

## THE REACTION OF AZIDE WITH *LIMULUS POLYPHEMUS* METHAEMOCYANIN

H. R. LIJNEN, R. WITTERS and R. LONTIE

*Laboratorium voor Biochemie, Katholieke Universiteit te Leuven, Dekenstraat 6, B-3000 Leuven, Belgium*

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### 1. Introduction

The oxyhaemocyanin of the arthropod-*Limulus polyphemus* showed no reaction with azide at pH 5.0, in contrast with the oxyhaemocyanin of the mollusc *Helix pomatia*, which yielded methaemocyanin [1]. By the action of stoichiometric amounts of hydrogen peroxide on arthropodan deoxyhaemocyanin, methaemocyanin was readily obtained [2]. The results presented here show that the methaemocyanin of *L. polyphemus* with azide gives a reaction analogous to that of the molluscan methaemocyanin [3].

### 2. Materials and methods

The horseshoe crabs *L. polyphemus* were received alive from the Marine Biological Laboratory (Woods Hole, MA). The haemolymph was collected by cutting a distal segment (tibia) of a leg and was allowed to clot. The solution was filtered over glass wool and centrifuged at 750 X g for 20 min. The haemocyanin was dialysed against 0.2 M NaCl and stored at 4°C under toluene. Samples of haemocyanin were brought to pH 5.0 by dialysis against 0.1 M acetate buffer, pH 5.0, and flushed with pure nitrogen overnight. Deoxyhaemocyanin was treated under nitrogen for 2 h with hydrogen peroxide in a molar ratio to copper of 10. The hydrogen peroxide in excess was removed by dialysis.

Protein concentrations were measured in a Beckman DU spectrophotometer (Munich):  $A_{278}$  (1%, 1 cm) 11.2. The absorption measurements were carried out with a Cary 16 spectrophotometer (Monroia, CA) in cells thermostated at 20°C. Circular-dichroic spectra were recorded in a Cary 61 spectropolarimeter in stoppered

cells of 1 cm pathlength, thermostated at 20°C. The molar circular dichroism  $[\Delta\epsilon]$  ( $M^{-1} cm^{-1}$ ) is expressed per mol copper. EPR spectra were measured with an E-109 spectrometer (Varian, Palo Alto) at 77°K, microwave frequency 9.12 GHz, field modulation amplitude 1 mT, microwave power 30 mW.

### 3. Results

The addition to methaemocyanin of *L. polyphemus* in 0.1 M acetate buffer, pH 5.0, of  $NaN_3$  (Merck, Darmstadt) yielded a green colour which turned to ochre within a few minutes. The latter spectrum showed an  $A_{495}$  max ( $\epsilon = 501 M^{-1} cm^{-1}$  expressed per mol copper).

In circular dichroism the methaemocyanin preparation showed a negative maximum at 339 nm, corresponding to the presence of 13.1% oxyhaemocyanin (fig.1). On addition of  $NaN_3$ , the circular dichroic spectra, recorded after 1 h, showed a negative maximum at 423 nm and a positive maximum at 356 nm, which partially compensated the residual oxygen band at 339 nm (fig.1). The positive maximum at 356 nm appeared clearly under CO, whereby  $O_2$  was expelled as in [4].

The reaction with azide was reversible, on dialysis against 0.1 M acetate buffer, pH 5.0, the absorption band at 495 nm and the circular dichroic bands at 423 nm and at 356 nm vanished.

The molar circular dichroism  $[\Delta\epsilon]$  at 423 nm as a function of the azide concentration allowed the determination of the association constant  $K_a$  according to:

$$\log K_a + n \log [N_3^-] = \log \frac{[\Delta\epsilon]_{\max} - [\Delta\epsilon]}{[\Delta\epsilon]}$$

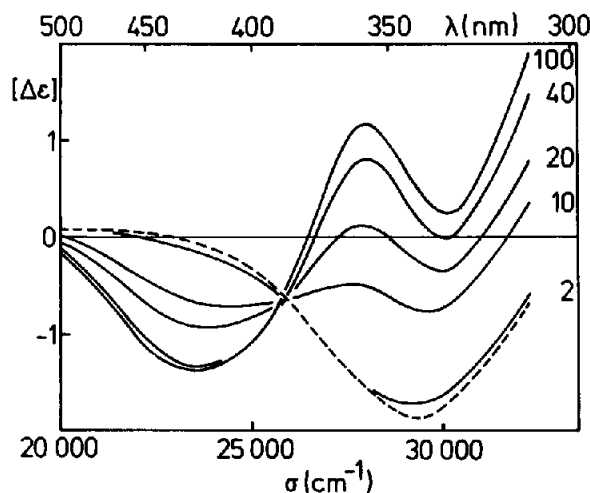


Fig.1. The influence of azide on the circular-dichroic spectrum of *L. polyphemus* methaemocyanin (22 mg/ml) in 0.2 M sodium acetate buffer, pH 5.0. Methaemocyanin blank (---), methaemocyanin in the presence of azide (—), the figures indicate the molar ratio azide to Cu. The data for a molar ratio of 200 were not presented, as they corresponded to those at a ratio of 100.

with  $[\Delta\epsilon]_{\max}$  the molar circular dichroism at the highest azide concentration (molar ratio to copper: 100 and 200) minus the blank value, and  $[\Delta\epsilon]$  the molar circular dichroism at the highest azide concentration minus the value at the considered azide concentration. For  $K_a$  a value of  $6.5 \times 10^{-5} \text{ M}^{-1}$  (correlation coefficient 0.971) was obtained and for  $n$  a value of 1.69. This fractional number might be due to differences in the reaction of the subunits of *L. polyphemus* methaemocyanin with azide.

The subunits were isolated by chromatography on DEAE-Sephadex A-50 with a sodium chloride gradient [5], brought to pH 5.0 by dialysis and treated with hydrogen peroxide as described. The methaemocyanin obtained with fractions I and II gave only a faint reaction with azide, the methaemocyanin of fraction IV treated with azide showed no circular-dichroic bands at 423 nm and 356 nm after 15 min, while the methaemocyanin from fraction III and V gave a pronounced reaction with azide.

As the circular-dichroic bands due to azide were lost on dialysis against 0.05 M borax-HCl buffer, pH 8.2, which contained azide in a ratio to copper of 50, the influence of pH on the binding was investigated.

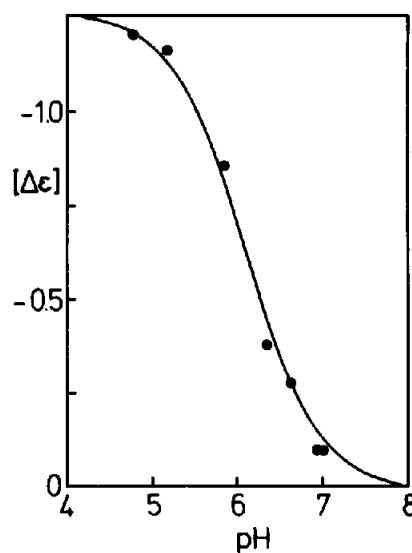


Fig.2. The variation of the molar circular dichroism  $[\Delta\epsilon]$  at 423 nm as a function of pH for *L. polyphemus* methaemocyanin (27 mg/ml) treated with azide in a molar ratio to Cu of 50. The curve was drawn according to the Henderson-Hasselbalch equation with a  $pK_a$  5.9.

The following buffer solutions were prepared: 0.1 M sodium acetate, pH 5.00, 5.07, 5.37 and 5.65; Tris-maleic acid-NaOH, pH 6.15; sodium phosphate, pH 6.81 and 7.22. The circular-dichroic spectra were measured after 15 min (fig.2). Through the experimental data a curve could be drawn according to the equation of Henderson-Hasselbalch with  $pK_a$  5.9.

Only a weak EPR signal of mononuclear copper near  $g$  2, amounting to 1–2% of the copper could be detected in the blank and in the azide-treated methaemocyanin solutions.

#### 4. Discussion

At a pH of 5.0 azide yields methaemocyanin with molluscan oxyhaemocyanin [1], likely by replacing peroxide, similarly to the transformation of oxyhaemerythrin in methaemerythrin [6]. An analogous reaction is shown by oxyhaemoglobin, where superoxide is expelled [7]. The ready displacement of peroxide by azide points to a bridging ligand between the two copper atoms and a lateral

binding of peroxide [8], with the restriction that the oxygen binding ought to be symmetrical [9]. In contrast arthropodan oxyhaemocyanin does not react with azide.

Molluscan methaemocyanin shows a weak signal near  $g$  4, indicating the presence of weakly-coupled Cu(II) pairs [3]. Arthropodan methaemocyanin, on the contrary, yields no EPR signal and is completely diamagnetic between 1.4 K and 200 K, indicating the presence of strongly coupled Cu(II) pairs with possibly direct interaction [10].

The methaemocyanin of *L. polyphemus* binds azide, the observed  $pK_a$  5.9 does not correspond to the dissociation of  $\text{HN}_3$  ( $pK_a$  4.76), so that the binding of azide seems to occur by a ligand exchange which is favoured by the binding of a proton to a protein ligand. The Cu(II) pairs in methaemocyanin remain strongly coupled.

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