THE REACTION OF AZIDE WITH *LIMULUS POLYPHEMUS* METHAEMOCYANIN

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Received 20 February 1978

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 stoicheiometric 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 × *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 (Monrovia, 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 (ϵ = 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:

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

Elsevier/North-Holland Biomedical Press

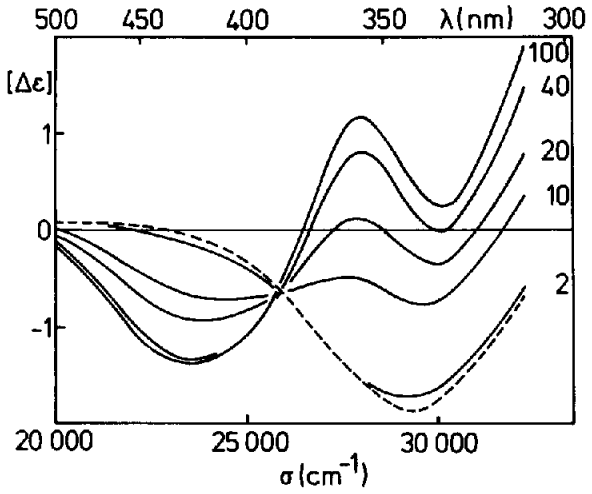

Fig.1. The influence of azide on the circular-dichroic spectrum of *L. polyphemus* methaemocyanin (22 mg/ml) in 0.2 M sodium acetate buffer, pH 5.0. Methaemocyanin blank (- - -), methaemocyanin in the presence of azide (———), the figures indicate the molar ratio azide to Cu. The data for a molar ratio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

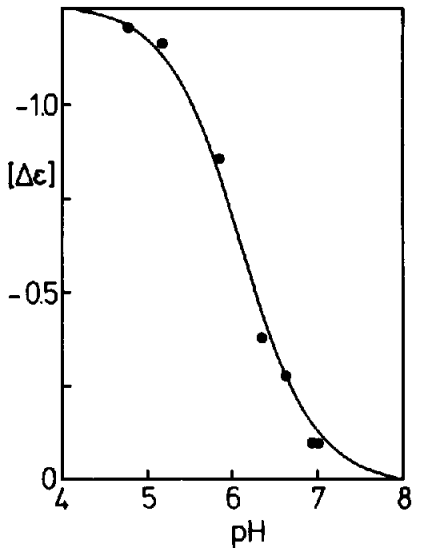

Fig.2. The variation of the molar circular dichroism [Δε] at 423 nm as a function of pH for *L. polyphemus* methaemocyanin (27 mg/ml) treated with azide in a molar ratio to Cu of 50. The curve was drawn according to the Henderson-Hasselbalch equation with a pK_a 5.9.

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 6.5×10^{-5} 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. 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 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.

Acknowledgements

We wish to thank the 'Instituut tot Aanmoediging van het Wetenschappelijk Onderzoek in Nijverheid en Landbouw' for a graduate fellowship (H.R.L.). We wish to express our gratitude to the 'Nationaal Fonds voor Wetenschappelijk Onderzoek, the Fonds voor Collectief Fundamenteel Onderzoek (Contract No 2.0016.76) and the 'Fonds Derde Cyclus, Katholieke Universiteit te Leuven', for research grants.

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