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Coördination Complexes and Catalytic Properties of Proteins and Related Substances. II. The Reactivity of Carbobenzoxy-L-prolyl-L-histidylglycinamide[1]

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The reactivity of carbobenzoxy-L-prolyl-L-histidylglycinamide, Cbz-PHG-NH_2, was measured kinetically by its ability to catalyze the hydrolysis of *p*-nitrophenyl acetate, NPA, and to interact with Zn(II) and Cu(II) ions as determined from simultaneous equilibrium *p*H values. The two methods of measuring the concentration of basic peptide were shown to be mutually compatible. The specific second-order rate constant k_2 for the hydrolysis of NPA by Cbz-PHG-NH_2, is 6.40 l. mole^{-1} min.$^{-1}$ at 25.3°. This value is in accord with that expected from the Brönsted catalysis law for imidazole derivatives where only the neutral molecules exhibit catalytic activity. It is concluded that the presence of the peptide bond confers no special reactivity on the side chain imidazole group with respect to its ability to split NPA. At 25.1° and ionic strength 0.16 the *pK'* is 6.42. The *p*H titrations in the presence of Zn(II) and Cu(II) showed that the number of molecules of Cbz-PHG-NH_2 bound to either of these ions, $\bar{\nu}$, approached an upper limit of 4, the value for imidazole itself. At 25.1° the logarithm of the first association constant, log k_1 was found to be 2.16 for Zn(II) and 3.28 for Cu(II). The subsequent steps for the combination of Cbz-PHG-NH_2 with Zn(II) follow the same general course as those of imidazole with Zn(II). With Cu(II), however, the peptide shows positive interactions whereas imidazole shows negative interactions. The absorption characteristics in the visible between 600–800 mμ of Cu(II)–Cbz-PHG-NH_2 and Cu(II)–imidazole complexes are presented. In both cases as $\bar{\nu}$ increases the extinction coefficient also increases and the absorption maximum is shifted to shorter wave lengths. The spectra of the individual Cu(II) complexes were computed. With both types of ligand the absorption maximum decreases approximately 50 mμ for each ligand molecule added to the complex. Cu(II)–Cbz-PHG-NH_2 complexes, however, absorb 30–40% more strongly than do Cu(II)–imidazole complexes.

Assessing the reactivity of the polar groups in proteins is complicated by their overlappin intrinsic reactivities. By simultaneously combining kinetic and equilibrium methods the reactivity of these groups may be defined more thoroughly than by relying upon one method alone. A previous report[4] described this combined approach on a model system containing imidazole, *p*-nitrophenyl acetate (NPA) and Zn(II) or Cu(II) ions. The reactivity of imidazole was measured kinetically by its ability to catalyze the hydrolysis of NPA and to interact with Zn(II) and Cu(II) as determined from simultaneous equilibrium *p*H values. The two methods of measuring the concentration of basic imidazole were shown to be mutually compatible.

A similar study is reported here on the synthetic tripeptide derivative, carbobenzoxy-L-prolyl-L-histidylglycinamide (Cbz-PHG-NH_2), as a further step toward studying the reactivity of the histidyl residue in proteins. This compound has been used for the synthesis of an analog of the natural vasopressins.[5] It was chosen for this study because the imidazole group is the only unprotected functional group.

Materials and Methods

Materials.—The preparation and physical properties of Cbz-PHG-NH_2 have been reported previously.[5] The nitrate salt, $H^+PNO_3^-$, was prepared by dissolving the peptide in an equivalent amount of standardized nitric acid and lyophilizing the solution. It was stored over P_2O_5. Prior to preparing solutions the last traces of H_2O, approximately 2%, were removed by drying at 56° *in vacuo* over P_2O_5 for 6 hr.

The *pK'* was determined at 25.1° by titrating a solution of 0.0496 *M* $H^+PNO_3^-$ in 0.11 *M* $NaNO_3$ with 0.4670 *N* NaOH. After each addition of base 2 minutes were allowed for equilibration. The *pK'* was computed to be 6.42 ± 0.01 in the *p*H range 5.00–7.30. That the salt was pure may be judged from the constancy of the *pK'* values for widely varying ratios of free base to conjugate acid, 0.058 to 7.59. Furthermore, 0.050 *M* solutions of $H^+PNO_3^-$ gave *p*H values of 3.86–3.88 whereas the theoretical *p*H of such a solution is 3.86. In certain experiments the chloride salt, H^+PCl^-, was used. Usually 0.100 *M* solutions, containing 0.155 g. of Cbz-PHG-NH_2 in 3.50 ml., were prepared by suspending the peptide derivative in water and adjusting the *p*H to 3.71 by the addition of standardized HCl solution. A solution of this material was subjected to paper electrophoresis (*vide infra*) and was found to give no ninhydrin test but instead a strong pink-orange band 6 cm. from the point of application when sprayed for the Pauly test.

Solutions of the chloride and nitrate salts of Cu(II) and Zn(II) were standardized as previously described.[4]

The *p*-nitrophenyl acetate (NPA) was the same as in a previous study.[4]

Measurement of *p*H.—All determinations were made with a Radiometer Type TTT 1a *p*H meter as described previously.[4] Some of the earlier measurements were carried out on solutions at room temperature (26–29°); however, most solutions were thermostated in a water-jacketed cell at 25.1 ± 0.1°. Unless stated otherwise all measurements reported were made at 25.1 ± 0.1°.

Kinetic Method.—The catalysis of the hydrolysis of NPA by Cbz-PHG-NH_2 was determined in the presence and absence of phosphate buffer. In one series of experiments 1 part of 1.0×10^{-3} *M* NPA in 1.90% ethanol–H_2O (v./v.) was added to 9 parts of a solution of phosphate buffer of varying *p*H and ionic strength containing varying concentrations of the peptide nitrate. The kinetic measurements were performed at 26.5 ± 1.0° and the *p*H measured at 26–29° at the conclusion of the kinetic run. In another series of experiments solutions were prepared containing peptide, peptide chloride, metal salt (as required) and sufficient NaCl to maintain a constant ionic strength of 0.16 during the reaction. After the *p*H was measured at 25.1°, 0.10 ml. of 1.0×10^{-3} *M* NPA in 1.90% ethanol–H_2O (v./v.) was added to 0.90 ml. of the solution. The smaller volumes were accommodated in the Beckman Model DU Spectrophotometer in fused silica cells of internal dimensions 3 × 10 × 25 mm. supplied by Pyrocell Manufacturing Co., New York, N. Y. These kinetic runs were carried out at 25.3 ± 0.3°. The *p*H was again measured at 25.1° at the end of the run. Details of the technique and computations have been described.[4]

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(2) Holder of a Summer Research Fellowship under a grant from the U. S. Public Health Service to Cornell University Medical College, 1957.

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(4) W. L. Koltun, R. N. Dexter, R. E. Clark and F. R. N. Gurd, THIS JOURNAL, **80**, 4188 (1958).

(5) P. G. Katsoyannis and V. du Vigneaud, *Arch. Biochem. Biophys.*, in press.

Paper Electrophoresis.—The Spinco Model R Paper Electrophoresis apparatus was used. The electrophoresis was performed using a buffer containing 0.02 *M* sodium acetate and 0.02 *M* acetic acid. Samples of 10 microliters of solutions containing about 0.2 micromole of peptide derivative were run on strips of Schleicher and Schull No. 2043 A paper. A current of 2.5 milliamperes was maintained for 6 hr. The ninhydrin reagent was composed of 180 ml. of *n*-butanol, 20 ml. of H_2O and 0.5 ml. of glacial acetic acid. The Pauly test (which may be made on strips previously sprayed with ninhydrin) was carried out by spraying with 0.02 *M* diazobenzene-*p*-sulfonic acid in 5 *N* HCl; after the paper had dried it was sprayed with 5% Na_2CO_3.

Results

Catalysis by Cbz-PHG-NH$_2$.—The observed first-order rate constants were taken to be equal to $k_1 + k_w$, where k_1 is the first-order rate constant for catalysis by Cbz-PHG-NH_2 and k_w is the rate constant for hydrolysis in a solution at the same *p*H, temperature and ionic strength in the absence of catalyst.[4] The apparent second-order rate constants, k_2', were obtained from the expression $k_2' = k_1/(P_0)$, where (P_0) is the total molar concentration of peptide derivative. The specific second-order rate constants were calculated from the expression $k_2 = k_1/(P)$ where (P) is the molar concentration of the basic form of the peptide derivative.

The results of kinetic measurements at 26.5 ± 0.5° in the presence of phosphate buffers and 0.2% ethanol are shown in Table I. The *p*H dependence of k_2', column 4, for any fixed value of (P_0) suggests that the only catalytic species is the basic form of the imidazole group. This assumption was made in the calculation of k_2, shown in column 5. The value of pK' of 6.42 was used to compute (P). The constancy of the values of k_2 in Table I supports the interpretation that the basic form of imidazole is the catalytic species and also shows that the concentrations of phosphate that were present had little or no effect. The grand average value of k_2 at 26.5° is 6.731. mole^{-1} min^{-1}.[6]

For purposes of standardization of the method as a measure of (P) in the systems containing Zn(II) and Cu(II), a similar group of measurements of k_2 was made at 25.3° at ionic strength 0.16 (adjusted by adding NaCl). The concentration of ethanol was 0.2%. No buffers were added. The average of 5 determinations over a range of (P) between 0.87×10^{-3} *M* and 3.39×10^{-3} *M* gave k_2 = 6.40 l. mole^{-1} min.$^{-1}$. The slightly lower value found at 25.3° compared with 26.5° is in accord with previous experience with imidazole.[4,8]

Measurement of Free Basic Cbz-PHG-NH$_2$ in the Presence of Zn(II) and Cu(II): (A) Kinetic Measurements.—The finding of a constant value of k_2 at constant temperature and ionic strength over a wide range of concentration of H^+P and P indicates that H^+P does not act as a catalyst and that kinetic measurements may be used to determine the concentration of P. Table IIA shows the results of kinetic measurements performed in the presence of Zn(II) and Cu(II) ions at 25.3°. The concentration of free basic peptide, expressed as the negative logarithm, was calculated using a value of k_2 of 6.40 and assuming that complexes with these cations do not act catalytically.[4]

(6) The magnitude of the temperature dependence of pK' observed with imidazole derivatives[7] indicates that only small errors are introduced by using a pK' value determined at 25.1°.

(7) J. T. Edsall, G. Felsenfeld, D. S. Goodman and F. R. N. Gurd, THIS JOURNAL, **76**, 3054 (1954).

(8) T. C. Bruice and G. L. Schmir, *ibid.*, **79**, 1663 (1957).

TABLE I

THE *p*H AND CONCENTRATION DEPENDENCE OF THE RATE OF CARBOBENZOXY-PROLYL-HISTIDYL-GLYCINAMIDE CATALYZED HYDROLYSIS OF *p*-NITROPHENYL ACETATE AT 26.5°

*p*H	$k_1 \times 10^3$ (min.$^{-1}$)	$k_w \times 10^3$ (min.$^{-1}$)[a]	k_2' (l. mole^{-1} min.$^{-1}$)	k_2 (l. mole^{-1} min.$^{-1}$)
A.	μ phosphate = 0.06, $(P_0) = 5.0 \times 10^{-3}$ *M*			
7.45	31.77	1.26	6.36	6.95
7.27	30.65	1.15	6.13	7.00
7.01	27.82	0.83	5.56	6.98
6.69	22.30	.63	4.46	6.86
6.41	15.48	.43	3.10	6.27
6.03	9.58	.12	1.92	6.60
B.	μ phosphate = 0.09, $(P_0) = 3.0 \times 10^{-3}$ *M*			
7.55	19.29	1.41	6.43	6.91
7.34	18.46	1.24	6.15	6.89
7.06	16.67	1.03	5.58	6.83
6.73	13.56	0.84	4.52	6.75
6.43	9.45	.50	3.15	6.25
6.05	5.97	.15	1.99	6.65
C.	μ phosphate = 0.11, $(P_0) = 1.5 \times 10^{-3}$ *M*			
7.63	9.80	1.57	6.54	6.94
7.40	9.55	1.44	6.36	7.01
7.11	8.53	1.21	5.69	6.84
6.75	6.44	0.90	4.29	6.30
6.46	4.93	.57	3.29	6.30
6.07	3.19	.19	2.13	6.89

[a] For these measurements made in the presence of buffers, the values of k_w were obtained by interpolating between values of the observed rates in a series of buffers of known *p*H and ionic strength, instead of by extrapolating to zero concentration of buffer.

(B) Equilibrium Measurements.—After the completion of the kinetic measurements the equilibrium *p*H values were determined at 25.1°. The concentration of the free basic peptide in each solution was calculated from the measured *p*H,[7] using the appropriate value for the concentration of H^+PCl^- and the pK' of 6.42. The results are shown in Table IIB.

A comparison of the two independent methods used to obtain the values of (P) may be made by comparing columns 5 and 8. The close agreement indicates (1) the validity of the assumption that the metal–peptide complexes are not catalytic agents to any measurable extent, and (2) the lack of interference by the hydrolytic products of NPA at the low concentrations present.

Association of Zn(II) and Cu(II) with Cbz-PHG-NH$_2$: (A) Zn(II).—From the data presented thus far, the first association constant of Zn(II) and Cbz-PHG-NH_2 may be determined. In Table II are listed the values of $\bar{\nu}$, the average number of peptide molecules bound per metal ion present, which were computed by the method previously employed with imidazole.[4,7,9] The data in Table II were analyzed by the method of Scatchard[7] from plots of log Q *versus* $\bar{\nu}$ where

$$Q = \bar{\nu}/(N - \bar{\nu})(P)$$

(9) Y. Nozaki, F. R. N. Gurd, R. F. Chen and J. T. Edsall, *ibid.*, **79**, 2123 (1957).

TABLE II

CATALYSIS OF HYDROLYSIS OF NPA BY CARBOBENZOXY-PROLYL-HISTIDYL-GLYCINAMIDE, (P), IN THE PRESENCE OF Zn(II) AND Cu(II) AT 25.3°

Composition: Initial total molar concn. of			A. Kinetic results: k_2 = 6.40 l. mole^{-1} min.$^{-1}$			B. Equilibrium results: pK' = 6.42		
$ZnCl_2$	H^+PCl^-	P	$k_1 \times 10^2$, min.$^{-1}$	−log (P)	$\bar{\nu}$	pH	−log (P)	$\bar{\nu}$
0.0098	0.04566	0.00434	1.216	2.72	0.249	4.98	2.80	0.282
.0098	.04180	.00820	1.716	2.57	.563	5.26	2.54	.542
.0098[a]	.03745	.01255	2.536	2.43	.899	5.44	2.41	.880
.00196	.04807	.00193	1.091	2.77	.114	4.90	2.84	.245
.00196	.04517	.00483	1.976	2.50	.868	5.28	2.48	.791
.00196	.04228	.00772	2.886	2.34	1.61	5.48	2.31	1.46
$CuCl_2$								
0.00198	0.04848	0.00145	0.215	3.47	0.562	4.26	3.49	0.569
.00198	.04708	.00290	.456	3.15	1.11	4.61	3.14	1.10
.00198	.04516	.00483	.962	2.82	1.68	4.91	2.85	1.73
.00198	.04324	.00675	1.529	2.62	2.20	5.17	2.61	2.18
.00198	.04035	.00965	2.885	2.35	2.60	5.46	2.35	2.65

[a] T = 26.6°, k_2 = 6.73 l. mole^{-1} min.$^{-1}$.

and N denotes the maximum number of sites available for binding the ligand. In the present system it was assumed that N = 4.[7,9] The results for Zn(II) are plotted in Fig. 1, closed circles. Since the kinetic and equilibrium measurements were in close agreement, only the values obtained from equilibrium measurements are shown. As previously demonstrated,[7,9] the value of log κ_1, the first intrinsic association constant, is equal to the limiting value of log Q when $\bar{\nu}$ approaches zero. Extrapolation yields a value of log κ_1 = 1.56. Using the statistical relation $\mathbf{k}_1 = 4\ \kappa_1$, where $\mathbf{k}_1$ is the first association constant, a value of log $\mathbf{k}_1$ = 2.16 is obtained.

The first association constant in such a system is of greatest interest as a guide to the possible behavior of imidazole groups in proteins.[10] The general course of the subsequent steps of the reaction was determined by the technique of stepwise titration to conserve the supply of the peptide derivative. For example, a solution containing 0.050 M $H^+P\ NO_3^-$, 0.002 M $Zn(NO_3)_2$ and 0.10 M $NaNO_3$ was titrated with standard NaOH solution containing sufficient $NaNO_3$ to maintain the ionic strength at 0.16. The NaOH was added from a microburet dipped below the surface in a titration vessel fitted with a magnetic stirrer. The same technique yielded results with imidazole itself that agreed well with previous measurements on separately prepared solutions.[4] The results with the peptide derivative are summarized in Table III and Fig. 1, open circles. Usually pH measurements were made 3 minutes after the addition of NaOH to allow the system to come to equilibrium. Above pH 5.80, corresponding to $\bar{\nu}$~2, transient precipitation occurred upon the addition of NaOH, and pH measurements were

TABLE III

TITRATION OF Cbz-PHG-NH_2 IN THE PRESENCE OF $Zn(NO_3)_2$ AT 25.1°

Total molar concn. of: P	$Zn(NO_3)_2$	NaOH	pH	−log(P)	$\bar{\nu}$
0.05049	0.00199	0.000909	4.50	3.23	0.173
.05044	.00199	.00134	4.70	3.03	.214
.05037	.00199	.00201	4.90	2.84	.282
.05026	.00198	.00303	5.10	2.65	.394
.05009	.00198	.00459	5.30	2.46	.580
.04997	.00197	.00573	5.40	2.37	.765
.04982	.00196	.00714	5.50	2.29	1.02
.04965	.00196	.00870	5.60	2.21	1.28
.04944	.00195	.01063	5.70	2.13	1.66
.04922	.00194	.01268	5.80	2.06	2.02
.04896[a]	.00193	.01508	5.90	1.99	2.51
.04868	.00192	.01762	6.00	1.93	3.03
.04842	.00191	.02000	6.10	1.87	3.35
.04815	.00190	.02253	6.20	1.81	3.73
.04789[b]	.00189	.02448	6.30	1.75	3.56

[a] Transient precipitate. [b] Permanent precipitate.

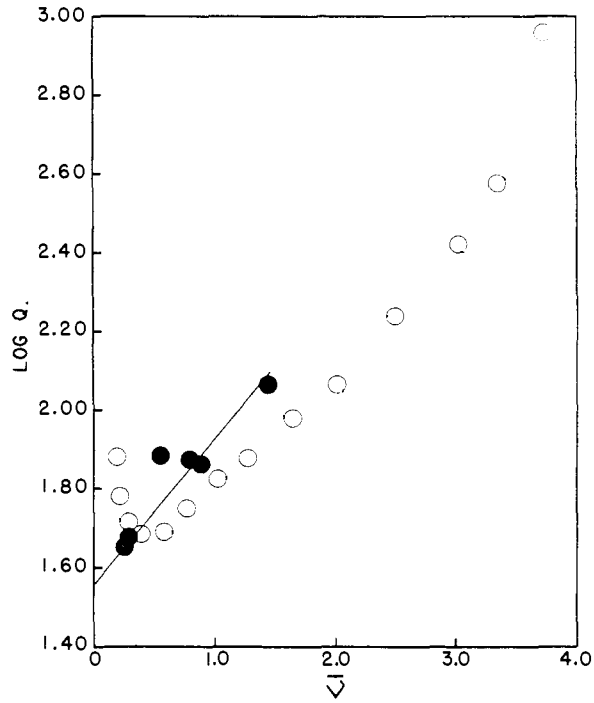

Fig. 1.—Plot of log Q *vs.* $\bar{\nu}$ for the binding of Zn(II) to Cbz-PHG-NH_2 at 25.1°. The closed circles, ●, represent points derived from pH measurements on individual solutions; the open circles, ○, represent points derived from stepwise titration.

(10) F. R. N. Gurd and P. E. Wilcox, *Adv. in Protein Chem.*, **11**, 311 (1956).

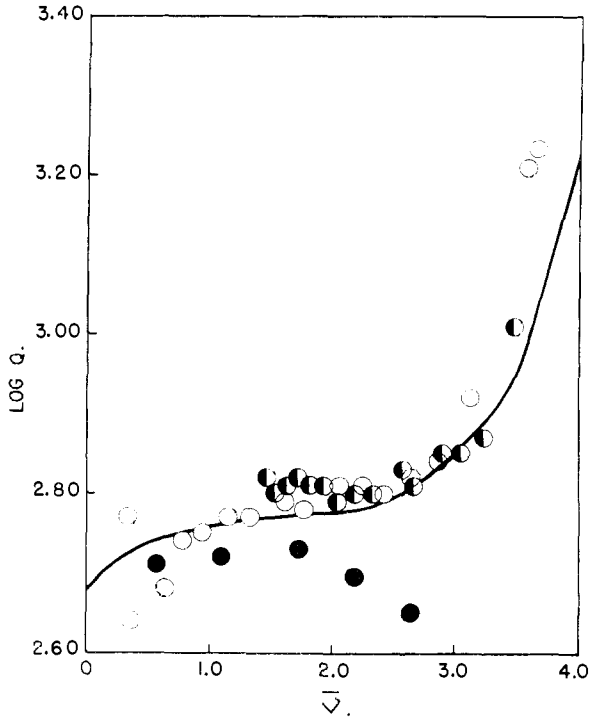

Fig. 2.—Plot of log Q *vs.* ν for the binding of Cu(II) to Cbz-PHG-NH_2 at 25.1°. Points derived from *p*H measurements on individual solutions are shown in closed circles, ●; points derived from stepwise titration with NaOH are shown in open circles, ○; points derived from back-titration with $Cu(NO_3)_2$ are shown in half-closed circles, ◐. The curve is computed as described in the text.

made only after the precipitate appeared to have redissolved. Permanent precipitation occurred above *p*H 6.20, $\bar{\nu} \sim 3.7$.

As may be seen from Fig. 1, the results of the stepwise titration approach much the same limiting value of log Q at $\bar{\nu} = 0$ as found with the individually prepared solutions (closed circles). The open circles in Fig. 1 could be fitted quite well by taking log $\mathbf{k}_2$ = log $\mathbf{k}_3$ = log $\mathbf{k}_4$ = 2.00 and constructing a theoretical curve. These constants give a curve slightly steeper than that needed to fit the results for complex-formation between Zn(II) ions and imidazole itself.[4] Because of the formation of transient precipitates during the stepwise titration, it seems wise to assume rather wide limits of uncertainty for the constants other than $\mathbf{k}_1$ and, perhaps, $\mathbf{k}_2$. Experimentally, it appears that transient precipitation introduces a bias toward low value of log Q.[11] It is possible that the stages of titration shown by the open circles in Fig. 1 represented only very close approaches to equilibrium and that the values of log $\mathbf{k}_3$ and log $\mathbf{k}_4$ taken above may be as much as 0.2 or 0.3 too low.[12]

(11) Similar stepwise titrations employing higher concentrations of Zn(II) were marked by onset of transient precipitation almost from the beginning; if the results were plotted in Fig. 1, they would fall below the open circles. Longer equilibration served to bring the results closer to those shown in the open circles.

(12) One other possible source of error could not be evaluated empirically. It was observed that releasing saturated KCl solution from the calomel electrode into solutions in which $\bar{\nu}$ was greater than about 2 often resulted in dense precipitation at the boundary of the KCl solution. This observation was made only in the presence of Zn(II) ions. Presumably such a phenomenon could occur at the interface at the tip of the salt bridge from the calomel electrode during normal operation, and might affect the accuracy of the measurements.

(B) **Cu(II).**—Measurements on individually prepared solutions are described in the lower part of Table II. The kinetic results (A) may be seen to be in good agreement with the equilibrium results (B) showing as before that the complexes with Cu(II) ions do not act catalytically.

The results of the equilibrium measurements from Table IIB have been used to compute values of log Q which are plotted against $\bar{\nu}$ in Fig. 2, closed circles. A stepwise titration also was performed, as shown in Table IV. The first part of the titration consisted of successive additions of NaOH solution to the solution containing $H^+PNO_3^-$, $Cu(NO_3)_2$ and sufficient NaCl to give an ionic strength of 0.16. The results are presented in Table IVA and are used to compute values of log Q which are plotted against $\bar{\nu}$ in Fig. 2 (open circles). A solution of 0.4890 *N* NaOH was used in order to minimize dilution. The second part of the titration consisted of adding $Cu(NO_3)_2$ solution (ionic strength 0.165) instead of NaOH (Table IVB) and had the effect of carrying $\bar{\nu}$ downward. It constitutes, therefore, a type of back-titration. The corresponding values of log Q are plotted against $\bar{\nu}$ in Fig. 2 (half-closed circles).

In the course of the forward titration transient precipitation was observed at $\bar{\nu} = 0.95$ and above. No precipitation was observed during the back-titration. The agreement between the results of the forward titration (open circles) and the back-titration (half-closed circles) indicates that the system remained close to equilibrium throughout the stepwise titration.

The curve shown in Fig. 2 is computed[7,9] using values of log κ_1, log κ_2, log κ_3 and log κ_4 of 2.68, 2.90, 2.45 and 3.25, respectively. The corresponding log **k**-values are 3.28, 3.08, 2.27 and 2.65.[4,7,9]

Absorption Spectra in the Visible of the Cu(II) Complexes of Cbz-PHG-NH_2 and Imidazole.—In view of the absorption in the visible of Cu(II) complexes with basic nitrogen atoms such as those in ammonia,[13] imidazole[7] or proteins,[10,14] it was felt advisable to examine the absorption characteristics of Cu(II)–Cbz-PHG-NH_2 complexes. Absorption spectra over the wave length range 600–800 mμ were measured for the conditions indicated in Table II. The pertinent results are shown in Table VA. All readings were made against a water blank and the observed optical densities corrected for the absorption due to free Cu(II) ion. The percentage distribution of Cu(II) between the free ion and the various species of complexes was calculated as a function of the concentration of basic ligand, expressed as −log (A), using the successive values of the association constants given above. The values shown in columns 4–8 correspond to the −log (A) values in column 3. The extinction coefficients ε are for a 1 cm. light path and are expressed in terms of the total molar con-

(13) J. Bjerrum, "Metal Ammine Formation in Aqueous Solution," P. Haase and Son, Copenhagen, 1941.

(14) I. M. Klotz in "The Proteins," H. Neurath and K. Bailey, eds., Vol. I, Part B, Academic Press, Inc., New York, N. Y., 1953, p. 727.

TABLE IV

TITRATION OF Cbz–PHG–NH_2 IN THE PRESENCE OF $Cu(NO_3)_2$ AT 25.1°

Total molar concn. of P	Total molar concn. of $Cu(NO_3)_2$	Total molar concn. of NaOH	pH	−log(P)	$\bar{\nu}$
A. Forward titration–addition of 0.4670 *N* NaOH					
0.04885	0.00213	0.000420	3.91	3.83	0.325
.04880	.00213	.000940	4.11	3.63	.369
.04872	.00212	.0016[illegible]	4.31	3.44	.636
.04868	.00212	.00206	4.40	3.35	.781
.04863[a]	.00212	.00253	4.50	3.26	.945
.04857	.00212	.00309	4.60	3.16	1.15
.04851	.00211	.00366	4.70	3.07	1.34
.04844	.00211	.00437	4.80	2.98	1.58
.04837	.00211	.00504	4.90	2.88	1.77
.04828	.00210	.00589	5.00	2.79	2.04
.04820	.00210	.00669	5.10	2.70	2.24
.04811	.00210	.00751	5.20	2.61	2.42
.04800	.00209	.00854	5.30	2.52	2.65
.04789	.00209	.00964	5.40	2.44	2.87
.04775	.00208	.01095	5.50	2.35	3.14
.04757	.00207	.01270	5.60	2.28	3.58
.04744	.00207	.01395	5.70	2.20	3.66
.04724	.00206	.01579	5.80	2.12	4.00
B. Back titration–addition of 0.0549 *M* $Cu(NO_3)_2$					
0.04679	0.00256	0.01563	5.73	2.18	3.49
.04635	.00306	.01549	5.68	2.26	3.23
.04591	.00355	.01534	5.59	2.35	3.05
.04548	.00396	.01520	5.51	2.43	2.90
.04507	.00449	.01506	5.43	2.51	2.67
.04465	.00495	.01492	5.38	2.57	2.59
.04425	.00541	.01479	5.30	2.65	2.34
.04385	.00585	.01465	5.23	2.73	2.18
.04346	.00629	.01452	5.19	2.77	2.04
.04308	.00671	.01439	5.12	2.84	1.93
.04270	.00713	.01427	5.08	2.89	1.82
.04233	.00755	.01415	5.03	2.94	1.72
.04197	.00795	.01402	5.00	2.97	1.63
.04161	.00835	.01390	4.98	3.00	1.54
.04126	.00875	.01379	4.93	3.05	1.48

[a] Transient precipitate above *p*H 4.50.

centration of bound Cu(II). It is clear that as $\bar{\nu}$ increases the extinction coefficient also increases (column 10) and the absorption maximum is shifted to shorter wave lengths (column 9).

For purposes of comparison spectral results obtained with Cu(II)–imidazole complexes are shown in Table VB. The percentage distribution of Cu(II) among the various forms was calculated using the successive association constants previously reported.[4] The characteristic trends of the wave lengths of maximum absorption and of maximum extinction coefficient again were observed.[13]

If spectral measurements are to be useful in determining the degree of complex formation between imidazole groups and Cu(II) ion, a knowledge of the absorption spectra of the individual complexes is essential. The individual spectra were computed from the calculated composition and the experimentally determined absorption spectra of the solutions. The wave lengths of maximum absorption and the maximum extinction coefficients are shown in Table VI. The close parallel between λ_{max} for Cu(II)–Cbz-PHG-NH_2 and for Cu(II)–imidazole is evident. In both cases the λ_{max} decreases approximately 50 mμ for each ligand molecule added to the complex.[15] The former complexes, however, absorb 30–40% more strongly than do the latter complexes. Because the solutions studied were relatively weakly absorbing, the extinction coefficients of the Cu(II)–Cbz-PHG-NH_2 complexes are considered reliable to within 10%. The values for the Cu(II)–imidazole complexes are probably reliable to within 5%. As a check on the validity of the procedures used the extinction of each solution was computed at λ_{max} from the composition of the solution and the calculated extinction coefficients of the individual complexes. The observed and computed values are in excellent agreement (Table V, columns 10 and 11). The extinction coefficients for $Cu(Im)^{++}$ and $Cu(Im)_4^{++}$, 19 and 56 l. $mole^{-1}$ $cm.^{-1}$, respectively, are in reasonable agreement with those reported earlier, 16.5 and 53.[7] The slight differences may reflect the use of chloride salts in the present study instead of the nitrate salts used previously. It is interesting that the addition of the third ligand to the metal ion is marked in both systems by a relatively small increase in extinction coefficient, even though the displacement of λ_{max} is equivalent to that which accompanies the addition of the second and fourth ligands.

Stability of Cbz-PHG-NH_2 in the Presence of Cu(II) Ion.—Following the spectral measurements, the last three solutions described in Table VA were pooled and brought to *p*H 5 with acetate buffer. The Cu(II) ion was removed by extraction with dithizone in chloroform and the aqueous solution subjected to paper electrophoresis. The material behaved exactly as did the stock preparation of Cbz-PHG-NH_2, which indicates that the peptide derivative was stable in the presence of Cu(II) ion.

Discussion

Catalytic Properties.—The value of k_2, the specific second-order rate constant for hydrolysis of NPA by Cbz-PHG-NH_2, is 6.40 l. $mole^{-1}$ $min.^{-1}$ at 25.3° in 0.2% ethanol and ionic strength 0.16. For the hydrolysis by imidazole under comparable conditions, k_2 is 31.3 l. $mole^{-1}$ $min.^{-1}$.[4] The lower value is to be expected in view of the lesser basicity of the peptide. In a study of solutions of various imidazoles in which the principal catalytic species is the neutral molecule, Bruice and Schmir showed that catalytic activity could be related by the usual Brönsted expression, $\log k_2 = apK' + b$.[16] The relation between the values of k_2 for Cbz-PHG-NH_2 ($pK' = 6.42$) and for imidazole ($pK' = 7.08$)[4] is rather close to that found by Bruice and Schmir for a series of imidazoles under somewhat different conditions.[8] It appears, therefore, that the presence of the peptide bonds linking the histidyl residue with its neighbors in Cbz-PHG-NH_2 confers no special reactivity on the side-chain imidazole group with respect to its ability to split NPA.

Complex Formation with Zn(II) and Cu(II) Ions.—Just as the imidazole group in Cbz-PHG-

(15) Bjerrum has reported similar displacements in the Cu(II)-ammonia system.[18]

(16) T. C. Bruice and G. L. Schmir, THIS JOURNAL, **80**, 148 (1958).

TABLE V

THE ABSORPTION CHARACTERISTICS IN THE VISIBLE REGION OF Cu(II) COMPLEXES OF Cbz-PHG-NH_2 AND IMIDAZOLE

pH	$\bar{\nu}$	−log(A)	% Cu	% CuA	% CuA_2	% CuA_3	% CuA_4	λ_{max}, mμ	ϵ_{max}, l. mole^{-1} cm.$^{-1}$ Expt.	Calcd.
A. Cbz-PHG-NH_2; total $CuCl_2$ = 0.0020 *M*										
4.26	0.57	3.49	53.0	33.0	13.3	0.7	0.0	735	37	32
4.61	1.10	3.14	26.0	36.0	32.0	4.3	1.0	720	35	36
4.91	1.73	2.85	10.2	26.8	44.2	12.0	7.0	700	43	42
5.17	2.18	2.61	3.9	14.3	41.5	19.0	22.0	685	47	47
5.46	2.65	2.35	0.5	5.0	26.8	22.5	45.2	650	53	53
B. Imidazole; total $CuCl_2$ = 0.0099 *M*										
4.49	0.50	4.29	52.0	42.0	5.5	0.3	0.0	730	21	21
4.92	0.99	3.86	25.0	52.5	20.0	2.5	.1	725	23	23
5.30	1.48	3.48	8.5	44.0	38.0	9.0	.3	715	26	26
5.62	1.95	3.16	2.5	25.0	46.0	24.0	2.0	700	30	30
5.97	2.46	2.81	0.3	9.5	38.0	44.0	8.7	670	35	35
6.30	2.90	2.48	.0	3.5	24.0	48.0	24.5	655	38	38
6.53	*3.16*	*2.25*	*.0*	*1.5*	*15.0*	*46.0*	*37.5*	*625*	41	40

TABLE VI

THE ABSORPTION CHARACTERISTICS IN THE VISIBLE REGION OF THE INDIVIDUAL Cu(II) COMPLEXES OF Cbz-PHG-NH_2 AND IMIDAZOLE

	Cbz-PHG-NH_2		Imidazole	
	λ_{max}, mμ	ϵ_{max}, l. mole^{-1} cm.$^{-1}$	λ_{max}, mμ	ϵ_{max}, l. mole^{-1} cm.$^{-1}$
CuA	735	27	735	19
CuA_2	685	49	690	35
CuA_3	635	55	635	39
CuA_4	600	71	600	56

NH_2 appears to behave as an individual group in its reaction with NPA, so also does it appear to be the sole ligand group in the binding of the peptide derivative to Zn(II) and Cu(II) ions. This is shown by the agreement between kinetic and equilibrium measurements (Table II) and by the fact that with both ions $\bar{\nu}$ approaches 4, the value of N for imidazole itself.[7,17] We may conclude that stable complexes are not formed that involve the metal ion simultaneously with more than one ligand group in each molecule of the peptide derivative.

The value of 2.16 for log $\mathbf{k}_1$ for the binding of Cbz-PHG-NH_2 to Zn(II) ions falls slightly below the corresponding values for imidazole and 4-methylimidazole, 2.52[4] and 2.44,[9] respectively. As mentioned earlier, the increases in affinity between Zn(II) and Cbz-PHG-NH_2 with each successive step of complex formation are probably at least as great as those shown by the Zn(II)–imidazole system.[4]

The value of 3.28 for log $\mathbf{k}_1$ for the binding of Cbz-PHG-NH_2 to Cu(II) ions falls decidedly below the corresponding values for imidazole and 4-methylimidazole, 4.20[4] and 4.13,[9] respectively. The reason is not apparent at present. It is possible that steric hindrance is responsible, but we have little information about the variability of $\mathbf{k}_1$ values for Cu(II) complexes of substituted imidazoles. On the other hand the course of the successive steps of complex formation (log κ_2 = 2.90, log κ_3 = 2.45 and log κ_4 = 3.25) is unlike the marked downward trend of the κ-values for other imidazole complexes with Cu(II) ions.[4,9] To interpret the course of these later steps it is tempting to postulate that the complexes are stabilized by interactions between parts of the peptide derivative other than the linkages between imidazole groups formed by the Cu(II) ion. Apparently the configuration of the Cu(II) complexes allows greater interaction between the ligand molecules than does that of the Zn(II) complexes. Study of molecular models shows that the imidazole groups of four molecules of the peptide derivative may combine at one time with the metal ion either in the tetrahedral configuration characteristic of Zn(II) complexes or the square coplanar configuration characteristic of Cu(II) complexes.[9,10] It must be borne in mind that the complex bearing two ligand groups per Cu(II) ion exists in two possible forms and that differences in distribution between these forms may account for part of the differences in the progression of κ-values for the systems containing Cbz-PHG-NH_2 and imidazole, respectively.[9,13] Confirmation of the role of secondary interactions and configurational effects in the stability of such complexes must await studies at other temperatures.

Studies on dipeptides such as glycylglycine have shown that Cu(II) ion may combine with an α-amino group and then proceed to displace a hydrogen ion from the peptide bond.[18–20] The possibility that the imidazole group might be able to play the role of the α-amino group and permit an analogous structure to form in the Cu(II)–Cbz-PHG-NH_2 system appears to be excluded. The agreement between the kinetic and equilibrium results described above allows little room for such a competing reaction. Furthermore, the spectral results fit the Cu(II)–imidazole system well but do not conform to the behavior of systems of the Cu(II)-glycylglycine type.[18,20]

Comment on Cbz-PHG-NH_2 as a Model for the Imidazole Group in Proteins.—A group comprising the side-chain of an amino acid residue in a protein may react individually or may form part of a

(17) N. C. Li, J. M. White and E. Doody, THIS JOURNAL, **76**, 6219 (1954).

(18) H. Dobbie and W. O. Kermack, *Biochem. J.*, **59**, 246 (1955).

(19) S. P. Datta and B. R. Rabin, *Trans. Faraday Soc.*, **52**, 1117 (1956).

(20) M. Fried, F. R. N. Gurd and W. L. Koltun, in preparation.

cluster of such side-chain groups. Inasmuch as it contains only the imidazole group free to take part in the reactions studied here, Cbz-PHG-NH_2 represents a model for the behavior of an individual imidazole group in a protein. It represents a model in this sense for the reaction with NPA and for the formation of the first complex with Cu(II) or Zn(II) ion. In the formation of the higher complexes secondary interactions appear to have a part, and it is unlikely that the values of k_2, k_3 or k_4 give a useful indication of the behavior of a cluster of imidazole groups in a protein.

Since identical sequences of three amino acid residues appear to be repeated only rarely in the peptide chain of a protein,[21] we may expect that each such residue will have different neighbors and so will be subject to different inductive effects transmitted through its peptide bonds. Strictly speaking, therefore, this peptide derivative represents a good model for the reactivity of the imidazole group only in the sequence -prolyl-histidyl-glycyl-. What range of properties to attribute to the individual side-chain groups of a given class in a protein, and how to correlate one measure of reactivity with another, are questions that must await the study of more model systems of this type.

(21) F. Šorm, B. Keil, V. Holeyšovský, V. Knesslová, V. Kosta, P. Mäsiar, B. Meloun, O. Mikeš, V. Tomášek and J. Vaněček, *Coll. Czechoslov. Chem. Commun.*, **22**, 1310 (1957).

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Coördination Complexes and Catalytic Properties of Proteins and Related Substances. III. Effect of Zinc and Cupric Ions on the Reaction of *p*-Nitrophenyl Acetate with Glycylglycine[1,2]

BY WALTER L. KOLTUN AND FRANK R. N. GURD

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Acetylglycylglycine is formed when *p*-nitrophenyl acetate (NPA) reacts with glycylglycinate (GG^-). When GG^- is present in excess the reaction follows first-order kinetics and provides a convenient means of determining the concentration of GG^- in systems containing Zn(II) or Cu(II) ions. The technique is compatible with measurements of complex formation by determinations of *p*H. The logarithms of the first and second association constants for GG^- with Zn(II) are 3.30 and 2.78, respectively, at 25° and ionic strength 0.16. The corresponding value for the formation of the complex denoted as $CuGG^-_2$ is 3.07.

Introduction

The potential advantages of studying the reactivity of polar side-chain and terminal groups in proteins by a combination of techniques applied concurrently were outlined in the first paper of this series.[3] It was shown by measurements on the Zn(II)–imidazole and Cu(II)–imidazole systems that kinetic and equilibrium measurements yielded identical results over a wide range of conditions. The rate of splitting of *p*-nitrophenyl acetate (NPA) by free basic imidazole was followed spectrophotometrically,[4,5] and the equilibrium concentration of free basic imidazole (Im) was determined from measurement of *p*H.[6] Detectable interference of one method of measurement with the other was avoided by using relatively small amounts of NPA.

The object of the present study is to apply the same combination of techniques to a model compound representing the N-terminal α-amino group of a peptide chain. In this case the simplest model is a peptide itself. We have chosen glycylglycine, GG, as a model because its metal complexes have been studied in some detail.[7–10]

We have again made use of the rate of splitting of NPA, in this case to measure the concentration of the unbound anion, (GG^-). Although this reaction leads to the irreversible formation of N-acetylglycylglycine, $AcGG^-$, such small quantities of GG^- are consumed in the reaction that a detectable change in the over-all equilibrium of GG with metal ion is avoided.[8] The kinetic and equilibrium measurements on the Zn(II)-GG system are mutually confirmatory in the same sense as the previous studies on the systems containing imidazoles.[3,11] The combined measurements on the Cu(II)–GG system make possible some unambiguous conclusions confirming part of the general picture originally suggested by Dobbie and Kermack.[8]

Materials and Methods

Glycylglycine was "chromatographically pure," supplied by Mann Research Laboratories, Inc., New York, N. Y. Other reagents were of analytical grade prepared and

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(2) Presented in part at the 133rd National Meeting, American Chemical Society, San Francisco, California, April 13–18, 1958.

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