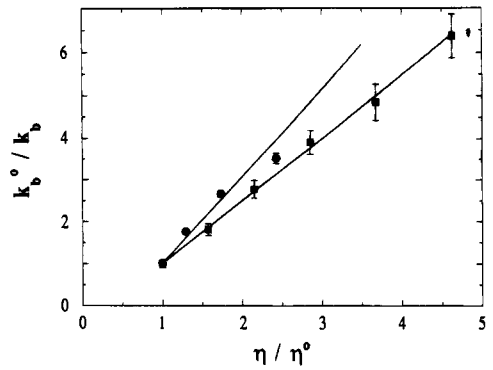

Figure 3. Dependence of the reciprocal of the relative rate constant for formation of the CNCbl–Hc complex, $k_b°/k_b$, on relative viscosity, at 25.0 °C and pH = 7.4 (0.1 M phosphate): (●) glycerol/water mixtures, slope 2.07 ± 0.17; (■) sucrose/water mixtures, slope 1.49 ± 0.02. The lines were fitted by a weighted least-squares method so they converge at the (1,1) coordinate.[37] Similar results at 2.0 °C (data not shown) gave slopes of 1.15 ± 0.10 and 1.66 ± 0.07 for glycerol/water and sucrose/water mixtures, respectively. For some data points the error bar falls within the symbol.

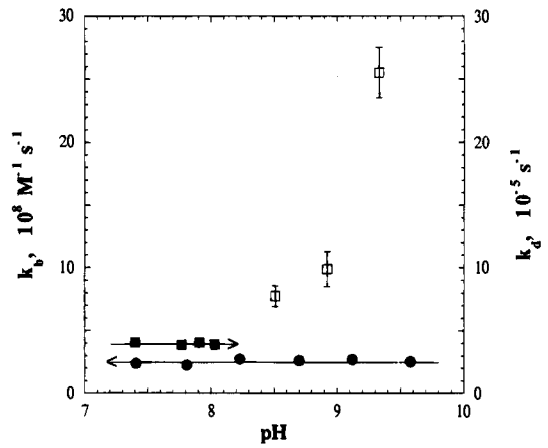

Figure 4. Dependence of (●) k_b, the second-order rate constant for the formation of the CNCbl–Hc complex at 25.0 °C (left ordinate), and (■, □) k_d, the first-order rate constant for dissociation of the CNCbl–Hc complex at 85.0 °C (right ordinate), on pH. The horizontal lines represent the weighted averages of the data points shown as solid symbols. For some data points the error bar falls within the symbol.

and care had to be exercised to provide sufficient excess of $[CN^-]$ over [CNCbl–Hc]. At such concentrations, cyanide became an active component of the buffer system, and eventually KCN replaced Na_2HPO_4 as a buffer component to avoid excessive ionic strength increases. Some cyanide decomposition at elevated temperatures was unavoidable. Cyanide and pH determinations revealed that under the conditions of the trapping experiments the decomposition could reach 40%, accompanied by an increase in pH up to 0.15 units. These properties of the buffer system required that the pH dependence of the binding and dissociation of the CNCbl–Hc complex be investigated. Using a series of Bicine buffers the binding rate constant at 25.0 °C was found to remain unchanged within the entire pH range studied, 7.41 to 9.58, as shown in Figure 4. Measurements of the dependence of the dissociation rate constant on pH were initially aimed at the possibility of studying the dissociation kinetics at higher pH where larger cyanide ion concentrations for an efficient trapping are easier to achieve. Using KCN, HCl, Na_2HPO_4, and NaOH a series of 8 buffers was prepared within a pH range of 8.5–10.7 at 85 °C, with concentrations adjusted to provide 0.1 M CN^- at 85 °C and ionic strength adjusted to 0.3 M with NaCl. These buffers were then used in measurements of the CNCbl–Hc dissociation rate constants at 80.0 and

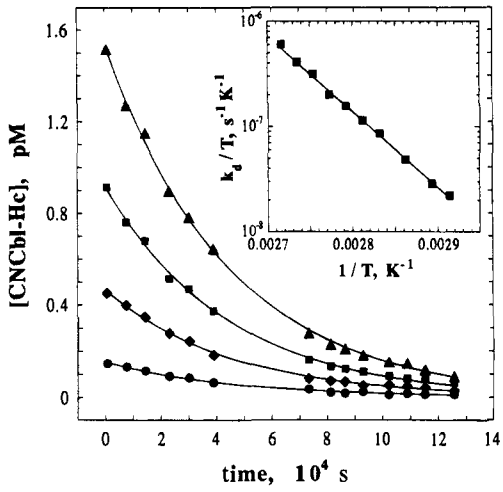

Figure 5. Representative plots of the decay of the CNCbl–Hc complex in four different starting concentrations at 85.0 °C and pH 7.4 (ionic strength 0.2 M) in the presence of 29 mM CN^- as a scavenger for the released CNCbl. The curves represent least-squares fits of the data (collected to 4 half-lives) to an exponential decay equation without offset. *Inset:* Plot of k_d/T *vs* $1/T$ for the rate constant for CNCbl–Hc complex dissociation in the temperature range 70.0–95.0 °C. The data were obtained for an initial pH = 7.40 ± 0.05 and an initial CN^- concentration in the 0.050–0.100 M range, except for the point at the highest temperature, where no cyanide was used. The solid line is a weighted, nonlinear least-squares fit to the Eyring equation, from which $\Delta S^{\ddagger}_d$ = 13.4 ± 1.6 cal mol^{-1} K^{-1} and $\Delta H^{\ddagger}_d$ = 32.9 ± 0.6 kcal mol^{-1}. The error bars fall within the symbols for all points.

85.0 °C which revealed a strong pH dependence of the dissociation rate constant starting at about pH 8.5 and leading to more than a 100-fold increase as the pH was increased to 10.7 at both temperatures. This presumably indicates that the ionization of an amino acid residue at the binding site strongly affects the affinity of the protein for CNCbl. Consequently, the pH range was narrowed to 7.40–8.03 at 85 °C, where 75 mM CN^- could be achieved using KCN/KH_2PO_4 buffers at ionic strength 0.5 M. Under these conditions, no pH dependence of k_d was observed (Figure 4), and pH = 7.4 was adapted as a standard condition for studying the dissociation kinetics as well.

To complete the testing of the cyanide trapping method, the decay of the [^{57}Co]-CNCbl–Hc was observed at various initial concentrations of the complex and cyanide. At 85.0 °C the observed first-order rate constant of the decay remained constant under each of the following experimental conditions: (a) pH = 7.4, $[CN^-]$ = 0.029 M and $1 \times 10^{-13} \leq$ [complex] $\leq 2 \times 10^{-12}$ M, (b) pH = 7.4, [complex] = 1×10^{-13} M and $0.0077 \leq [CN^-] \leq 0.0307$ M, (c) pH = 9.0, [complex] = 2×10^{-13} M and $0.104 \leq [CN^-] \leq 0.190$ M. Thus, the measured rates of decay of the [^{57}Co]-CNCbl–Hc complex can be taken as the dissociation rate constant of the cyanocobalamin–haptocorrin complex, k_d.

The temperature dependence of k_d was studied in the 70.0–92.5 °C range in buffer solutions containing 0.248 M KH_2PO_4 and 0.252 M KCN that provided an initial pH = 7.40 ± 0.05 and an initial CN^- concentration in the 0.050–0.100 M range. The decay of the complex followed strictly first-order kinetics until completion (Figure 5). However, due to considerable scatter resulting from low sample radioactivities, the experiments were designed to follow 2–3 half-times of the decay. In the Eyring plot of the temperature dependence of the dissociation rate constant (Figure 5 inset), a data point obtained at 95 °C in the *absence* of cyanide has been included. Apparently, at this temperature the thermal denaturation of the released haptocorrin was sufficiently fast to cause the observed decay of the complex

Table 1. Summary of Kinetic and Thermodynamic Parameters for Binding of Cyanocobalamin to Chicken Serum Haptocorrin at pH 7.4

constant[a]	ΔH, kcal mol^{-1}	ΔS, cal mol^{-1} K^{-1}
$k_b = 2.36 \times 10^8$ M^{-1} s^{-1}	4.71 ± 0.10	−4.48 ± 0.34
$k_d = 4.3 \times 10^{-9}$ s^{-1} [b]	32.9 ± 0.6	13.4 ± 1.6
$K_b = 5 \times 10^{16}$ M^{-1}	−28.2 ± 0.6	−17.9 ± 1.7

[a] 25 °C. [b] Extrapolated to 25.0 °C *via* the Eyring equation, from data collected at 70–95 °C.

to follow first-order kinetics with the rate constant equal to k_d in the absence of trapping cyanide. A weighted least-squares fit of the k_d values within the 70.0–95.0 °C range to the Eyring equation yielded $\Delta S^{\ddagger}_d$ = 13.4 ± 1.6 cal mol^{-1} K^{-1} and $\Delta H^{\ddagger}_d$ = 32.9 ± 0.6 kcal mol^{-1}. The values of the entropies and enthalpies of activation for binding and dissociation of the cyanocobalamin–haptocorrin complex allowed calculation of the equilibrium constant as log K_b = 16.7 ± 0.6 or $K_b = 5 \times 10^{16}$ M^{-1} at 25.0 °C, and ΔS° = −17.9 ± 1.7 cal mol^{-1} K^{-1} and ΔH° = −28.2 ± 0.6 kcal mol^{-1} for the binding equilibrium. The kinetic and thermodynamic parameters for CNCbl binding to Hc are summarized in Table 1.

Discussion

For the cyanocobalamin–Cbl-binding protein system, binding and dissociation rate constant values have not been available until now. Nevertheless, theoretical considerations of diffusion control of reactions involving proteins and conclusions from enzyme kinetics measurements[38] indicate that the second-order rate constant for the formation of the CNCbl–Hc complex should not reach the 10^9 M^{-1} s^{-1} mark, likely being 1 or 2 orders of magnitude lower. As the direct binding constant determination for a 1:1 complex requires the use of concentrations around K_b^{-1}, such experiments require special caution to allow the system to reach the equilibrium when a high binding constant is expected, even for a diffusion-controlled binding. For equal or similar concentrations of both reactants the binding follows second-order rather than pseudo-first-order kinetics that require even longer time to reach completion. Since this region of concentrations is crucial for a valid set of experimental data in binding constant measurement,[34] incomplete binding could result in an apparent K_b value several orders of magnitude below the true value. As a rule, measurements of high binding constants involving proteins rely on quantitation of the ligand only, whether free or protein bound. Thus, protein losses that are likely in samples in which the protein remains unbound for a prolonged time can lead to low values of K_b as well.

The literature reports of K_b determinations for Cbl-binding proteins have been reviewed for the possible origin of the substantial difference between the range of the reported values (10^9–10^{12} M^{-1}) and the value obtained in this work. While the use of unpurified or only partly purified proteins could be the cause in many cases (many of these studies were dated before the introduction of efficient purification techniques), some other factors may affect the reported results as well. Where enough experimental detail to assess the data have been revealed, it can be seen that binding equilibrium may have not been reached in all samples before separation.[10,39] Moreover, some of the separation procedures used could significantly disturb the previously established equilibrium, if the reported K_b values were correct.[6]

The equilibrium dialysis method attempted in this study, combined with appropriate statistical treatment of the radioactiv-

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ity data, appeared well suited for determination of an equilibrium constant within or somewhat above the range of K_b values reported so far, allowing for sufficient equilibration time with simultaneous separation that does not disturb the chemical equilibrium, until subsequent kinetic measurements revealed a K_b value that rendered any direct equilibrium determination impossible. Even with an analytical method capable of quantitating the species at 10^{-16}–10^{-17} M levels, the unbound protein would likely not withstand the required equilibration times without significant decomposition.

Another indication of a CNCbl–Hc binding constant higher than the range of literature reports came from our experience with the UCBC assays. For an assumed $K_b = 1 \times 10^{11}$ M^{-1} the use of CNCbl and Hc concentrations specified in the assay procedure would allow for up to 7% of protein to remain unbound at equilibrium. However, the results of the assays remained unaffected when these concentrations were varied within 2 orders of magnitude around their standard levels, indicating a significantly higher binding constant.

Regardless of the method used to separate the protein-bound and unbound ligand (equilibrium dialysis, size-exclusion chromatography, or adsorption onto charcoal), any equilibrium method of measuring such large binding constants has an inherent upper limit due to the specific radioactivity of the labeled ligand, limitations on the size of a sample that can be counted, and the minimum radioactivity of a sample needed to produce acceptable counting statistics in the presence of a finite background. For carrier-free [^{57}Co]-CNCbl, depending on the exact experimental protocol, this limit is on the order of K_b = 10^{11}–10^{13} M^{-1}. In the most detailed previous study of the thermodynamics of Cbl binding to B_{12}-binding proteins, Hippe and Olesen[6] studied the equilibrium binding of CNCbl to human intrinsic factor and transcobalamins I and II using [^{57}Co]-CNCbl and separating bound and unbound ligand by gel filtration. These authors reported a binding constant of 6×10^9 M^{-1} for intrinsic factor at 26 °C, characterized by a substantial enthalpy change ($\Delta H^\circ = -22.7$ kcal mol^{-1}, $\Delta S^\circ = -31.0$ cal mol^{-1} K^{-1}). The thermodynamic parameters seem to be reasonable in light of the current data although the binding is some 9.3 kcal weaker due to both a lower exothermicity and a larger negative entropy change. However, these authors also reported binding constants of about 3×10^{11} M^{-1} for transcobalamins I and II, with both equilibria being isoenthalpic. The virtually identical binding constants for these two proteins combined with the lack of any observable temperature dependence strongly suggests that the value of 3×10^{11} M^{-1} simply represents the upper limit of the method employed and is not a true measurement of the binding constant for these equilibria.

The value obtained for the binding rate constant for CNCbl–Hc complex formation ($k_b = 2.36 \times 10^8$ M^{-1} s^{-1}) is close to that expected for diffusion-controlled protein–ligand associations.[40–43] Measurements of the viscosity dependence of k_b were undertaken not only to further characterize the binding, but also to learn indirectly what kind of binding can be expected for other cobalamin derivatives. If the binding of cyanocobalamin to Hc was fully diffusion controlled, the same would be expected for most other Cbl derivatives, making it plausible to estimate equilibrium constants for complexation of such analogs with Hc from their dissociation rate constants. Such an assumption regarding the binding rate constants has been applied to the biotin–avidin system, although without detailed studies of the binding process.[44–46]

The viscosity dependence of the binding rate constant found in this study seems to confirm the expected diffusion-controlled process. However, the degree of diffusion control is difficult to determine due to the apparent interference of medium-related effects on the rate constant that cause an excessive viscosity dependence so that the slopes of the k_b°/k_b *vs* η/η° plots are larger than 1. The change in temperature from 25 to 2 °C seems to influence these effects rather than the degree of diffusion control of k_b because this temperature change affected the slopes quite differently, depending on whether glycerol or sucrose was used as the viscogen. The observed dependence on viscosity can be corrected for such medium effects, as has been done in several studies on enzyme kinetics,[36,47,48] once a cobalamin derivative can be found that binds to haptocorrin with a k_b low enough to be safely regarded as diffusion independent. Kinetic studies with such an analog will permit quantitation of the viscosity-independent medium effects so that these can be factored out of the kinetics of CNCbl association.

The observed enthalpy of activation for k_b ($\Delta H^\ddagger_b = 4.71 \pm 0.10$ kcal mol^{-1}) can also be regarded as indicative of a diffusion-controlled binding. The low enthalpy of activation found for k_b is close to that for the second-order rate constant for diffusion in water calculated from the temperature dependence of H_2O viscosity, using the Smoluchowski and the Stokes–Einstein equations for the same temperature range ($\Delta H^\ddagger_D = 3.27 \pm 0.07$ kcal mol^{-1}). It is also lower than the reported value for binding of chicken cystatin to papain, $\Delta H^\ddagger_b = 7.53 \pm 0.60$ kcal mol^{-1}, which was concluded to approach the diffusion-controlled limit.[49] The observed pH independence of the binding rate constant in the present study is also consistent with diffusion control. Thus, k_b is pH independent up to pH 9.58 even though dissociation kinetics clearly reveal a binding site ionization that substantially reduced binding affinity above pH 8.5. Consequently, the diffusionless (*i.e.* in-cage protein–ligand combination) rate constant for binding must be substantially larger than the observed rate constant, which in turn must be limited by diffusion to form the caged pair.

Scavenging of the released [^{57}Co]-CNCbl with the excess of CN^- ions has permitted determination of the dissociation rate constants over a range of temperatures and, consequently, the high equilibrium constant. The validity of the cyanide trapping method has been confirmed in the preliminary tests and is additionally supported by the agreement of the dissociation constant determined in the absence of cyanide at 95 °C (apparently by rapid denaturation of the released apo-Hc) with the result of extrapolation of the temperature dependence of the rate constant obtained by cyanide trapping. The CNCbl–Hc complex itself has been shown to be exceedingly thermally stable, as 10-min incubation at 100 °C has no effect on its CD spectrum.[11] Thus, the high temperatures needed to induce measurable dissociation kinetics do not cause thermal denaturation of the starting CNCbl–Hc complex.

To our knowledge the binding constant obtained in this study is the highest equilibrium constant ever measured for a protein–ligand system, exceeding by at least an order of magnitude that for the biotin–avidin complex.[44] The previously reported K_b

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values for all three major vitamin B_{12}-binding proteins, intrinsic factor, transcobalamin, and haptocorrin, in the 10^9-10^{12} M^{-1} range[2,6,24] may need to be properly regarded as lower limits of the actual binding constants, at least for haptocorrin and transcobalamin. The possibility that the binding constants for all three types of vitamin B_{12} binders are not as close to each other as suggested by earlier work could help in understanding the significant differences between these three proteins regarding their properties and the roles they play in living organisms.[2,50]

The interaction of CNCbl with chicken serum haptocorrin provides a large amount of binding free energy for immobilization of the cobalamin to the protein binding pocket ($\Delta G° = -22.9$ kcal mol^{-1} at 25 °C). It is clear, at least for AdoCbl-dependent ribonucleotide reductase from *Lactobacillus leichmannii*,[51,52] that AdoCbl-dependent enzymes bind AdoCbl much less tightly. If similar binding contacts are exploited in both systems, the binding energy discrepancy suggests that in the AdoCbl-dependent enzymes, a substantial amount of binding energy may be utilized to physically distort the coenzyme, raising its ground state free energy and activating it for Co−C bond homolysis. At any rate, establishment of the thermodynamics of the association of CNCbl with haptocorrin will now permit a detailed dissection of the structural basis for this very tight binding *via* studies of the thermodynamics of binding of structurally altered Cbl analogs. Such studies are currently in progress.

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