Acid–Base Titration of Hemocyanin from *Octopus vulgaris* Lam.†

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ABSTRACT: Potentiometric and spectrophotometric titrations of native (oxygenated and deoxygenated) and copper-free hemocyanin of *Octopus vulgaris* have been carried out under several experimental conditions. The results of acid–base continuous titration at 25° in KCl solutions at different ionic strengths indicate that each of the two copper atoms in the functional subunit (50.800) is bound to four non-carboxyl groups in a strong hydrophobic environment. These groups have been identified as histidines by titrating at equilibrium the native and copper-free proteins in the presence of 3 M urea and 0.5 M guanidine at 10, 25, and 30°. The spectrophotometric titration of native and apohemocyanin in KCl, as well as in urea and guanidine, thiourea, and thiocyanate, has shown that oxygen, copper, and other ligands have a strong stabilizing effect on the conformation of the protein.

This work is part of a long-term program to investigate the nature of the active site of hemocyanin with emphasis on the reactive groups of the protein molecule which chelate the metal and confer on hemocyanin its peculiar property as an oxygen-carrying pigment.

Since the beginning of the work we realized that an investigation of hemocyanin as polyelectrolyte has to face several difficulties due to the complexity of this copper protein. It is known (Eriksson-Quensel and Svedberg, 1936) that the aggregation state of hemocyanin, *i.e.*, its molecular weight, is strongly dependent on the hydrogen ion concentration, remarkable changes of the molecular species present in solution occurring on both sides of the pH stability region. Moreover, irreversible denaturation and copper removal take place at low pH values (Henze, 1901).

In this paper the results are reported on potentiometric and spectrophotometric titrations of the hemocyanin from *Octopus vulgaris* in KCl solutions at different ionic strengths. With the purpose of increasing the accessibility of the protein molecule to the titrating agents, experiments have been carried out also in 3 M urea and 0.5 M guanidine-HCl. Native (oxygenated and deoxygenated) hemocyanin and protein preparations from which most of the copper had been removed have been used throughout the work. Experiments in the presence of ligands such as thiourea and thiocyanate in conditions in which the metal is not detached from the protein are also reported.

Experimental Section

Materials. The preparation and storage of hemocyanin have been described (Ghiretti-Magaldi *et al.*, 1966). The protein was dialyzed against phosphate buffer (pH 7.5–8.0) containing 0.01 M EDTA and used within a few days. Copper-free hemocyanin was prepared by dialyzing for 36 hr in the cold the native protein (25 mg/ml) against 0.025 M KCN in Tris buffer (pH 7.5–8.0) (μ = 0.05) and then against the appropriate medium with repeated changes. After this treatment the hemocyanin of *Octopus vulgaris* still holds 20–25% of its original copper (Ghiretti-Magaldi and Nardi, 1963). This we call apohemocyanin.

For several experiments hemocyanin containing 45% copper has been prepared by limiting the duration of dialysis against cyanide to 6–7 hr. The solutions are colorless and the copper–oxygen band has almost completely disappeared. All operations were carried out at 4°.

Potentiometric Titration. pH measurements were done under oxygen or argon in a water thermostated jacketed cell with a M-26 pH meter with expanded scale and a TTT1 titration unit from Radiometer. Titrations of hemocyanin in KCl solutions were carried out at constant velocity using the complex syringe-recorder SBU1 and SBR2. A G202B glass electrode and a K101/B2 calomel electrode with a KCl saturated bridge at open liquid contact were also used. The registration rate was 25 cm/28 min, the rate of titration was 0.1 pH/cm, and the temperature was 25 ± 0.1°. In each experiment 10 ml of the hemocyanin solution containing 150–200 mg of protein was placed in the cell and a flow of oxygen or argon (for native and apohemocyanin, respectively) was gently maintained over the fluid to avoid contamination by carbon dioxide. The gases were previously washed with 50% H_2SO_4 and 40% NaOH, and then passed through the titration medium so that no volume change of the sample would occur. The hemocyanin solution was stirred for 30 min for temperature and gas equilibration, and then titrated with 0.100 N solutions of acid or base. The starting pH value was selected so as to have overlapping of the acid and basic regions in the titration curves. Blank experiments were done in the same conditions using equal amounts of solvent. This is a continuous titration except for the region of pH in which equilibrium is reached instantaneously.

Equilibrium titrations in the presence of urea and guanidine-HCl were carried out manually using a micrometric syringe (Chemetron) of 1.000 ml at different temperatures (10, 25, and 30 ±0.1°). Buffers of the International Bureau of Standards at pH 4.65, 4.68, and 9.18 ± 0.01 at 25° were used to standardize the pH meter at the beginning and the end of titration.

Analysis of the Experimental Data. The titration curves were calculated by the method of Cannan (1942) and analyzed according to the model of Linderstrom-Lang as described by Tanford (1962). For all ionizable groups intrinsi-

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cally identical of a given type, it is possible to write eq 1,

$$\mathrm{pH} - \log[n_i/(n_t - n_i)] = \mathrm{p}K_i - 0.868Zw \quad (1)$$

where n_t and n_i are the total and the dissociated groups of the ith kind at a given pH value, respectively. pK_i is the intrinsic dissociation constant at the ionic strength used and Z is the net charge of the protein molecule at a given pH. $0.868Zw$ is proportional to the electrostatic work which is required to remove one hydrogen ion from the molecule surface to infinite. In the absence of electrostatic interactions pK_i is equal to $\mathrm{pH} - \log[n_i/(n_t - n_i)]$.

The application of eq 1 to hemocyanin is complicated by the fact that the value of w depends on the size and shape of the protein molecule. It will change, therefore, with the pH. No quantitative data are available on the molecular weight distribution as a function of pH in our conditions. We have referred, therefore, the experimental values to the functional unit of the protein (50.800) containing two copper atoms. Moreover, nothing is known on the adsorption to the protein of the ions present in the medium. That is why in our calculations Z_{H^+} has been used instead of Z. Z_{H^+} is the number of protons dissociated by or bound to the protein at a given pH value. The number of residues of each kind available for titration was calculated according to eq 1 and using the empirical procedure described by Tanford (1962).

Spectrophotometric Measurements. Spectra were taken using a DK-1 Beckman spectrophotometer with a thermostated sample-cell holder at 25 ± 0.1° (unless differently stated). Quartz 1-cm light-path cells were used for all experiments. For the difference spectra the reference solution was at neutral pH. The values have been corrected for the light scattering using the equation $\Delta A = K/\lambda^4$. Hemocyanin preparations containing different amounts of copper have been used: 100% (native), 45%, and 20–25%. In all the experiments the protein concentration ranged from 0.5 to 1.0 mg/ml. The sample solution, previously dialyzed against 0.1 M KCl, was brought to known pH values with small amounts of 0.1–10 N NaOH so that the protein concentration was not affected appreciably. Titrations were also carried out on native hemocyanin in the presence of 0.1 M SCN^- and 0.01 M thiourea + 0.1 M KCl. Back titrations were done by adding to the sample small amounts of HCl solutions at different concentrations (from 8.0 to 0.1 N).

Protein, Amide Nitrogen, and Copper Analysis. The concentration of hemocyanin was estimated spectrophotometrically at 290 nm in 0.3 M KOH using an experimentally determined extinction of $E_{290}(1\%)$ 1.67. The OD values were corrected for the light scattering as mentioned before. In several cases the protein concentration was estimated with the biuret method previously calibrated on hemocyanin solutions whose concentration had been measured by dry weight.

Amide nitrogen was determined titrimetrically with the Conway (1962) microdiffusion technique; 2 mg of protein was hydrolyzed at 37° with 2 M NaOH in the Conway vessel for 12, 24, and 36 hr. Ammonia was recovered on 1% boric acid and was titrated in the presence of a mixed indicator. Titrations have also been carried out after hydrolysis of the protein with 2 N HCl according to Leggett Bailey (1967) and ammonia was estimated as before.

Copper was measured by atomic absorption (Perkin-Elmer spectrophotometer, Model 300). On several occasions the reaction with 2,2′-biquinoline was also used.

Chemicals. All reagents were analytical grade. Normex solutions of HCl and KOH were titrated before use with sodium tetraborate (twice recrystallized from water) or potassium acid phthalate (dried for 2 hr at 120°), respectively. Analytical grade urea was used without further purification; the results were the same when urea twice recrystallized from absolute ethyl alcohol was used. Commercial guanidine-HCl was recrystallized from benzene–ethyl alcohol and then from methyl alcohol by the method of Nozaki and Tanford (1967). Deionized or glass-distilled water was used in making up all solutions. These were bubbled with nitrogen or argon for avoiding contaminations by carbon dioxide.

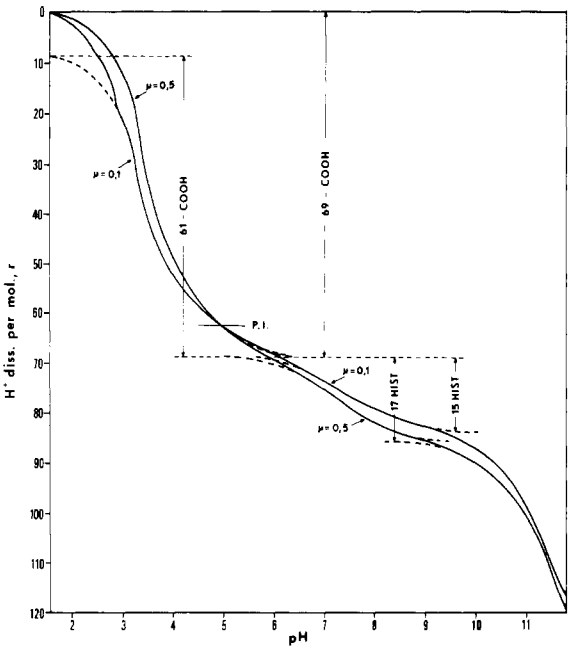

FIGURE 1: Titration curves of native (deoxygenated) hemocyanin in KCl at 0.1 and 0.5 ionic strengths. The broken lines indicate the calculated titration of each kind of groups in the region of overlapping. T = 25 ± 0.1°.

Results

I. Potentiometric Titrations

Titration in KCl. When titrated in 0.1 μ of KCl, the hemocyanin from *Octopus* remains in solution in the whole range of pH except from pH 4.3 to 5.3. In 0.5 μ of KCl the protein is insoluble to all pH values below 5.3. However, as indicated by titrations carried out at different speeds, in the conditions used equilibrium is attained at a higher rate than that of the titration. For the isoionic point of native and copper-free hemocyanin the values of 4.7 and 4.8 have been used, respectively (Davson and Mallette, 1945).

The reference point for the construction of the plots was fixed in the ordinate scale at the value of minimum proton charge, *i.e.*, at the acidic side of the titration curve. All calculations have been referred to 50.800, which is the molecular weight of the functional subunit of *Octopus vulgaris* hemocyanin on the basis of the Cu/O_2 and the Cu/protein ratios. The thermodynamic reversibility of deoxygenated and copper-free hemocyanin lies in the range from pH 4.5 to 10.8. Two hemocyanin solutions were brought to these pH values and after 30 min were back titrated. Apohemocyanin is titrated more "easily" than the native protein, whereas deoxygenated hemocyanin binds more H^+ and OH^- ions in the acidic and basic regions, respectively. As shown in Figures 1 and 2, there is a sharp difference be-

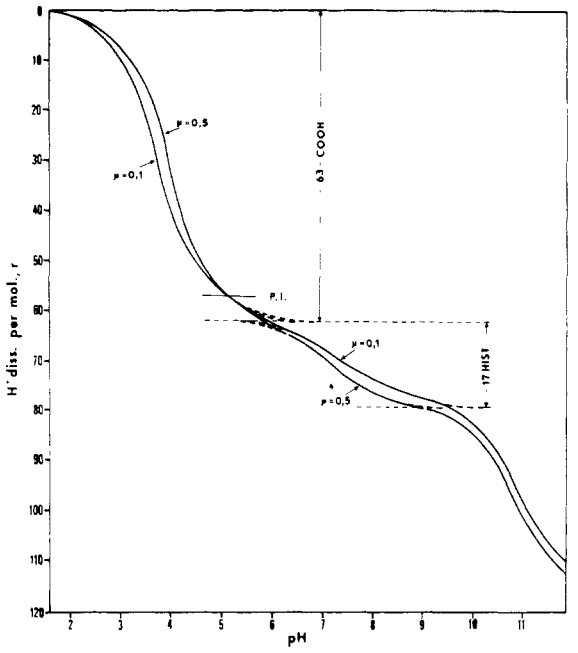

FIGURE 2: Titration curves of copper-free hemocyanin in KCl at 0.1 and 0.5 ionic strengths. The broken lines indicate the calculated titration of each kind of groups in the region of overlapping. T = 25 ± 0.1°.

tween native and apohemocyanin in the acidic region: 69 carboxyl groups are titrated in deoxygenated hemocyanin and 63 in the copper-free protein.

The amino acid analysis of the hemocyanin from *Octopus vulgaris* indicates the presence of 49 aspartic acid and 40 glutamic acid residues per mole (Ghiretti-Magaldi *et al.*, 1966). By assuming one or two carboxyl end groups, the total acidic groups would amount to 90–91 of which 30 ± 1 are present as asparagine and glutamine as shown by the amide nitrogen analysis. The good agreement between the analytical data and the results of potentiometric titration indicates that, in spite of its high molecular weight, the protein contains very few if any permanently masked carboxyl groups.

The pK_i value for the carboxyl groups is the same for deoxy- and apohemocyanin and equals 4.0, a value which is lower than that found for other proteins (4.6–4.7). In fact the calculations have been made using the total number of carboxyl groups (61) and not the number which is actually in equilibrium with the solvent at any pH value.

As for the neutral region, in deoxygenated hemocyanin

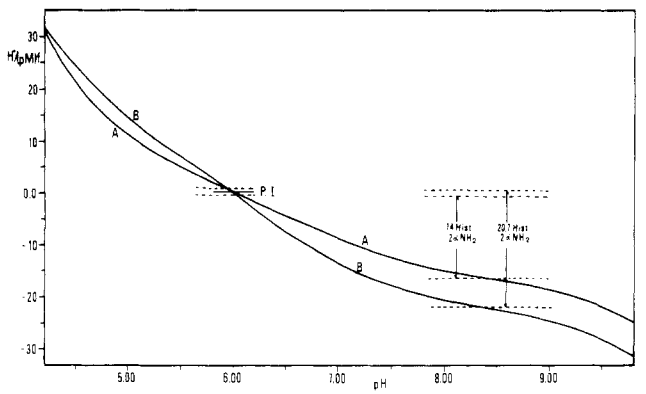

FIGURE 3: Equilibrium titration of native (curve A) and apohemocyanin (curve B) in 3 M urea and 0.5 M guanidine-HCl in the range of pH 4.2–9.8. T = 25 ± 0.1°.

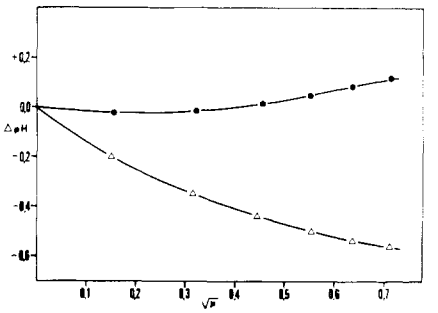

FIGURE 4: Effect of KCl (●) and of guanidine-HCl (△) on the isoionic point of hemocyanin in 3 M urea as a function of the square root of the ionic strength. T = 25 ± 0.1°.

15 histidyl residues are titrated at 0.1 μ and 17 groups at 0.5 μ. In copper-free hemocyanin 17 imidazole groups were found at both ionic strengths. These values are much lower than those obtained by analytical methods: 22 histidines and one or two NH_2 terminals, an indication that about 30% of the total imidazole groups are masked in the region of pH in which they are normally titrated. The pK_i value of the histidine residues is equal to 6.7 for both deoxygenated and copper-free hemocyanin.

Titration in Urea and Guanidine. The titration curves of deoxygenated and copper-free hemocyanin in 3 M urea and 0.5 M guanidine-HCl at 25° are reported in Figure 3. Each curve has been obtained from three experiments with different protein samples. The results are expressed as moles of H^+ ions linked to or dissociated from the protein, starting from the isoionic point. This was found equal to 6.0 for both native and copper-free hemocyanin. Whereas in the absence of small ions less than 1% of the protein dissolves, the solubility greatly increases in 3 M urea. The isoionic point, therefore, was measured after deionization on a Dintzis (1952) column in the presence of 3 M urea.

In Figure 4 the effect of KCl and of guanidine-HCl on the isoionic point of hemocyanin in 3 M urea is reported in function of the square root of the ionic strength. K^+ and Cl^- ions are slightly adsorbed to the protein whereas 25 guanidine ions per mole are adsorbed at 0.5 μ. The titration was carried out from pH 3.8 to 10.0. In two experiments it was extended from pH 1.8 to 12.5. The ability of hemocyanin to bind protons apparently decreases below pH 3 and above pH 11.5. In fact, after addition of the same amount of titrant, the pH of the sample equals that of the blank solution. The equilibrium with H^+ ions is reached instantly both with deoxy- and apohemocyanin from pH 5 to 10. In this range the titration is entirely reversible even after 30 min. On the contrary, at lower and higher pH values, the titration is time dependent and the reversibility is influenced

TABLE I: Number of Titrated Groups and Their pK_i in Native and Apohemocyanin of *Octopus vulgaris* in 3 M Urea and 0.5 M Guanidine-HCl.

Titrated Groups	Native Hemocyanin		Apohemocyanin	
	n	pK_i	n	pK_i
COOH	61	4.6	61	4.7
Histidines	14	6.7	14	6.6
Histidines			6.7	6.2
α-NH_2	2	7.5	2	7.5

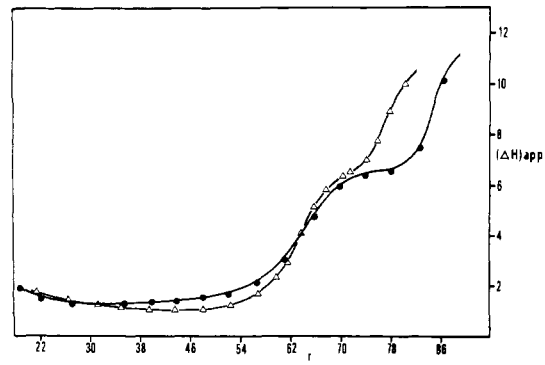

FIGURE 5: Apparent heat of ionization of native (△) and of copper-free (●) hemocyanin calculated from the titration curves at different temperatures as a function of r.

by the extent of exposure to that pH value.

The data were analyzed using a value of 61 for the carboxyl groups as for the acid-base titration in KCl. A pK_i value of 4.5 was calculated for both deoxy- and apohemocyanin. The angular coefficient of the function which is proportional to w is equal to 0.020 ± 0.003 in native hemocyanin and to 0.016 ± 0.03 in the copper-free protein. These values have been used for calculating the dissociation function of the imidazole and α-NH_2 groups. n_t was tentatively evaluated from the inflection points of the titration curve at pH 6.0 and 8.5 and the final data have been elaborated according to the method of successive approximation. The best results are those obtained by assuming the values reported in Table I. It can be seen that 14 histidine residues with pK_i equal to 6.7 are titrated both in deoxy- and apohemocyanin. Moreover, in apohemocyanin 6.7 basic groups are titrated with a pK_i value of 6.2 which also, on the basis of their apparent heat of ionization, are interpreted as histidine residues.

The Apparent Heat of Ionization. Deoxy- and copper-free hemocyanin were titrated at different temperatures and the apparent heat of ionization was calculated from the curves at 10 and 30° according to the equation

$$\Delta H_{app} = 2.303R\frac{T_1T_2}{T_1 - T_2}(pH_2 - pH_1)_r \qquad (2)$$

In Figure 5 the values obtained are reported as a function of r. The titration values at 40° were discarded: at this temperature the apohemocyanin is partially denatured as indicated by its higher viscosity and by the decrease (20–30%) of the titrated groups. The ΔH_{app} value is equal to 6.5 cal/mol for both hemocyanins.

II. Spectrophotometric Titrations

The titration curves of the phenolic groups have been taken at 300, 295, 290, and 288 nm according to Tanford and Wagner (1954) and the results obtained in all the experimental conditions used are shown in Figure 6. The curves are independent from the wavelength: in fact the same curve is found when calculated from the spectral changes at different values. With native hemocyanin in KCl the titration is reversible up to pH 11.35 whereas in the partially resolved protein as well as in apohemocyanin the reversibility reaches about 10.8. The pK_i of tyrosyl groups has been found equal to 10.5 in all the conditions used.

The number of tyrosine residues per mole of protein was calculated by using the usual extinction coefficient for phenolic ionization of 2.33 × 10^3/mol (Beaven and Holiday,

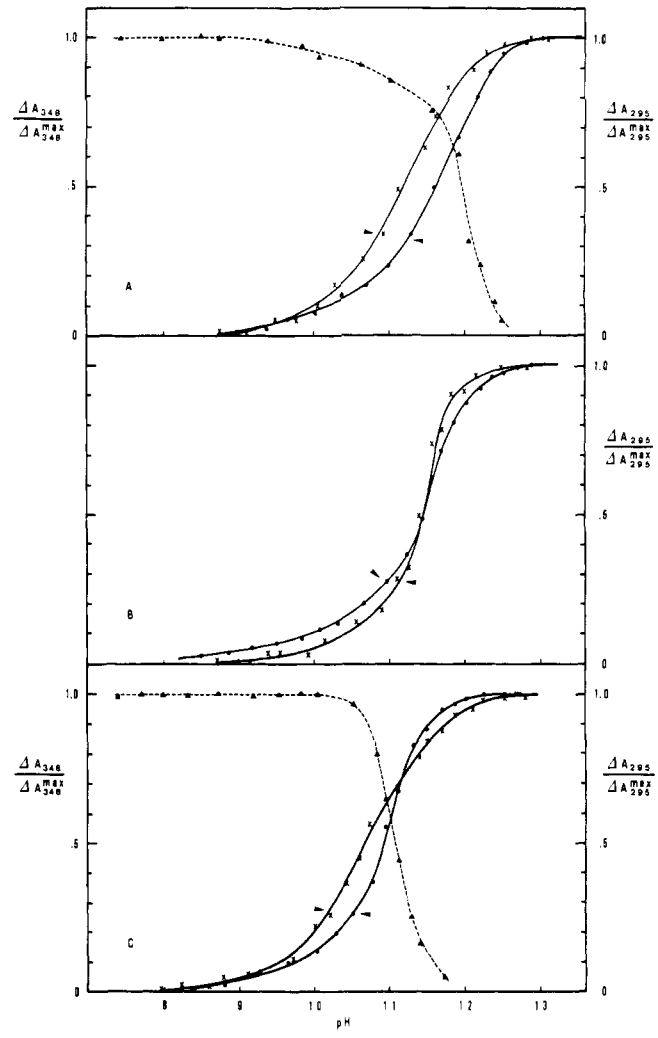

FIGURE 6: Spectrophotometric titration curves of the phenolic groups of hemocyanin in different conditions. The degree of ionization, as determined at 295 nm, is plotted *vs.* pH. (A) In KCl 0.1 ionic strength. Native (oxygenated) hemocyanin (●); copper-free hemocyanin (X). The titration curve (not reported) of the partially resolved hemocyanin (45% Cu) is practically coincident with that of the apoprotein. (B) In 0.1 M thiourea and 0.1 M KCl (●) and in 0.1 M SCN^- (X). (C) In 3 M urea and 0.5 M guanidine-HCl. Native (oxygenated) hemocyanin (●); copper-free hemocyanin (X). The arrows indicate the limit of reversibility of the titration. In A and C the molar extinction at 348 nm of native (oxygenated) hemocyanin as function of pH is reported. T = 25 ± 0.1°.

1952). In the presence of urea and guanidine 18 tyrosines are titrated both in native and copper-free hemocyanin, in agreement with the analytical data (Ghiretti-Magaldi *et al.*, 1966). In other conditions less tyrosyl groups are titrated. Also the pH of half-ionization is different in the different experimental conditions and the phenolic difference peak is shifted to 295 nm in the presence of urea and guanidine and SCN^- (Table II). As shown in Figure 6, the copper-oxygen band falls to 50% of its original value at pH 12 in KCl and at pH 11 in urea and guanidine.

Discussion and Conclusions

The unusual stability of the copper complex in hemocyanin suggested the participation of cysteine or imidazole residues in the active site. The presence of SH groups as ligands of copper in hemocyanin was discarded when it was demonstrated that the protein reconstitutes from apohemocyanin even after blocking of the sulfydryl groups with spe-

TABLE II: Spectral Properties of Native and Apohemocyanin of *Octopus vulgaris*

	Molar Difference Extinction Coefficients × 10^{-3}					λ_{max}	N.Tyr.	pH Half-ionization
	ΔE_{348}	ΔE_{300}	ΔE_{295}	ΔE_{290}	ΔE_{288}	(nm)	295 nm	
Hcy-ox	17.5 ± 0.2	38.8 ± 0.5	38.4 ± 0.5	33.5 ± 0.6	24.3 ± 1.0	297	16.5 ± 0.3	11.6
Hcy-apo		36.6 ± 0.5	38.9 ± 0.5	33.3 ± 0.6	26.0 ± 1.0	297	16.7 ± 0.3	11.2
in thiourea		36.4 ± 0.5	37.3 ± 0.5			297	16.0 ± 0.3	11.4
in SCN^-		39.7 ± 0.5	41.7 ± 0.5			295	17.9 ± 0.3	11.4
Hcy-ox in 3 M urea + 0.5 M Gdn·HCl	17.0 ± 0.2	40.0 ± 0.5	41.6 ± 0.5	35.0 ± 0.6	25.7 ± 1.0	295	17.9 ± 0.3	10.9
Hcy-apo in 3 M urea + 0.5 M Gdn·HCl		41.0 ± 0.5	42.5 ± 0.5	33.4 ± 0.6	23.6 ± 1.0	295	18.2 ± 0.2	10.6

cific SH reagents (Lontie, 1958). It was also shown that after removal of copper by cyanide, none or one SH group per four copper atoms is made available for titration (Nakamura and Mason, 1960; Ghiretti-Magaldi and Nuzzolo, 1965).

The participation of imidazole groups in the active site of hemocyanin has been investigated by several authors. From the comparison of the titration curves of native and copper-free hemocyanin of *Helix pomatia,* Lontie (1958) found a difference of one histidine per copper atom in the region where these groups are normally titrated. That histidines could be involved in the chelation of copper was claimed also by Van Holde (1967) by comparing the dichroic spectra of the hemocyanin of *Octopus vulgaris* and *Loligo pealei* with the CD spectra of peptide–copper complexes containing histidine. More recently Wood and Bannister (1968) have reported that photooxidation in the presence of Methylene Blue of *Murex trunculus* hemocyanin causes a rapid loss of the oxygen binding capacity which parallels the destruction of histidine residues.

The results of acid–base titration in KCl at different ionic strengths reported in this paper show that in native hemocyanin from *Octopus vulgaris* more "carboxyl" groups are titrated than in the copper-free protein. Similar results were obtained by Tanford and Epstein (1954) on zinc–insulin and by Breslow and Gurd (1962) on myoglobin in the presence of copper and zinc. According to these authors more acidic groups are titrated when these cations are present. Actually they are basic groups which bind the metal and are titrated when the metal atoms are displaced by hydrogen ions. In hemocyanin two copper atoms are bound per subunit of 50.800 and they are released at pH below 3. Keeping in mind that apohemocyanin from *Octopus* still contains 20–25% of its original copper, the difference of six carboxyl groups found in the acidic region must be extrapolated to eight, *i.e.,* each of the two copper atoms in the functional subunit of 50.800 is bound to four basic groups.

In the neutral region an equal number of histidines is titrated both in native and copper-free hemocyanin which accounts for only 65–70% of the imidazole groups present in the protein. The absence of significant differences above pH 6 can be ascribed to the stabilization of the metal ligands as nonprotonated form in an hydrophobic region of the protein. The finding that these ligands are not titrated even in apohemocyanin indicates that they are so "buried" in the hydrophobic region that no interaction with the solvent can occur in any pH region. That this interpretation is correct is shown by the results of acid–base titration in the presence of urea and guanidine. In these conditions after the removal of copper, 6–7 more groups are titrated in the neutral region which were masked in the native hemocyanin. The participation of ϵ-NH_2 groups has been discarded on the basis of the results of chemical analysis after exhaustive acetylation of the protein followed by the removal of copper (Salvato and Zatta, 1972). The pK_i and the ΔH_{app} ionization value are further proofs that the new groups are the histidine residues which were missing when the titration was carried out in KCl solutions. The lower value (6.2) of the pK_i indicates that the extra histidines found in apohemocyanin are stabilized as nonprotonated forms according to their location in an hydrophobic environment.

Recent studies on the action of these denaturing agents (unpublished) indicate that native and copper-free hemocyanin in 3 M urea and 0.5 M guanidine are structurally identical except for the binding site. The CD spectra show that in these conditions both proteins maintain almost the same per cent of ordered structure. Moreover, the sudden increase of about twofold the original value of the intrinsic viscosity which was observed by Costantino *et al.* (1971) as occurring in 2.5 M urea has been equally observed in copper-free hemocyanin.

The same results have been obtained by titrating native and apohemocyanin in 3 M urea and 0.5 M KSCN, thiourea, or KCl as well as in 3 M urea and 0.5 M guanidine at different temperatures. The constancy of the results in so many different conditions, none of which involving the removal of copper (Rombauts, 1968; Rombauts and Lontie, 1960), can be explained only by assuming that the difference of the imidazole residues found in native and copper-free hemocyanin is due to the presence of the metal in the protein.

As shown by the spectrophotometric titration, oxygen, copper, and other ligands have a strong stabilizing effect on the structure of hemocyanin. The pH of half-ionization of phenolic groups is lower in the partially resolved and copper-free protein. The titration curves shown in Figure 6 are obviously not normal titration curves. In the presence of the metal the titration of all the phenolic groups require conformational changes of the protein. The observation that the calculated ionization curves are not dependent on the wavelength is interpreted as being due to the low content of tryp-

tophan residues in the hemocyanin molecule.

The difference of phenolic groups which is found in different conditions indicates the presence of "buried" tyrosine residues (Donovan, 1964). This seems to be confirmed by the shift of the spectrum into the 295-nm region in the presence of urea and SCN^- solutions. Whether this effect is structural or is due to a few anomalous tyrosine residues only is not known. Work is in progress to solve this particular problem.

Acknowledgments

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