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# DIFFERENTIAL HYDROGEN ION TITRATIONS OF THE HISTIDINE RESIDUES IN *HELIX POMATIA* HAEMOCYANIN

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The properties of the histidine residues in *Helix pomatia* haemocyanin have been studied by differential hydrogen ion titrations. In oxy- and deoxyhaemocyanin $31 \times 10^{-5}$ histidine residues per g protein are titrated in contrast to $35 \times 10^{-5}$ residues in apohaemocyanin. The difference corresponds to a stoichiometry of one histidine residue per copper atom bound. Even in apohaemocyanin about $6 \times 10^{-5}$ histidine residues per g protein are not titrated in their normal pH region.

In the presence of sufficient calcium to displace the dissociation completely out of the titration region, the titration curve of apohaemocyanin could be linarized according to the model of Linderstrøm–Lang. In oxy- and deoxyhaemocyanin, however, a distinct deviation from linearity was found under the same conditions. In the absence of calcium the effect of the dissociation adds up to this deviation.

The electrostatic interaction factors were determined for the protein at 0.1 M KCl and for the dissociation products: halves and tenths at 1.0 M KCl. The electrostatic interaction factor for the wholes and the halves are much smaller than the values calculated from the Linderstrøm–Lang equation, using the radius of the equivalent sphere either obtained from electron microscopy or from the partial specific volume. This is probably due to solvent penetration. For the tenths at 1.0 M KCl, this effect is small.

## 1. Introduction

The haemocyanin of *Helix pomatia* has a molecular weight of $9 \times 10^6$ [1] between pH 4.5 and 7.3. Outside this pH region a dissociation occurs into halves and tenths [2] and even further into twentieths. The tenths and twentieths exist in a compact and loose conformation [3] depending on the ionic strength. Within the pH-stability region a partial dissociation into halves can be obtained under the influence of alkali halides [4], revealing the presence of two components, the α-haemocyanin that dissociates completely and the β-component that does not. The α-haemocyanin itself seems to show microheterogeneity as the dissociation does not follow the law of mass action and gives an anomalous pattern in the analytical ultracentrifuge [4, 6]. Inside the pH-stability region a dissociation into halves and tenths can be obtained by ethoxyformylation of the histidine residues [7]. Alkaline earth ions extend the stability region to higher pH values [8]. The protein binds 360 copper atoms. The ligands of copper are not known with certainty. Evidence from photooxidation, ethoxyformylation and titration [7–13] points to histidine residues. The protein binds also 180 molecules of oxygen. This binding creates an absorbance at 346 and at 580 nm.

The binding of oxygen shows cooperative saturation curves at pH values higher than 7.5 but only in the presence of calcium [8]. All these different functional aspects of haemocyanin show a strong influence of pH and occur in a region where the histidine residues are titrated.

In the present study differential hydrogen ion titrations were used to accurately determine the number of accessible histidine residues in both haemocyanin and apohaemocyanin.

The titration curves were also analysed according to the model of Linderstrøm–Lang. Although this model is very simplified, it is in general very successful in the linearization of titration curves. The effect of dissociation, which is expected to have a profound influence on the titration, is analysed as well as the effect of calcium which extends the pH-stability region.

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## 2. Materials and methods

Haemocyanin was prepared from the haemolymph of *Helix pomatia* according to Heirwegh et al. [14]. The stock solution was stored at pH 5.7 (0.1 M sodium acetate buffer) under carbon monoxide at 4°C [15]. The protein concentration was measured at 278 nm in 0.1 M sodium borate buffer, pH 9.2, to reduce the influence of turbidity. The specific extinction coefficient $A$ (1%, 1 cm) = 14.16 was used [14].

The α-component was separated by preparative ultracentrifugation in a Spinco model L centrifuge at 4°C (rotor 30, 35 000 revs/min, 120 min) in 1 M NaCl at pH 8.5 (0.1 M sodium acetate buffer).

The apoprotein was prepared at pH 8.5 (0.1 M Tris–HCl buffer, 0.1 M $CaCl_2$, 10 mM $NH_2OH$·HCl) by the dropwise addition of 0.1 M KCN solution in the same buffer, until the blue colour disappeared. The protein concentration was about 10 mg/ml. The solution was then dialysed against the same buffer, 50 mM KCN, and afterwards against 0.1 M sodium acetate buffer, pH 5.7.

Twice distilled water was boiled to remove the dissolved $CO_2$. During cooling $CO_2$-free $N_2$ was passed through the water and it was stored under $N_2$.

The titrants were HCl, standardized against $Na_2CO_3$ by titration with bromocresol green as indicator, and carbonate-free NaOH, both about 0.1 M.

As isoionic haemocyanin is not soluble, the protein solutions were prepared by dialysis against the appropriate salt solutions, made of analytical grade salts.

The titration procedure and the apparatus is described in detail by Janssen et al. [16]. An apparatus analogous to this one was built. It was equipped with a Radiometer pH meter PHM 72. The pH meter was calibrated with Beckman phosphate buffer (pH 7.0) and 50 mM potassium hydrogen phthalate (pH = 4.00).

The titration vessel consisted of an optical cell with a section of 2 cm by 2 cm. It was placed in a thermostated copper holder. A piece of polyvinylchloride containing the electrodes and a glass stirrer was mounted upon it. The vessel was continuously flushed with $N_2$ or $O_2$. The titration vessel was placed on a photometer PL4 (Carl Zeiss, Oberkochen, W. Germany). In this way a continuous control during titration of the aggregation state of the protein was possible by measurement of turbidity. Blank titrations were performed to control the procedure and the electrodes.

## 3. Analysis of titration curves

### 3.1. Numerical differentiation

In differential hydrogen ion titration curves $-\Delta pH/\Delta ZH$, the numerical derivative of the titration curve, is plotted vs ZH, the mean proton charge of the protein. With very small increments in the amount of added acid or base, $-\Delta pH/\Delta ZH$ approaches the reciprocal of the buffer capacity, which is the analytical derivative of the titration curve. This has been shown to be [16]:

$$-\frac{d\,pH}{d\,\overline{ZH}} = \frac{1}{2.3\,\Sigma_i n_i \alpha_i (1-\alpha_i)} + 0.868w\,. \qquad (1)$$

Herein $n_i$ is the number of amino-acid residues in group $i$, $\alpha_i$ the degree of ionization of group $i$, and $w$ the electrostatic interaction factor. This function becomes very large at these values of ZH where every $\alpha_i$ is either 1 or 0, and thus allows the accurate determination of the number of amino acids in one group. In the derivation of eq. (1) the electrostatic interaction factor was assumed to be a constant through the whole titration region. This is not the case when dissociation of the protein occurs during titration. Conformational changes, such as unfolding, can also alter the electrostatic interaction factor. Such a protein is treated as an equilibrium mixture and the titration curve can be described as follows:

$$\overline{ZH} = \sum_j \phi_j\, ZH_j\,. \qquad (2)$$

Herein $ZH_j$ is the mean proton charge of the protein in state $j$, $\phi_j$ the weight-fraction of the protein in that state, and $\overline{ZH}$ the mean proton charge of the mixture. For reasons of simplicity and generality the proton charge is calculated per g protein. The different $\phi_j$ are related by the law of mass action and by conservation of mass:

$$\sum_j \phi_j = 1\,. \qquad (3)$$

The differential titration curve can now be calculated by the following procedure:

$$\frac{d\,\overline{ZH}}{d\,pH} = \sum_j \phi_j \frac{d\,ZH_j}{d\,pH} + \sum_j ZH_j \frac{d\,\phi_j}{d\,pH}\,. \qquad (4)$$

The factor $d\,ZH_j/d\,pH$ corresponds to the reciprocal

of formula (1). The second term of eq. (4) gives the change of $\overline{ZH}$ due to a change in the concentrations of the different states of the protein. The factor $d\phi_j/d\,pH$ can be determined from an independent measurement of the distribution of the protein as a function of pH. This pH dependence and the titration curves are coupled through the linked function concept [17]:

$$-d \log K_j/d\,pH = ZH_j - ZH_{j-1}\,, \qquad (5)$$

where $K_j$ is the equilibrium constant for the transition of state $j-1$ to state $j$. Eq. (4) can only be used for the simulation of the differential titration curve and is too complex for parameter analysis.

### 3.2. Linearization

Titration curves are usually linearized according to the model of Linderstrøm–Lang, as described by Tanford [20]. For state $j$:

$$pH - \log[\alpha_{i,j}/(1-\alpha_{i,j})] = pK_{int,i} + 0.868 w_j ZH_j\,. \qquad (6)$$

Although the total charge of the protein should be used in this equation, in the absence of information concerning the binding of counterions, $ZH_j$ is used instead. In general it is assumed that this binding increases proportionally to ZH and therefore the apparent electrostatic interaction factor will be smaller than the true one.

Multiplying all terms of eq. (6) with $\phi_j$ and summing over all states $j$ gives:

$$pH - \sum_j \phi_j \log[\alpha_{i,j}/(1-\alpha_{i,j})]$$

$$= pK_{int,i} + 0.868 \sum_j \phi_j w_j ZH_j\,. \qquad (7)$$

Here it is assumed that $pK_{int}$ is independent of state $j$. It is clear that the term $\Sigma_j \phi_j \log[\alpha_{i,j}/(1-\alpha_{i,j})]$ is not simply related to the experimentally accessible value of $\alpha_{app.}$. Linearization is thus in general not possible in the transition region. However, when the transconformation occurs in a very narrow pH region, as a consequence of high cooperativity, sufficient data might be obtained before and after the transition so as to determine the parameters of the initial and the end state. If only these states occur $ZH_j$ can be calculated. Together with $\overline{ZH}$ this allows to determine $\phi_j$. If intermediates occur the values of $\phi_j$ have to be determined independently.

## 4. Results and discussion

In fig. 1 the differential hydrogen ion titration curves are shown for oxy- and apohaemocyanin in 0.1 M KCl, 5 mM $CaCl_2$. $\overline{ZH}$ was assumed to be zero at the pH of dialysis (5.6). The titration is limited to the region of pH 5 to 9.3. Outside this region irreversible effects occur. Due to the presence of 5 mM $CaCl_2$ the protein does not dissociate within the titration region. As no free α-amino groups were found in haemocyanin [19] the assumption was made that the residues titrated from pH 6 to 9 were histidine residues. Evidence for this identification comes from the intrinsic pK (7.2), the influence of temperature which allows the calculation of an apparent heat of ionization of 6.5 kcal/mole typical for histidine residues, and from the fact that ethoxyformylation [7] decreases the number of titratable residues. This identification is however not absolute and a number of other groups

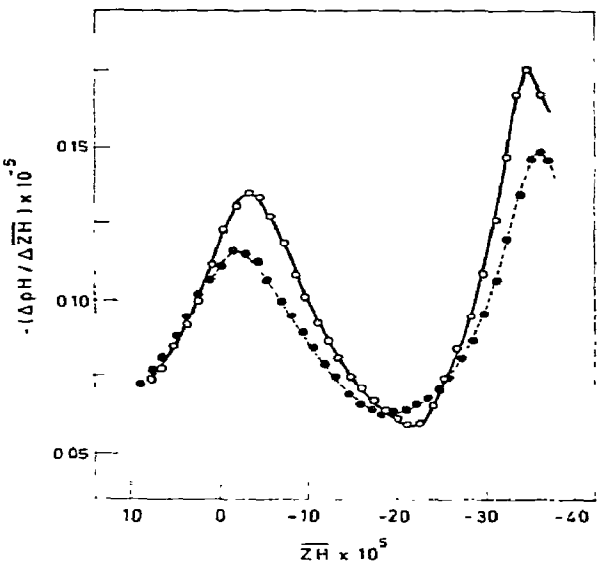


Fig. 1. Differential hydrogen ion titration curves of oxyhaemocyanin (○) and apohaemocyanin (●). Titrations were done in 0.1 M KCl, 5 mM $CaCl_2$ at 20°C under oxygen. Protein concentration was in both cases 10 mg/ml.

with anomalous pK values might be present. From fig. 1 a number of 35 ($\pm$ 0.5) $\times 10^{-5}$ residues per g protein can oe estimated to be titratable in apohaemocyanin and 31 ($\pm$ 0.5) $\times 10^{-5}$ in the holoprotein. From aminoacid analysis [2] 44 $\times 10^{-5}$ histidine residues per g protein are expected. This number is too high due to the neglect of the sugar content. Correcting for this, the number reduces to 41 $\times 10^{-5}$ residues per g protein. This means that even in apohaemocyanin 6 $\times 10^{-5}$ residues per g protein are not titrated in their normal pH region. In the absence of independent information, it is not possible to give an explanation for this inaccessibility. The difference between the holo- and the apoprotein of 4 $\times 10^{-5}$ histidine residues per g protein corresponds to a stoichiometry of one histidine residue per copper atom bound. This result is in excellent agreement with the data obtained for haemocyanin from *Octopus vulgaris* [13] and, as the evidence from other experiments points to the binding of copper to histidine [7,9–13]. A single histidine residue is, however, insufficient to explain the stability constant of $10^{20}$ [20]. The identity of the other ligands is unknown. Tyrosine residues are possible candidates, as 12 $\times 10^{-5}$ residues per g protein are becoming accessible for titration upon the removal of copper [7].

Typical dissociation patterns of haemocyanin are shown by Witters & Lontie [2]. The midpoints for the dissociation of the wholes into the halves and the halves into the tenths are around pH 5.5 and 7.7, respectively. In the presence of calcium these transition points shift to higher pH values and 5 mM is sufficient to displace the dissociation out of the titration region of the histidines (as shown by the turbidity measurements). Under these conditions one would expect the plot of $\{pH - \log[\alpha/(1-\alpha)]\}$ vs $\overline{ZH}$ to be linear, as no change in the aggregation state of the protein occurs. This is approximately the case with apohaemocyanin, as shown in fig. 2A. When oxy- or deoxyhaemocyanin (the latter shown in fig. 2B) is titrated in 0.1 M KCl, 5 mM $CaCl_2$ and the data are plotted in the same way, a distinct deviation from linearity was found, which cannot be attributed to dissociation. This deviation must be due to a shift in apparent pK which does not occur, or to a much smaller extent, in apohaemocyanin. It is interesting to note that in the same pH region and under the same conditions, the binding of oxygen shows cooperatively. The difference in titration behaviour of oxy- and deoxyhaemocyanin is however very small and is most apparent in the differential titration curves (fig. 3). These show that oxyhaemocyanin has its maximal buffer capacity at a lower $\overline{ZH}$ value, and thus higher pH, than deoxyhaemocyanin. This corresponds with the decrease in $P_{50}$ (the oxygen pressure at 50% saturation) or the increase in oxygen affinity with increasing pH in the same region (Vannoppen–Ver Eecke, unpublished results).

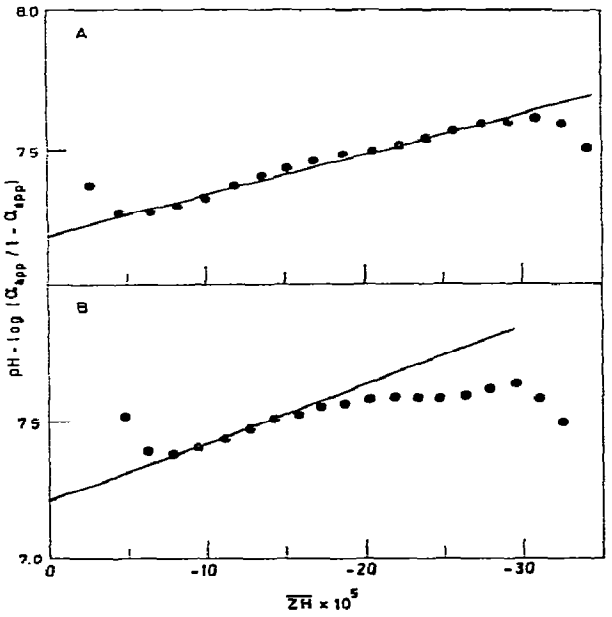


Fig. 2. Linearized titration curves: A. Apohaemocyanin in 0.1 M KCl, 5 mM $CaCl_2$. B. Deoxyhaemocyanin in 0.1 M KCl, 5 mM $CaCl_2$. A distinct deviation from linearity occurs in the region $-17 > \overline{ZH} > -25$.

Fig. 4A shows the linearized titration curve of $\alpha$-haemocyanin at 0.1 M KCl, in the absence of calcium. In the region of dissociation a similar but even larger deviation from linearity is observed. This is probably due both to dissociation and to the pK-shift mentioned above. In principle it is possible to determine the number of protons involved in the dissociation independently with the linked function concept and the pH-dependence of dissociation. In practice this is, however, impossible as the values of $\phi_i$ are independent

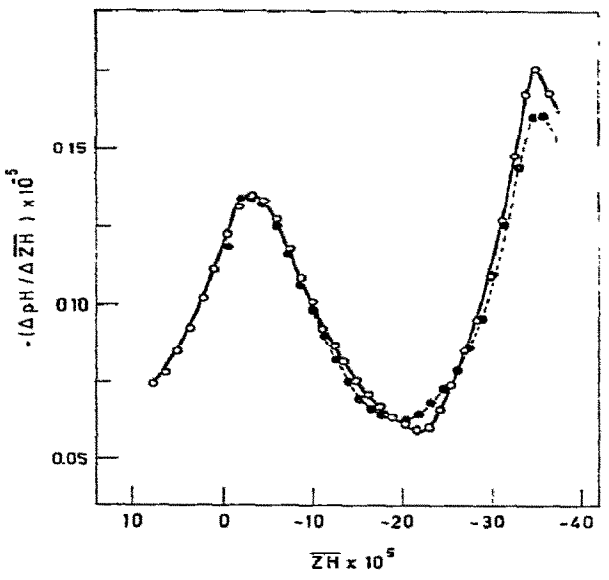


Fig. 3. Differential hydrogen ion titration curves of oxyhaemocyanin (○) and deoxyhaemocyanin (●). Titrations were done in 0.1 M KCl, 5 mM $CaCl_2$ at 20°C under oxygen and nitrogen respectively. The protein concentration was in both cases 10 mg/ml.

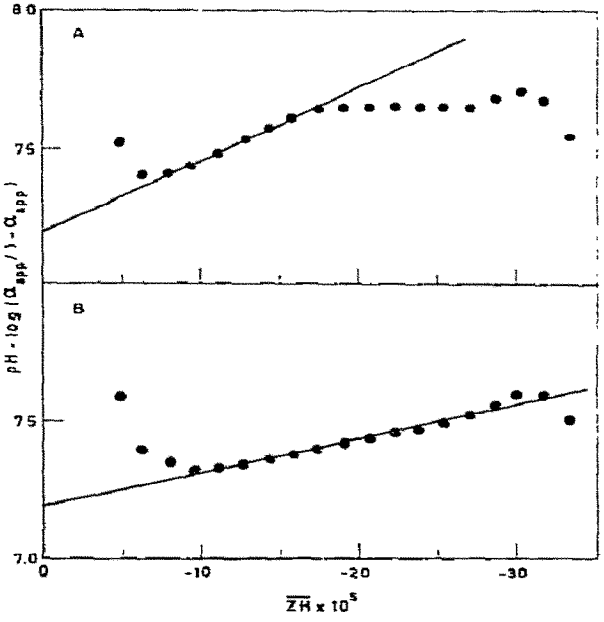


Fig. 4. Linearized titration curves: A. α-heamocyanin in 0.1 M KCl, in the absence of calcium. A deviation from linearity occurs in the region $-16 > \overline{ZH} > -26$, where dissociation into halves and tenths occurs. B. α-haemocyanin in 1.0 M KCl.

on the total protein concentration, in constrast to what is expected from the law of mass action [4,6].

In 1 M KCl α-haemocyanin is present as halves and the dissociation into tenths shows a midpoint at pH 7.4 [4]. The titration curve under these conditions could completely be linearized as shown in fig. 4B. This indicates that no pK shifts are apparent and that the titration curve is not sensitive to the dissociation, probably as a consequence of the very effective shielding by the salts.

The electrostatic interaction factor can be determined from the slope of the plot $\{pH-\log[\alpha/(1-\alpha)]\}$ vs $\overline{ZH}$. For the wholes in the absence of calcium a value of $3 \times 10^{-4}$ can be estimated. This experimentally obtained electrostatic interaction factor can be compared with the theoretical value, which is calculated with the Landstrøm–Lang equation [18]. The volume of the equivalent sphere, is either obtained from the dimensions given by electron microscopy [21] or calculated from the partial specific volume [18,22]. These theoretical values, $9 \times 10^{-4}$ and $17 \times 10^{-4}$ respectively, are much larger. This can be accounted for by solvent penetration of the hollow structure of haemocyanin. For the wholes in the presence of calcium a slightly lower value of $2 \times 10^{-4}$ was found.

At 1 M KCl the value of $\Delta\{pH-\log[\alpha/(1-\alpha)]\}/\Delta\overline{ZH} = 1500$, which corresponds to $w_{halves} = 3.3 \times 10^{-4}$ and $w_{tenths} = 16.6 \times 10^{-4}$. The theoretical values, calculated on the basis of the dimensions obtained by electron microscopy, are 6.6 and $18.6 \times 10^{-4}$ respectively.

The closer agreement between the calculated and experimental electrostatic interacion factors for the tenths is understandable, as no solvent penetration effects are expected under these conditions.

According to Siezen [3] the tenths are at the ionic strength of 1.0 in a C(compact) conformation, in contrast to a L(loose) conformation at 0.1. The electrostatic interaction factor for the tenths in the L conformation cannot exactly be determined. As the subunit dissociation occurs in a very narrow pH region,

a second linear part in the plot $\{pH - \log[\alpha/(1-\alpha)]\}$ vs $\overline{ZH}$ is expected to represent the titration of the tenths. This is the case, as shown in fig. 4A, but the region is too small to determine the electrostatic interaction factor.

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