# ON THE ACTIVE SITE OF HEMOCYANIN

B. SALVATO, P. ZATTA,
Anna GHIRETTI-MAGALDI and F. GHIRETTI
*Centre of the Italian National Research Council for the Physiology and Biochemistry of Hemocyanins, Institute of Animal Biology, University of Padua, Italy*

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The results of acid–base titration in KCl solutions of native and copper-free hemocyanin (Hcy) from *Octopus vulgaris* (Cephalopoda) have been interpreted as an indication that each of the two copper atoms in the active site is bound to four non carboxylic groups of the protein [1]. The participation of amino groups can be discarded on the basis of chemical analyses after exhaustive acetylation of the native protein followed by the removal of copper [2].

These experiments, however, give no indication about the nature of the amino acids involved. In fact, in the neutral region an equal number of histidines are titrated in native and copper-free Hcy which accounts for only 65–70% of the total groups present; moreover, in the basic region there are too many groups which cannot be differentiated. The absence of a significant difference between the titration curves of native and copper-free Hcy above pH 6 suggests that the metal ligands are stabilized in a hydrophobic region of the protein even after acid denaturation.

On the basis of the finding that in low concentrations of urea and guanidine Hcy dissociates in subunits with a low molecular weight without losing its ability to combine reversibly with oxygen, new acid–base titration experiments of native and copper-free protein from *Octopus vulgaris* have been carried out from pH 4 to 10 in the presence of 3 M urea and 0.5 M gua-HCl.

In these conditions 14 histidyl groups with a $pK_{int}$ of 6.7 are titrated in native Hcy per functional unit (50,800 daltons) containing two copper atoms (the same occurs in KCl solutions). After removal of copper, besides 14 histidyl groups with the same $pK_{int}$ value, 6.8 more histidines are titrated which have a $pK_{int}$ equal to 6.2. Equal results are obtained in the presence of 3 M urea plus 0.5 M thiourea or thiocyanate. Reminding that 15–20% of the total copper is still present in the apo-Hcy preparations used [3], these results indicate that, over 22 histidyl groups present per functional unit [4], 8 are involved in the active site.

This conclusion is supported by C.D. spectra which show that native and apo-Hcy are structurally identical except for the binding site [5]. In 3 M urea native Hcy maintains the same structure as in KCl solutions; the little differences given by apo-Hcy in the same experimental conditions cannot be ascribed to the exposure of the residual 35% histidines but are better interpreted as due to structural modifications at the level of the binding site following the removal of copper.

That no conformational changes of the whole Hcy molecule occur in the conditions used, is also inferred from other measurements. In the presence of increasing amounts of urea, the intrinsic viscosity of the protein solution remains unchanged up to 2.5 M urea. At this value a sudden increase is observed in the native as well as in the copper-free Hcy [6, 7]. Since a dissociation of the protein would diminish the hydrodynamic volume, this increase can be explained only by assuming a swelling of the molecule which, being the same in native and copper-free Hcy, would produce the same effects in both proteins.

On the basis of these data, it is assumed that in Hcy each copper atom of the active site is bound to four histidines in a strongly hydrophobic environment. As shown by the spectrophotometric titration of native

and apo-Hcy in KCl as well as in thiourea and thiocyanate [8], two tyrosyl groups are involved in the oxygenation reaction. This also seems confirmed by the C.D. spectra in KCl and in 3 M urea solutions [5].

Identical results have been obtained with the Hcy of *Carcinus moenas* and *Limulus polyphemus* (Arthropoda).

## References

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