Complexation and Oxygenation Equilibria of Cobaltous Chelates of Dipeptides with Coordinating Side Groups

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Abstract: Chelate formation, amide deprotonation, and oxygenation reactions of 1:1 cobaltous complexes of glycyl-L-histidine, glycylhistamine, glycyl-L-aspartic acid, L-histidyl-L-histidine, L-histidylglycine, and L-aspartylglycine have been investigated, and the corresponding thermodynamic equilibrium constants are reported. The influence of the peptide side chains on these equilibria is a function of several variables, including the steric arrangements of side-chain substituents on the basic glycylglycine framework, the strength of coordination of the metal ion by side-chain liganding groups, and the inductive effects of the side chains on the acidity of the amide nitrogen. These effects are described in detail for ligands containing 4-methylimidazole and carboxymethyl side groups.

Cobalt-dioxygen complexes have received considerable attention as models for the binding of molecular oxygen in biological oxygen carriers.[1b] For this reason a basic understanding of systems which incorporate biologically significant liganding groups, e.g. histidine, is of considerable importance. Although some of the earliest investigations of oxygen complexes involved cobaltous chelates of dipeptides,[2-4] the accurate characterization of such systems has been achieved only fairly recently.[5-10] This paper reports the first investigation of the oxygenation equilibria of the 1:1 cobaltous chelates of the dipeptides glycyl-L-histidine (Gly-His), glycyl-L-aspartic acid (Gly-Asp), glycylhistamine, L-histidylglycine (His-Gly), L-aspartylglycine (Asp-Gly), and L-histidyl-L-histidine (His-His). In some cases the presence of an additional ligating group results in cobalt-promoted amide nitrogen deprotonation at comparatively low pH. For some time it was debated whether the cobalt(II) ion was capable of effecting the ionization of the amide nitrogen,[11-15] but the necessity of considering this equilibrium in cobalt-peptide systems has now been well established.[7,16,17] However, the interaction of cobalt(II) is much weaker than that in the more extensively studied cupric-dipeptide systems, often requiring fairly high pH before deprotonation is observed. For this reason, hydrolysis of the free metal is a serious problem in the investigation of 1:1 cobalt(II)-dipeptide systems. The first hydrolysis constant of the hexaaquocobalt(II) ion was included in the calculations, but precipitation of $Co(OH)_2$ often restricts the pH range over which equilibrium measurements can be made.

Experimental Section

All ligands were obtained commercially except for glycylhistamine, which was kindly provided by Dr. Rod O'Connor. In all cases the purity of the ligand was determined by titration with KOH. Stock metal solutions were prepared from reagent grade cobalt nitrate and were standardized by titration with EDTA. Potentiometric equilibrium measurements were carried out using carbonate-free KOH in an air-tight titration vessel under an atmosphere of nitrogen which had been passed through two alkaline pyrogallol scrubbers and bubbled through 0.10 M KNO_3 prior to entry into the vessel. Measurements were made using a Beckman Research Model pH meter equipped with glass and calomel electrodes, which were standardized with dilute HCl to read $-\log$ of concentration rather than activity. Solutions were adjusted to 0.10 M ionic strength by the addition of KNO_3 and were maintained at 25.0 ± 0.05 °C.

Base uptake experiments were conducted using the apparatus described above, except that an atmosphere of oxygen was used rather than nitrogen. In a typical experiment, a solution of glycylhistidine was adjusted to pH 8 using a measured volume of standard KOH. One equivalent of cobalt nitrate was added with continuous bubbling of oxygen, and sufficient base was added to bring the pH back to 8. From the total volume of base used, one can calculate the sum of the number of protons released during both chelation and oxygenation. By use of the known ligand protonation constants and the volume of base necessary to readjust the pH subsequent to addition of metal, the net number of protons released by oxygenation of the chelate was calculated.

Oxygenation equilibria were determined by the use of a Yellow Springs Instruments biological oxygen monitor standardized with air-saturated 0.10 M KNO_3 solution. Because of the fairly small oxygenation constants and the need to work at low pH to prevent precipitation, equilibrium measurements were carried out with oxygen saturated solutions. Hydrogen ion concentrations were determined using a Corning Model 1400 pH meter equipped with a combination semimicro electrode, which was standardized as described above.

Procedure. The ligand protonation constants, expressed as shown in eq 1, were calculated from potentiometric equilibrium measurements on metal-free systems. Values of K_n were calculated using an iterative computer program previously described,[18] and are listed in Table I.

$$H_{n-1}L + H^+ \rightleftharpoons H_nL \qquad K_n = \frac{[H_nL]}{[H_{n-1}L][H^+]} \tag{1}$$

Normal chelate stability constants were expressed as shown in eq 2:

$$M + L \rightleftharpoons ML \qquad K_{ML} = \frac{[ML]}{[M][L]} \tag{2}$$

where ML represents the chelate formed by coordination through the terminal amino and peptide carbonyl oxygen groups.[13,15,19] Values of K_{ML} were determined from potentiometric equilibrium measurements on 1:1 cobalt(II)-ligand systems. However, it was necessary to consider two equilibria in addition to those shown in eq 1 and 2, amide dissociation and free-metal hydrolysis. These were expressed as shown in eq 3 and 4:

$$MOH^+ + H^+ \rightleftharpoons M^{2+} \qquad K_{OH} = \frac{[M^{2+}]}{[MOH^+][H^+]} \tag{3}$$

$$M(H_{-1}L) + H^+ \rightleftharpoons ML \qquad K_A = \frac{[ML]}{[M(H_{-1}L)][H^+]} \tag{4}$$

where $H_{-1}L$ refers to the amide deprotonated form of the ligand. Thus, the conversion of ML to $M(H_{-1}L)$ involves a change in coordination sites from the peptide carbonyl oxygen to the ionized peptide nitrogen. A value of K_{OH} was obtained from the literature.[20] Polymerization equilibria of the cobaltous ion were neglected, as rough calculations indicated that under the conditions used in this study, i.e. dilute solutions, pH ≤8, these species are present only in trace amounts.

It is impossible to determine the value of K_{ML} independently of K_A. Therefore, the calculation of K_{ML} was performed using a modified version of a computer program written by Dr. R. J. Motekaitis which varies the value of K_A such as to minimize the differences between calculated and observed pH values at each point in the titration which is within 2 pH units of the value of log K_A. In the Asp-Gly system it was found that no amide ionization occurs concurrently with normal chelate formation, and precipitation of cobaltous hydroxide prevents the study of this reaction at higher pH. It was also necessary to include

Table I. Ligand Protonation Constants for a Series of Dipeptide Ligands[a-c]

Ligand	Log K_1	Log K_2	Log K_3	Log K_4
His-His	7.52 (2)	6.70 (1)	5.64 (1)	2.58 (6)
Gly-Asp	8.35 (1)	4.29 (1)	2.81 (1)	
Asp-Gly	8.03 (1)	3.67 (1)	2.82 (1)	
Gly-His	8.16 (1)	6.72 (1)		
Glycylhistamine	8.04 (1)	6.78 (1)		
His-Gly	7.60 (1)	5.80 (1)		
Gly-Gly	8.11 (1)	3.16 (1)		
Gly-Ser	8.17 (1)	3.02 (1)		

[a] All values determined at 0.10 M KNO_3 and 25 °C. [b] Constants defined in eq 1. [c] Values in parentheses represent standard deviation in least significant digit.

a chelate protonation constant of $10^{6.20\pm0.04}$ in the His-His calculation in order to satisfactorily reproduce the observed titration curve. Values of K_{ML} and K_A are listed in Table II.

In order to determine oxygenation equilibrium constants, it is necessary to know X, the number of protons released into solution when two molecules of the cobaltous chelate react with molecular oxygen to form the peroxo-bridged dimer. It has been observed that oxygenation of cobaltous chelates of peptides usually involves the deprotonation of the amide nitrogens,[5,7,21] which contributes 2 units to the overall value of X. In addition, most of the 1:1 dipeptide complexes contain aquated coordination sites, and one would expect an olation reaction to occur, forming a μ-hydroxo bridge and giving a value of $X = 3$.[7] This prediction was confirmed by base uptake experiments on the His-His and Gly-His systems. Histidylhistidine has a total of five potential coordinating groups, so the presence of a hydroxo bridge in the dioxygen complex indicates that one of these groups, presumably the carboxylate, is not coordinated to the metal.

Oxygenation constants were determined by oxygen uptake. The percent oxygen saturation of a ligand solution was measured both prior and subsequent to the addition of 1 equiv of cobalt(II). The change in the oxygen concentration directly gives the concentration of the μ-peroxo-μ-hydroxo complex, since it is well established that there is 1 mol of oxygen per mol of complex. Since a 1:1 ratio of ligand to metal was maintained throughout this study, one may assume that 1 mol of the dioxygen complex contains 2 mol of both ligand and cobalt. Therefore, one may define the variables L_0 and M_0 as shown in eq 5 and 6:

$$L_0 = T_L - 2[Co_2(H_{-1}L)_2(O_2)(OH)] \quad (5)$$

$$M_0 = T_M - 2[Co_2(H_{-1}L)_2(O_2)(OH)] \quad (6)$$

where T_L and T_M refer to the analytical concentrations of ligand and metal. One may now set up the following series of equations:

$$L_0 = A_1[L] + X_1[ML] \quad (7)$$

$$M_0 = Y_1[M] + X_1[ML] \quad (8)$$

$$[ML] = K_{ML}[M][L] \quad (9)$$

where A_1, Y_1, and X_1 are the usual functions of protonation, hydrolysis, and amide dissociation constants. The value of K_{ML} was obtained as described above. It is now possible to solve algebraically for [M], [L], and [ML], and to evaluate the oxygenation constant K_{O_2}, expressed as:

$$K_{O_2} = \frac{[M_2(H_{-1}L)_2(O_2)(OH)][H^+]^3}{[ML]^2[O_2]} \quad (10)$$

The quantity $[O_2]$ is known from the final reading of percent oxygen of the equilibrium solution of ligand and metal. Values of log K_{O_2} are listed in Table III.

Discussion

The ligand protonation constants follow the expected patterns. Substitution at the α carbon of the carboxy amino acid residue is too remote from the free amino group to appreciably affect its log K_n, so that for the series of glycyl-R-peptides, log $K_1 \simeq 8.2$. The only significant variation in log K_1 occurs when the amino residue is histidine, for which log K_1 drops to ~7.5, due to the inductive effects of the imidazole group. Also, the value of log K_n of the imidazole group itself is markedly affected by proximity to the protonated amino group. The log K_n for an imidazole group of an N-terminal histidine residue is about 5.7, whereas the value of a C-terminal histidine is 6.7. From these results the protonation scheme of His-His becomes apparent, since the two imidazole log K_n values of this ligand closely match the values observed for the isolated imidazoles of Gly-His and His-Gly. Thus, the order of protonation of His-His is as follows: N-terminal amino group, C-terminal imidazole, N-terminal imidazole, carboxylate.

Table II.[a,b] Logarithms of the Normal Stability and Amide Protonation Constants for the 1:1 Complexes of Cobalt(II) with a Series of Dipeptides

Ligand	Log K_{ML}[c]	Log K_A[d]
His-His	5.49 (3)	7.8 (2)
His-Gly	5.19 (2)	7.15 (9)
Asp-Gly	4.10 (3)	
Gly-Asp	3.57 (1)	9.26 (5)
Gly-His	3.32 (2)	7.24 (3)
Gly-Ser	3.08 (2)	8.77 (5)
Gly-Gly	3.07 (2)	9.35 (8)
Glycylhistamine	2.94 (1)	7.93 (3)

[a] All values determined at 0.10 M KNO_3 and 25 °C. [b] Numbers in parentheses indicate standard deviation of least significant digit. [c] Constant defined in eq 2. [d] Constant defined in eq 4.

Table III.[a,b] Logarithms of the Normal Oxygenation Equilibrium Constants (K_{O_2}) for the Series of 1:1 Cobaltous Dipeptide Complexes

Ligand	Log K_{O_2}[c]
His-His	−8.2 (2)
Gly-His	−13.5 (2)
His-Gly	−16.6 (2)
Glycylhistamine	−15.5 (1)
Gly-Asp	−20.1 (1)
Asp-Gly	−20.7 (2)

[a] All values determined at 0.10 M KNO_3 and 25 °C. [b] Numbers in parentheses indicate standard deviation of least significant digit. [c] Constant defined in eq 10.

Values of log K_{ML} for the series of cobalt(II)-dipeptide complexes investigated are listed in Table II. Stability constants for the Gly-Gly and Gly-Ser complexes are included to indicate the strength of binding in the absence of coordinating side groups. Complexes of these two ligands have nearly identical values of log K_{ML}, as expected due to the similarity in structure. The values for the remaining glycyl-R-peptides bracket the constants for the Gly-Gly and Gly-Ser complexes, indicating that in all cases coordination occurs through the free amino and peptide oxygen groups, as shown by I, with little influence from coordination of the free carboxylate or side groups. This result is readily explained, since molecular models show that coordination of a C-terminal side group or the free carboxylate is virtually impossible if the metal remains coordinated to the peptide carbonyl oxygen. It is only after amide deprotonation, with the shifting of one coordination site from the amide carbonyl to the amide nitrogen, that coordination of the C-terminal side chain and free carboxylate becomes possible, as illustrated by II. Even in this configuration there is some steric hindrance to the simultaneous coordination of both the free carboxylate and the side-chain ligating group.

A completely different situation arises when the side-chain coordinating group is substituted at the α carbon of the N-terminal amino acid. In this case a sterically unhindered, six-membered chelate ring may form without amide ionization.

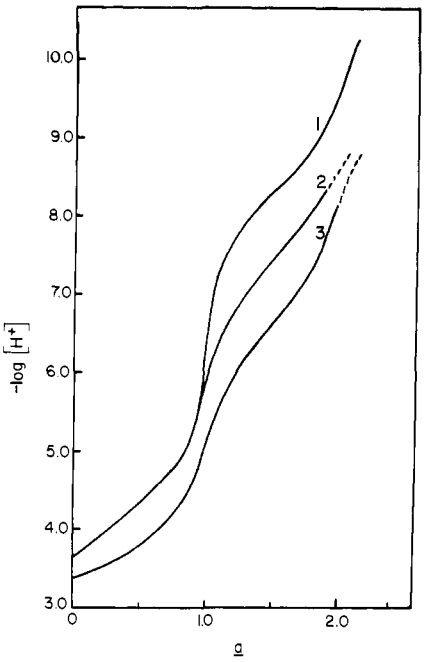
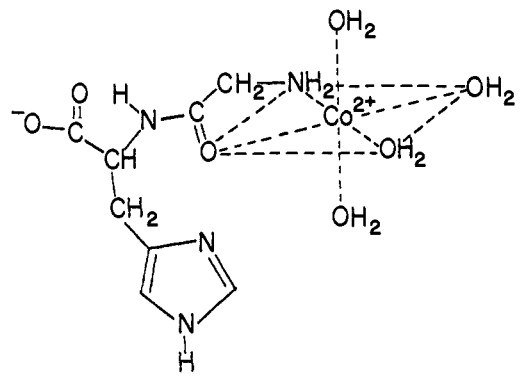


Figure 1. Potentiometric equilibrium curves for glycyl-L-aspartic acid in the absence of metal ion (curve 1), and for 1:1 solutions of cobalt(II) to glycyl-L-aspartic acid (curve 2), and L-aspartylglycine (curve 3); μ = 0.10 M KNO_3; T = 25 °C.



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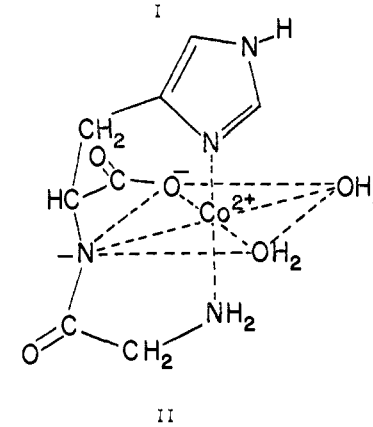

II

Thus one observes a definite increase in log K_{ML} when aspartic acid or histidine is employed as the N-terminal residue. This effect is evident from the potentiometric equilibrium curves shown in Figure 1, for which the Asp-Gly curve is substantially lower in pH than the Gly-Asp curve, even though the log K_1 values are similar.

Values of the amide protonation constants of the cobalt(II)-dipeptide complexes are listed in Table II. There do not appear to be any simple trends which will explain all the data. Motekaitis and Martell[16] have proposed that the ability of a metal ion to promote amide dissociation should decrease as it becomes more effectively ligated by other coordinating groups. Their conclusions were based largely on results obtained from a series of *N,N*-diacetic acid derivatives of polyglycines. A portion of their results is reproduced in Table IV. It clearly shows that in every case addition of the acetate groups lowers the tendency toward amide dissociation. However, a significant difference between these polyglycine derivatives and the ligands reported here is that in the present case the substitutions on the glycylglycine framework are in the more immediate vicinity of the amide group, and the same inductive effects which alter the log K_1 values of the free amino group would also be expected to exert some influence on the acidity of the amide nitrogen.[15] Therefore, the value of log K_A is a function of at least two variables: (1) the strength of the liganding groups about the metal, with strongly coordinating groups tending to decrease the ability of the metal ion to effect amide dissociation, and (2) the inductive effects of substituents near the amide linkage itself. The very low values of log K_A observed in the Gly-His and His-Gly systems indicate that this latter effect can be very important. These two complexes have the lowest log K_A values yet reported for cobalt-promoted amide dissociation.

Table IV. Values of Log K_A for Cupric Complexes of Polyglycines and Their *N,N*-Diacetic Acid Derivatives

Ligand	Log K_A
Gly-Gly	4.58
2GDA[a]	6.61
Triglycine	5.20
3GDA[a]	6.91
Tetraglycine	5.40
4GDA[a]	7.05

[a] Abbreviations used are: 2GDA, diglycine-*N,N*-diacetic acid; 3GDA, triglycine-*N,N*-diacetic acid; 4GDA, tetraglycine-*N,N*-diacetic acid.

The inability to determine a K_A for the Asp-Gly complex is due to the lack of sufficient overlap between the complexation and amide dissociation equilibria. While one would expect the K_A of the Asp-Gly complex to be similar to that of the Gly-Asp complex, normal chelate formation occurs at 1 pH unit lower in the Asp-Gly system. Thus, chelate formation is essentially complete prior to any appreciable degree of amide deprotonation. Precipitation of cobaltous hydroxide prevents the investigation of the amide dissociation at higher pH.

Although amide dissociation constants have been previously reported for some 2:1 cobaltous complexes of dipeptides,[5,7,10] this is the first time that log K_A values have been determined for 1:1 complexes of simple dipeptides. Of the ligands included in this study, equilibrium data have been previously reported only for the Gly-Gly complex.[4,19,22,23] In all cases the effects of amide dissociation were simply ignored. However, even in the Gly-Gly calculations the neglect of this equilibrium has a noticeable effect on the standard deviation between the calculated pH values and the observed pH values in the buffer region where the degree of chelate formation is appreciable. In the Gly-Gly case, the standard deviation drops from σ = 0.050 to 0.015 when amide dissociation is taken into consideration. Even though this is approaching the limit at which this method of calculation is effective, the results are very reproducible and the improvement in σ is considered significant. Of course, in systems where log K_A is lower and complexation and deprotonation equilibria overlap to a greater degree, the improvement in the pH fit is much greater. In the Gly-His calculations σ drops from 0.159, when it is assumed K_A = 0 to 0.005 when log K_A = 7.24. This method of calculation is much more sensitive than older procedures which involve calculation of values of K_A on the basis of changes in calculated quantities,

Table V. Value of Log K_{O_2}' for a Series of 1:1 Cobaltous Dipeptide Complexes

Ligand	Log K_{O_2}'[a]
His-His	7.3
Gly-His	1.0
Glycylhistamine	0.4
Gly-Asp	−1.6
His-Gly	−2.3

[a] Equilibrium constant defined in eq 11.

such as log K_{ML}, rather than changes in a measured quantity such as pH.

The His-His system is the only case where metal chelate protonation was found to be important. Protonation equilibria are absent from the Gly-His and glycylhistamine systems even though these ligands also contain essentially noncoordinating imidazole groups. However, the formation region for Gly-His and glycylhistamine is at a much higher pH than that of the His-His complex. A direct comparison of Gly-His and His-His is shown in Figure 2. Therefore, one would expect protonation equilibria to be much less important in the Gly-His and glycylhistamine systems. In addition, the presence of a strongly coordinating imidazole group on the N-terminal residue of His-His lessens the inductive influence of the cobalt ion and makes the C-terminal imidazole group more susceptible to protonation.

Values of the oxygenation constants are listed in Table III. The values of log K_{O_2} are difficult to compare directly with other systems because the amide dissociation reaction introduces the unique $[H^+]^3$ term into the numerator of the equilibrium quotient. Oxygen complexation by the aspartyl complexes is very weak, as indicated by values of log K_{O_2} of 10^{-21}. Such systems represent the lower limit of oxygenation equilibrium which can be accurately studied by these methods. These complexes are important, however, because they constitute only the third reported example of oxygenation of a complex which contains only two coordinated nitrogen atoms. Previously reported violations of Fallab's "three nitrogen rule"[24] are the isomeric ethylenediaminediacetic acids[25] and the 2:1 amino acid complexes.[5]

The effect that imidazole groups have on the oxygenation equilibria can be seen by comparing values of Gly-Asp (log K_{O_2} = −20.1), Gly-His (log K_{O_2} = −13.5), and His-His (log K_{O_2} = −8.2). The addition of successive imidazole groups to the basic Gly-Gly framework increases the value of the oxygenation constant by several orders of magnitude. The obvious explanation for this is that coordination of each imidazole group involves strong donation of electron density from the ligand to the metal and facilitates the partial transfer of an electron from the metal to oxygen, forming a complex which consists of formally cobalt(III) and peroxide. However, when peptide ligands are involved there is a second factor which must be considered, which is the influence of the substituents on amide dissociation. The loss of the amide proton appears to be a necessary condition for oxygenation of peptide complexes,[5,7,21] so that an increase in the acidity of the amide proton can have a substantial effect on the oxygenation equilibrium. This effect can be demonstrated by a simple treatment of the equilibrium results from Tables II and III. One can combine values of K_{O_2} and K_A as shown below to obtain a new constant K_{O_2}':

$$K_{O_2}' = \frac{[Co(H_{-1}L)(O_2)(OH)Co(H_{-1}L)][H^+]}{[Co(H_{-1}L)]^2[O_2]} = K_{O_2}K_A^2 \quad (11)$$

which is a measure of the oxygen affinity of the amide-deprotonated complex. From the values of K_{O_2}' shown in Table V, it can be seen that the His-Gly complex actually has a rather low affinity for oxygen. Its efficiency at coordinating oxygen at relatively low pH is due to its very low value of log K_A, which results in an unusually high concentration of the oxygen active species.

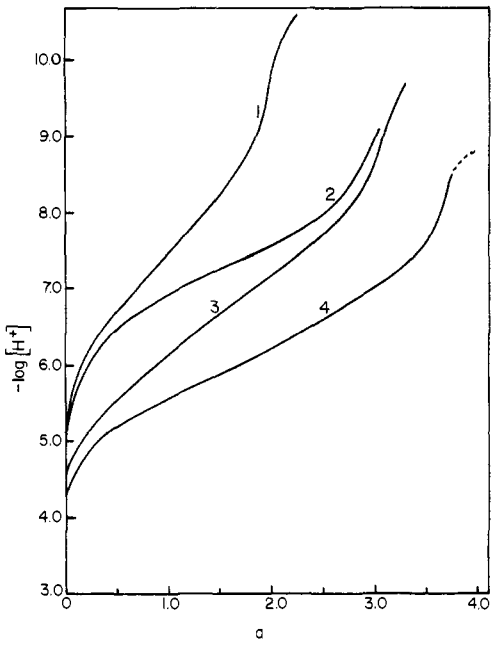

Figure 2. Potentiometric equilibrium curves for the following systems: glycyl-L-histidine, ligand only (1); 1:1 ratio of glycyl-L-histidine to $Co(NO_3)_2$ (2); L-histidyl-L-histidine, ligand only (3); 1:1 ratio of L-histidyl-L-histidine to $Co(NO_3)_2$ (4); μ = 0.10 M KNO_3; T = 25 °C.

The difference in the values of log K_{O_2}' for the Gly-His and His-Gly complexes is quite large considering that they each contain identical coordinating groups. The difference in ligand geometry is such that the three nitrogen donors of His-Gly are restricted to facial coordination positions, while those of Gly-His may assume meridional positions. This difference in geometry is apparently quite important. Significant differences have been observed between the K_{O_2}' values for the trien-tren systems[26] and the S-EDDA and U-EDDA complexes.[25] In each instance the branched isomer has the lower equilibrium constant. In this particular case, Gly-His is not actually linear, due to the carboxylate group. However, a comparison of the K_{O_2}' values of Gly-His and glycylhistamine indicates that coordination of this carboxylate group has a minor effect on the oxygenation reaction. Studies on the trien-tren[26] and S-DTMA-U-DTMA[27] systems have shown that the rate of formation of the oxygen complexes is slower for the branched isomers. The slower rate of formation of the dioxygen complexes of the branched isomers is believed due to an equilibrium between the octahedral cobalt(II) complexes and a pentacoordinate, trigonal-bipyramidal complex which is essentially inert toward reaction with molecular oxygen.[28] Such an equilibrium could also account for the observed trend in the oxygenation equilibrium data as well.

The values of K_{O_2}' listed in Table V are typical for hydroxo-bridged oxygen complexes. Previously reported values range from $10^{-5.3}$ for the ethylenediamine-*N,N*-diacetic acid complex to $10^{10.8}$ for the 2:1 ethylenediamine complex. It does not appear that the deprotonated amide group makes an unusually large contribution to log K_{O_2}'.

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References and Notes

(1) (a) Abstracted in part from a dissertation to be submitted by Wesley R. Harris to the faculty of Texas A&M University in partial fulfillment of the requirements for the degree of Doctor of Philosophy. (b) For recent reviews, see G. McLendon and A. E. Martell, *Coord. Chem. Rev.*, **19**, 1 (1976), and F. Basolo, B. Hoffman, and J. A. Ibers, *Acc. Chem. Res.*, **8**, 384 (1975).
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Communications to the Editor

$^{13}C-^{1}H$ Coupling Constants in Cyclohexane

Sir:

Recently much interest has been shown in the study of $^{13}C-^{1}H$ coupling constants.[1] The data on $^{13}C-^{1}H$ couplings in flexible saturated systems are, however, rather limited, owing to extreme complexity of their proton-coupled ^{13}C NMR spectra.

We wish to report here the experimental values of *all* possible $^{13}C-^{1}H$ coupling constants in cyclohexane which is a classical example of a conformationally flexible system. The data discussed below were obtained from the low- and high-temperature proton-coupled ^{13}C spectra of cyclohexane-d_{11}.

Cyclohexane-d_{11} was synthesized by chlorination[2] of cyclohexane-d_{12} (Isocommerz, Leipzig, isotope purity 99%) followed by Grignard replacement of the chloro atom with hydrogen.[3]

Fast inversion of the cyclohexane ring at ambient temperatures leads to the complete averaging of the NMR parameters characterizing axial and equatorial proton positions.[4] Thus, the high-temperature (+34 °C) $^{13}C-\{^{2}D\}$ spectrum (Figure 1a) should be regarded as a superimposition of four AX spectra (A = ^{1}H, X = ^{13}C) from the four possible $^{13}C^{12}C_5D_{11}H$ isotopomers, which differ in the relative positions of ^{13}C and ^{1}H nuclei and types of coupling constants (i.e., $^{1}J_{av}$, $^{2}J_{av}$, $^{3}J_{av}$, and $^{4}J_{av}$). Analysis of both the $^{13}C-\{^{2}D\}$ NMR spectrum (25.16 MHz) and the ^{13}C satellites in the ^{1}H NMR spectrum (100.1 MHz) makes it possible to assign the signals to the individual isotopomers. This allows immediate identification of four coupling constants two of which (with wt 1) are equal to 124.56 and 0.44 Hz and the other two (with wt 2) are equal to 3.81 and 5.06 Hz. The largest value of 124.56 Hz should be assigned to $^{1}J_{av}$.[5] This gives $^{4}J_{av}$ = 0.44 Hz. In order to assign $^{2}J_{av}$ and $^{3}J_{av}$, we have used the isotope effects on ^{13}C chemical shifts caused by the replacement of ^{2}D by ^{1}H. These isotope shifts, $\Delta\nu(^{13}C)$, observed as displacements of the doublet centrums from the ^{13}C signal of cyclohexane-d_{12}, are equal to 2.70 and 0.64 Hz for the doublets spaced 3.81 and 5.06 Hz, respectively, which favor the following assignment: $^{2}J_{av}$ = 3.81 Hz, and $^{3}J_{av}$ = 5.06 Hz.[6]

At low temperatures (−104 °C in our experiments) the ring inversion slows down and the sharp lines from the individual conformers appear in the spectrum (Figure 1b).[4] A total of eight nonequivalent cyclohexane-d_{11} conformers can be resolved into two groups[7] depending on whether the proton is in an axial or in an equatorial position. The values of two ^{1}J couplings (i.e., $^{1}J_e$ and $^{1}J_a$) are equal to 126.44 and 122.44 Hz

Table I. Coupling Constants J_{CH},[a] ^{13}C Isotope Chemical Shifts Caused by the Replacement of ^{2}H with ^{1}H, $\Delta\nu(^{13}C)$,[a-c] Proton Isotope Chemical Shifts Caused by the Replacement of ^{12}C with ^{13}C, $\Delta\nu(^{1}H)^{a,c,d}$ in Cyclohexane-d_{11} (10% v/v Solution in CS_2 Containing ~10% v/v TMS)

Parameter			Proton	
		H_{av}[e]	H_e[f]	H_a[f]
$^{1}J_{CH}$	Exptl	124.56	126.44	122.44
	Calcd		118.7[g]	123.6[g]
			122.9[h]	119.4[h]
$^{2}J_{CH}$	Exptl	−3.81	−3.69	−3.94
	Calcd		−6.35[g]	−6.90[g]
			−6.67[h]	−6.79[h]
$^{3}J_{CH}$	Exptl	5.06	8.12	2.12
	Calcd		8.34[g]	1.44[g]
			8.78[h]	1.36[h]
$^{4}J_{CH}$	Exptl	(−)0.44[i]	(−)0.50[i]	(−)0.31[i]
	Calcd		−0.83[g]	−0.47[g]
			−0.88[h]	−0.46[h]
$^{n}\Delta\nu(^{13}C)$	$^{1}\Delta\nu$	10.58	10.03	11.22
	$^{2}\Delta\nu$	2.70	2.91	2.59
	$^{3}\Delta\nu$	0.64	1.00	0.31
	$^{4}\Delta\nu$	0.01	0.06	−0.03
$^{n}\Delta\nu(^{1}H)$	$^{1}\Delta\nu$	−0.18	−0.26	−0.11
	$^{2}\Delta\nu$	−0.05	−0.10	−0.09
	$^{3}\Delta\nu$	−0.02	−0.04	

[a] In hertz. The accuracy is within 0.05 Hz. [b] At 25.16 MHz for ^{13}C nuclei. [c] Positive values correspond to the downfield shifts. [d] At 100.1 MHz for ^{1}H nuclei. [e] +34 °C. [f] −104 °C. [g] The FP INDO calculations with the geometry taken from ref 14. [h] The FP INDO calculations with $r(C-H_a)$ = 1.101 and $r(C-H_e)$ = 1.141 Å. Other geometric parameters as in ref 14. [i] For the assignment and the signs of $^{4}J_{CH}$s, see text.