Kinetics and Activation Parameters of the Reaction of Cyanide with Free Aquocobalamin and Aquocobalamin Bound to a Haptocorrin from Chicken Serum*

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The kinetics of the reaction of cyanide with free aquocobalamin (H₂O₇Cbl) and with aquocobalamin bound to a vitamin B₁₂-binding protein (haptocorrin) from chicken serum (HC-H₂O₇Cbl) have been investigated as a function of temperature and pH. The mechanism of replacement of H₂O from protein-bound and free H₂O₇Cbl is apparently the same and involves attack of both CN⁻ (k₁) and HCN (k₂). The reactions with HC-H₂O₇Cbl are somewhat slower than those with free H₂O₇Cbl, k₁ being 22-fold smaller and k₂ 7-fold smaller at 25 °C. The relatively small effect of the protein on the rate constants supports the view that the metal in HC-H₂O₇Cbl is readily accessible to solvent. Activation parameters suggest that the transition states for ligand substitution are stabilized by nucleophilic participation (at least by CN⁻) and that ligand substitution on the protein-bound cobalamin proceeds through more ordered, concerted transition states. The latter effect suggests that the Co–O bond of H₂O₇Cbl is strengthened upon binding to the haptocorrin.

One of the unresolved problems in the biological chemistry of cobalamin-dependent enzymes is the mechanism whereby such enzymes “activate” 5’-deoxyadenosylcobalamin (AdoCbl)¹ to produce a 5’-deoxyadenosyl (Ado) radical and cob(II)alamin (1–17). Binding of AdoCbl to such enzymes in the presence of substrate is now known to increase the rate of Co–C bond homolysis by at least 10⁶-fold (18). While it is generally agreed that activation of AdoCbl is sterically in-

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¹ The abbreviations used are: AdoCbl, 5’-deoxyadenosylcobalamin; H₂O₇Cbl, free aquocobalamin; HC-H₂O₇Cbl, aquocobalamin bound to the chicken serum haptocorrin; Ado,5’-deoxyadenosyl; Bz, 5,6-dimethylbenzimidazole; MP-8, the heme octapeptide from cytochrome c which contains His-18 as proximal ligand; CAPS, 3-(cyclohexylamino)propanesulfonic acid; MES, 4-morpholineethanesulfonic acid.

duced by a conformational change in the protein upon binding substrate, few specific hypotheses of how such steric activation might arise have been advanced. One such hypothesis, the “mechanochemical trigger” mechanism is quite attractive on several grounds. It envisages an enzyme-mediated compression of the long (2.24 Å) axial Co–N(Bz) bond (19) leading directly to homolytic fission of the Co–C (Ado) bond. This would provide rationalizations for the presence of a pendent axial ligand in this unique pentadentate chelate as well as its steric bulk, and for the relatively tight binding of AdoCbl to most AdoCbl-requiring enzymes. Model systems also exist for the labilization of sterically strained alkylcobalamins upon decreasing the distance between the benzimidazole of the nucleotide loop and the cobalt ion (20–28). For example the slow homolysis of the base-off form of neopen
tylocobalamin in acid solution under aerobic conditions (tₙₐ₅ = 28 days) is increased some 500-fold when the reaction is conducted at neutral pH, even though the benzimidazole nucleotide appears to be only about 30% coordinated (26).

Derivatives of vitamin B₁₂ are also very tightly bound by a wide range of proteins including the gastric intrinsic factors, the serum transcobalamins, and the haptocorrins (also known as R-binders or cobalophilins) obtained from diverse sources (see, for example, Refs. 29 and 30). A variety of cobalamins with different β axial ligands (CN⁻, H₂O, OH⁻, Cl⁻, CH₃₂ and Ado) have similar affinities for such proteins (31–34). However, since alteration of the nucleotide loop significantly decreases this affinity (35–37) it is generally believed that the cobalamins bind to such proteins with the β face pointing toward the solvent. Although the binding of many B₁₂ derivatives to such proteins has been studied, little work has been done to determine the effects of the protein moiety on the chemical and physical properties of bound cobalamins.

We are currently involved in studies of the chemistry of cobalamins bound to such B₁₂-binding proteins as models for the interaction of AdoCbl with AdoCbl-requiring enzymes. Recently using a 70-kDa glycoprotein haptocorrin from chicken serum, we have found that the ³¹P NMR resonances of protein-bound cobalamins are shifted some 0.7–1.0 ppm downfield from the positions of the free cobalamin resonances (38). This is apparently due to changes in phosphodiester conformation which may involve steric compression of the axial Co–N(Bz) bond (38).

In the current work we have attempted to use cyanide as a probe of the chemistry of protein-bound cobalamins. Cyanide has been shown to be a useful probe ligand for the metal center of metalloproteins. For instance, the kinetics of the reaction of cyanide with microperoxidase-8 (MP-8), a heme octapeptide obtained by proteolysis of cytochrome c which
retains His-18 as proximal (axial) ligand, have recently been studied (39). Comparison of the results with the kinetics of the reaction of cyanide with hemoproteins containing a single His ligand, such as myoglobin, hemoglobin, various peroxidases, and cytochrome c oxidase, has shown how the protein moiety controls and modifies the ligand binding properties of the metallo prosthetic group (39). Since the kinetics and mechanism of the reaction of free H2O-Cbl with cyanide in aqueous solution at 25 °C have been reported (40), we have extended this approach to the complex of H2O-Cbl with the chicken serum haptocorrin (HC-H2O-Cbl) and have determined the kinetics of its reaction with cyanide as a function of pH and temperature. The results of these investigations are the subject of this paper.

EXPERIMENTAL PROCEDURES

Chicken serum haptocorrin saturated with H2O-Cbl (HC-H2O-Cbl) was prepared and characterized as previously described (38).

The kinetics of cyanide addition to HC-H2O-Cbl to produce HCN-Cbl were followed by the absorbance change at 564 nm (close to the y-band of the product (38) using 1.0-cm path length semi-micro quartz cuvettes on a Cary 219 recording spectrophotometer fitted with a thermostatted cell block. The temperature was monitored in the cuvettes with a thermistor device (Yellowsprings Instruments) calibrated against NBS calibrated thermometers. Pseudo-first-order conditions were maintained with HC-H2O-Cbl (1.2 × 10^-3 M) and total cyanide at least 10-fold that concentration. pH was maintained with 0.1 M appropriate buffer (acetate, phosphate, bicine, or CAPS), and ionic strength was maintained at 1.0 M with KCl. Reactions were initiated by addition of 100 μl of temperature-equilibrated HC-H2O-Cbl stock solution to temperature-equilibrated cuvettes containing buffer, KCl, and cyanide for a final volume of 1.0 ml. Cyanide solutions were prepared fresh daily and their pH adjusted to match that of the reaction solutions. pH was measured using a Radiometer PHM-4 pH meter and a Radiometer Type B combined glass electrode with electrode, samples, standards, and rinse water incubated at the measurement temperature.

Kinetic traces were monitored for at least five half-times and analyzed by plotting ln(Ao - At) versus t using standard linear regression analysis to obtain a pseudo-first-order rate constant, kobs. The relevant second-order rate constants, kapp, were obtained from the slope of plots of kobs versus cyanide concentration in which the latter varied by a factor of 20-500, also by linear regression analysis. A similar technique was used to study the reaction of free H2O-Cbl (~3.2 × 10^-4 M) with cyanide, but reactions were monitored at 560 nm, the isoelectric point for conversion of CNCbl to dicyanocobalamin (40).

TABLE I

| Species       | Temperature °C | pH   | ∆H kcal mol^-1 | ∆S cal mol^-1 K^-1 |
|---------------|--------------|-----|----------------|--------------------|
| HCN           | 5.0          | 9.62 ± 0.03 |                 |                    |
| 15.0          | 9.34 ± 0.02  |     |                |                    |
| 25.0          | 9.04 ± 0.02  | 10.8 ± 0.1 | -5.2 ± 0.5      |                    |
| 45.0          | 8.56 ± 0.03  |     |                |                    |
| H2O-Cbl       | 5.0          | 8.46 ± 0.01 |                 |                    |
| 15.0          | 8.28 ± 0.01  | 6.84 ± 0.07 | -14.1 ± 0.3     |                    |
| 25.0          | 8.1          |     |                |                    |
| HC-H2O-Cbl    | 5.0          | 8.69 ± 0.03 |                 |                    |
| 25.0          | 8.39 ± 0.02  | 7.17 ± 0.17 | -13.9 ± 0.5     |                    |
| 45.0          | 7.97 ± 0.02  |     |                |                    |

* Ref. 41.  
* Ref. 40.

RESULTS

The variation with temperature in the kobs for HCN, H2O-Cbl, and HC-H2O-Cbl, and the enthalpy and entropy of ionization, obtained from plots of ln kobs versus 1/T (not shown), are listed in Table I. The spectral changes associated with the kobs values of H2O-Cbl and HC-H2O-Cbl were found to be completely reversible in the pH range 5.3 to 11.5. Consequently, the haptocorrin appears to be stable in alkaline solution at least up to pH 11.5.

Two determinations of kobs at 25 °C, for the reaction of free H2O-Cbl with cyanide at pH 4.00 and 10.53, were performed to confirm the previously reported values (40). The values obtained were 0.523 ± 0.002 M^-1 s^-1 at pH 4.00 and 0.998 ± 0.018 M^-1 s^-1 at pH 10.53. These were in adequate agreement with values calculated from the results of Reenstra and Jencks (40) of 0.253 and 0.907 M^-1 s^-1.

The reaction was found to proceed too rapidly at 45 °C for reliable determination of second-order rate constants by conventional spectroscopy. Therefore, the variation of kobs with pH for the reaction of free H2O-Cbl with cyanide was studied at 5 °C and 15 °C. The results are shown in Fig. 1. In agreement with a previous report (40), no evidence of saturation kinetics was found at high cyanide concentration (up to 0.5 M at pH 3.99, 5 °C).

The mechanism of reaction of cyanide with H2O-Cbl is well established (40). The reaction can occur via either of two routes. The first route involves the attack of CN^- on H2O-Cbl (Equation 1), hydroxocobalamin being substitution inert (42).

$$\text{HC-H}_2\text{O-Cbl} + \text{CN}^- \rightarrow \text{HC-CN-O-Cbl}$$

FIG. 1. Variation of the observed second-order rate constant, kobs, with pH for the reaction of free H2O-Cbl with cyanide at 5 °C (○), 15 °C (△), and 25 °C (●), ionic strength 1.0 M (KCl). The solid lines are the theoretical curves calculated via Equation 11 and the data in Tables I and II. The results from Ref. 40 were used to calculate the solid line at 25 °C.
cyanide concentration are statistically indistinguishable from zero over the entire pH range studied, $k_{-1} \ll k_1$ and $k_{-1}$ may be neglected. The rate law for this route is given by Equation 2. Defining

$$\frac{d[NCCbl]}{dt} = k_1[H_2Ocbl][CN^-]$$

(2)

$K_{HOCbl}$ and $K_{HCN}$ as in Equations 3 and 4 leads to the rate law of Equation 5, where [Co]$_T$ is the total cobalamin concentration and $[cyanide]_T = [CN^-] + [HCN]$.

The second route involves attack of HCN on $H_2Ocbl$ to form an N-bound intermediate which deprotonates and isomerizes to the C-bound product (40) (Equations 6–8). It is

$$H_2Ocbl \xrightarrow{K_{HOCbl}} HOcbl + H^+$$

(3)

$$HCN \xrightarrow{K_{HCN}} CN^- + H^+$$

(4)

$$\frac{d[NCCbl]}{dt} = \frac{k_1[Co][cyanide]_T}{(1 + K_{HOCbl}/[H^+])(1 + [H^+]/K_{HCN})}$$

(5)

the ion product of water. Since $k_{als}$ is defined as in Equation 10, the complete rate law of

$$\frac{d[NCCbl]}{dt} = k_{als}[Co][cyanide]_T$$

(9)

the results, which were nearly identical using either minimization routine, are summarized in Table II.

Alternatively (40), the second-order rate constants may be expressed in terms of addition of CN$^-$ to H$_2$Ocbl by dividing $k_{als}$ by the fraction of cyanide present as CN$^-$ and the fraction of cobalamin present as the aquo species (Equation 12). Data

$$k_{als} = \frac{k_1}{(1 + K_{HOCbl}/[H^+])(1 + [H^+]/K_{HCN})}$$

(10)

$$k_{als} = \frac{k_1}{(1 + K_{HOCbl}/[H^+])(1 + [H^+]/K_{HCN})} + \frac{k_2}{(1 + K_{HOCbl}/[H^+])(1 + K_{HCN}/[H^+])(1 + aK_a/[H^+]})$$

(11)

iterative, nonlinear least squares program using either a simple minimization algorithm or a Newton–Raphson procedure using Marquartd’s algorithm. The results, which were nearly identical using either minimization routine, are summarized in Table II.

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(13)

the unusual pH rate profiles of Fig. 2 clearly show a pH-independent region at high pH (representing rate-limiting addition of CN$^-$ to H$_2$Ocbl), an acid-catalysed region at intermediate pH (representing rate-limiting addition of HCN to form the N-bound species), and a pH-independent region at low pH (due to rate-limiting isomerization of the N-bound species to the C-bound species, Equation 8).

Values of $k_{als}$ for reaction of HC-H$_2$Ocbl with cyanide were determined over the pH range 3.5 to 11.2 at 5, 25, and 45 °C. As with free H$_2$Ocbl, no evidence was found for saturation kinetics up to cyanide concentrations as high as 0.5 M. The
Cyanide Reaction Kinetics

**FIG. 4.** Variation of $k_{corr}$ (Equations 12 and 13) the second-order rate constant for reaction of HC-H$_2$OCl with cyanide at 5 °C (穸), 25 °C (黴), and 45 °C (ريس). The solid lines are theoretical curves calculated via Equation 13 and the data in Tables I and II.

**FIG. 3.** Variation of the observed second-order rate constant, $k_{obs}$, with pH for the reaction of HC-H$_2$OCl with cyanide at 5 °C (電子信箱), 25 °C (黴), and 45 °C (ريس), of ionic strength 1.0 M (KCl). The solid lines are theoretical curves calculated via Equation 11 and the data in Tables I and II.

**TABLE III**

| Complex     | $\Delta H^\circ$ | $\Delta S^\circ$ | $\Delta H^\circ$ | $\Delta S^\circ$ |
|-------------|-------------------|-------------------|-------------------|-------------------|
|             | kcal mol$^{-1}$   | cal mol$^{-1}$ K$^{-1}$ | kcal mol$^{-1}$ | cal mol$^{-1}$ K$^{-1}$ |
| H$_2$OCl    | 12.4 ± 0.3        | -6.05 ± 0.91      | 18.4 ± 0.1        | 12.0 ± 0.2        |
| HC-H$_2$OCl | 13.6 ± 0.1        | -8.01 ± 0.06      | 17.2 ± 0.4        | 3.82 ± 1.5        |

pH rate profiles obtained are shown in Fig. 3 and the values of the kinetic parameters obtained by curve-fitting to Equation 11 are given in Table II. pH rate profiles for $k_{corr}$ (Equations 12 and 13), i.e. corrected for ionization of the starting materials, are shown in Fig. 4.

Activation parameters for addition of CN$^-$ (i.e., $k_1$, Equation 1) and HCN (i.e., $k_2$, Equation 6) to free H$_2$OCl and HC-H$_2$OCl were determined from the temperature-dependence of $k_1$ and $k_2$ via plots of ln$(kh/k_BT)$ versus $1/T$ (not shown) where $h$ and $k_B$ are Planck’s constant and Boltzman’s constant, respectively. The values obtained are listed in Table III.

**DISCUSSION**

The enthalpy and entropy of ionization of the aquo ligand of free H$_2$OCl has not been reported previously. As can be seen from the temperature dependence of the equilibrium constant for the self-ionization of water ($\Delta H = 13.3$ kcal mol$^{-1}$, $\Delta S = -19.4$ cal mol$^{-1}$ K$^{-1}$, calculated from the data in Ref. 45), ionization of water entails a positive enthalpy change but a negative entropy change. The latter value suggests that the products of ionization cause substantial ordering of solvent as would be expected. Coordination to the metal center causes a substantial reduction in the enthalpy of ionization and a smaller reduction in the magnitude of the entropy change. Both effects can be reasonably attributed to stabilization and charge delocalization of the conjugate base (OH$^-$) by coordination.

On binding of H$_2$OCl to the haptocorrin, the pK$_a$ is raised from 8.10 to 8.29 at 25 °C. This is a consequence of somewhat higher enthalpy of ionization; the entropies of ionization of free and protein-bound H$_2$OCl are not significantly different. Binding of ferric porphyrins to various proteins can increase the pK$_a$ of Fe(III)-coordinated water, decrease it, or leave it relatively unchanged. For instance, the pK$_a$ of aquo-MP-8 is 8.90 (46), while the pK$_a$ values of human metHb (47), horse metMb (48), and sperm whale metMb (47) are 8.05, 8.93, and 8.99, respectively. Whether these differences are due to enthalpic or entropic factors is not yet known, and there appears to be at present no means of predicting the effect of the protein on the ionization of a metalloaquo center. However, in the case of HC-H$_2$OCl the small effect of the protein on the pK$_a$ implies that the microenvironment of the coordinated water is not significantly different from that of free H$_2$OCl in bulk water, i.e. the upper axial ligand position of the cobalamin is not buried in a protein region of significantly different dielectric than solvent.

The temperature dependence of the pK$_a$ of HCN has been reported (49) but at a low ionic strength (<0.01 M) from which the values $\Delta H = 11.4$ kcal mol$^{-1}$ and $\Delta S = -6.7$ cal mol$^{-1}$ K$^{-1}$ can be calculated with pK$_a = 9.21$ at 25 °C. Our results show that HCN is a stronger acid at higher ionic strength as would be anticipated for a neutral Bronsted acid.

The good agreement obtained between the theoretical curves and the experimental data in Figs. 3 and 4, as well as the similarity of the reactant ionization-corrected pH rate...
profiles for free H$_2$OCl (Fig. 2) and the protein-bound species (Fig. 4) suggest that HC-H$_2$OCl and free H$_2$OCl react with cyanide by the same mechanism. However, at 25 °C, the reaction of CN$^-$ is 10.8 times slower and that of HCN 2.3 times slower with HC-H$_2$OCl than with free H$_2$OCl. When compared with the effect which some hemoproteins have on such reactions, this effect is modest. The second-order rate constants for reaction of CN$^-$ and HCN with ferric MP-8 are $6.0 \times 10^5$ M$^{-1}$ s$^{-1}$ and $4.8 \times 10^4$ M$^{-1}$ s$^{-1}$, respectively (39). In contrast the rate of reaction of metMb with HCN is insignificant compared with its rate of reaction with CN$^-$ (50). And the rate constants for reaction of CN$^-$ with metMb and metHb are of the order of $2 - 5 \times 10^5$ M$^{-1}$ s$^{-1}$ (51, 52), i.e. the protein slows down the reaction by about 20,000-fold. This has been ascribed both to poor ligand accessibility to the metal and to steric crowding of the heme distal side (39). We may conclude, in agreement with previous suggestions about cobalamin-binding proteins (35, 36), that the cobalamin-binding site of the haptocorrin from chicken serum leaves the cobalamin relatively open to the solvent. This, of course, explains the success of affinity chromatography procedures in which the cobalamin moiety is immobilized by attachment to the upper axial ligand position (38).

The present results, together with those of Reenstra and Jencks (40) show that both CN$^-$ and HCN (presumably reacting through N) act as nucleophiles toward the Co(III) center of H$_2$OCl. A similar effect has been shown with the ferric heme center of MP-8 (39). Although we were unable to observe the postulated N-bound intermediate cyanide complex, precedents do exist, as has been pointed out (40). We may further add that Betterton (53) observed that when HCN reacts with diaquocobinamide, isosteric points are not immediately established but shift with time in a subsequent irreversible reaction, indicating formation of an intermediate. In addition the transient formation of what is probably an N-bound cyanide species on reaction of hemin with cyanide has been observed spectroscopically (54, 55). There would, therefore, appear to be little doubt about the validity of the proposed reaction scheme.

It is generally agreed that the mechanism of ligand substitution reactions of six-coordinate cob(III)alaminas in aqueous solution is a predominately dissociative (i.e. $L_0$) process (40, 56, 57). Hence, the rate of substitution of H$_2$O by H$_2$OCl by small anions such as $I^-$, SCN$^-$, and CN$^-$ is relatively insensitive to the nature of the incoming ligand (58). However, substitution rates have been found to vary by nearly two orders of magnitude for a larger series of ligands in which equilibrium constants vary by 11 orders of magnitude (59). This observation prompted Reenstra and Jencks (40) to suggest that there is some stabilization of the transition state by the incoming ligand (i.e. nucleophilic participation) at least for some ligands. On the other hand some of the variability in ligand substitution rates may be due to other factors. If the incoming ligand is capable of hydrogen bonding to the acatimide side chains directed upwards toward the $\beta$ face of cobalamin, rate constants can decrease significantly. For example, the second-order rate constant for substitution of H$_2$O by SCN$^-$ is $7.1 \times 10^3$ M$^{-1}$ s$^{-1}$ (60), but 21.5 and 1.08 M$^{-1}$ s$^{-1}$ for NH$_2$OH and CH$_3$NH$_2$, respectively (61). It thus appears that factors such as ligand charge, and steric, hydrophobic-, and hydrogen-bonding effects between the ligand and the corrin ring side chains play a role in the variability of ligand substitution rates.

Nonetheless, our results on the activation parameters for substitution of H$_2$OCl by CN$^-$ and HCN provide support for the importance of nucleophilic participation in the ligand substitution transition state. The transition state for substitution by CN$^-$ is enthalpically stabilized by 6 kcal relative to that for HCN substitution (Table III). As CN$^-$ must be expected to be a far stronger nucleophile than HCN (CN$^-$ is more basic toward the proton than HCN by over 11 orders of magnitude (61)), this observation provides strong support for nucleophilic stabilization. Entropies of activation, however, are much more difficult to interpret due to the importance of differential solvation of ground and transition states. The positive entropy of activation for HCN substitution suggests that the entropy gain due to loss of the leaving ligand is largely uncompensated by entropy loss due to bonding of the incoming ligand. This is consistent with a strictly $L_0$ mechanism with little or no nucleophilic participation and a symmetric transition state. However, the value of $\Delta S^*$ for HCN substitution is very similar to that expected for a simple gas phase dissociation reaction (62) suggesting that there is little change in solvation of the departing ligand in the transition state. This makes it difficult to rationalize the negative entropy of activation for CN$^-$ substitution, since the entropy loss due to participation of the incoming-ligand in the transition state cannot be expected to exceed the entropy gain due to dissociation of the leaving ligand. Nonetheless, the substantial decrease in $\Delta S^*$ for CN$^-$ substitution relative to HCN substitution is consistent with a very much more ordered transition state in which significant nucleophilic participation has made the mechanism somewhat more concerted (40).

On binding to the protein, the reaction rates change considerably (Table II) and at 25 °C CN$^-$ and HCN react with virtually the same rate constants with HC-H$_2$OCl. The activation parameters show that this is due to (i) an increase in $\Delta H^*$ and an even more unfavorable $\Delta S^*$ for replacement of H$_2$O by CN$^-$ and (ii) a slightly smaller $\Delta H^*$ but significantly less favorable $\Delta S^*$ for replacement of H$_2$O by HCN. The transition states for replacement of H$_2$O by CN$^-$ and HCN are thus both more ordered in protein-bound than in free H$_2$OCl. This suggests that there is less Co–O bond breaking in both transition states and that both displacement reactions have become somewhat more concerted (40). This would be the anticipated result if binding of H$_2$OCl to the protein leads to a strengthening of the Co–O bond. Excellent structural evidence has been obtained for a mutual dependence of Co–C and trans Co–L bonds in organocobalamines (63) and related cobalamin models (64). Thus, shortening of the axial Co–N bond leads to a strengthening of the trans Co–C bond in such organocobalt species (63). A similar effect has recently been shown to operate in organocobalamins (65). An analogous effect may be responsible for the decreased reaction rate of HC-H$_2$OCl with cyanide. Recent $^{31}$P NMR measurements (38) of cobalamins bound to the haptocorrin from chicken serum show that the nucleotide loop phosphodiester resonance is shifted downfield as much as 1 ppm upon binding of cobalamins to the protein. This effect has been definitely attributed to a change of phosphodiester conformation upon binding. Earlier NMR studies with free cobalamins (66–69) have shown that nucleotide loop conformation is dependent on the axial Co–N(Bz) bond length. Thus, a possible explanation for the more ordered, and somewhat concerted ligand substitution transition states for protein-bound H$_2$OCl is steric compression of the axial Co–N(Bz) bond leading to a strengthened Co–O bond via the anticipated electronic trans effect. Further studies to clarify this question are in progress.

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