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THE X-RAY MOLECULAR WEIGHT OF HEMERYTHRIN FROM *PHASCOLOSOMA GOULDI* AND SOME NOTES ON THE OXYGENATION REACTION*

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Hemerythrin is a protein which combines reversibly with oxygen and is found in erythrocyte-like cells. Representatives of three phyla possess it. It is present in the nucleated coelomic cells of all the gephyrean annelids examined[1], the priapulids *Halicryptus spinulosus* and *Priapulus caudatus*[2], and the escardine brachiopod *Lingula unguis*[3]. It is also found in the non-nucleated corpuscles of the polychaete annelid *Magelona papillicornis*[4]. The European gephyrean, *Sipunculus nudus* has been used for most of the studies to date. One of the purposes of the present paper is to compare *Sipunculus* hemerythrin with that from *Phascolosoma gouldi*, the common gephyrean of Woods Hole.

Improvements in the determination of dissociation curves by spectrophotometry[5] and in the theory of multiple equilibria[6,7,8] made it of interest to look into some of the properties of hemerythrin. The available data from the early 1930's on *Sipunculus* hemerythrin indicated the ratio Fe/O_2 to be 3/1 (mole/mole)[1], and the molecular

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weight to be 66,500[9] with 4 oxygen-binding sites per molecule[1]. However, there is no *a priori* reason why these values should hold for *Phascolosoma* hemerythrin. Since knowledge of the number of binding sites per molecule is an indispensable prerequisite to the study of binding reactions, the necessary constants for *Phascolosoma* hemerythrin were determined.

The values obtained for both the amount of oxygen and iron per molecule of pigment differ from the earlier ones cited above. The ratio Fe/O_2 was found to be 2.38/1 (mole/mole). The accompanying paper of BOERI AND GHIRETTI-MAGALDI[10] reports 2/1 for hemerythrin from *Sipunculus nudus*. In addition a molecular weight of 120,000 with 19 Fe atoms and 8 O_2 molecules is indicated by the present data. As expected from the work of KUBO[11] and MARRIAN[12], the affinity for oxygen was found to be quite high. However, a full interpretation of the dissociation curves was prevented by the rather large number of binding sites, and primarily by the new and unexpected observation that the fractional change in optical density of *Phascolosoma* hemerythrin solutions is not proportional to the degree of saturation.

EXPERIMENTAL

Materials

1. *Oxyhemerythrin*: Packed "erythrocytes" were prepared[13] and lysed by the addition of 10 volumes of distilled water. The lysate was spun to remove cellular debris and dialysed against cold 20% (by volume) ethanol. The protein crystallized overnight in the refrigerator. The next day the crystals were gently washed in cold 20% ethanol until the supernatant fluid was quite clear, and then they were packed by centrifugation. The sediment was dissolved in 0.5 *M* NaCl buffered to pH 8 with a mixture of boric acid and borax. The NaCl solution was 0.05 *M* in total borate. Three or four volumes of this salt solution were used per volume of crystals, yielding a strong oxyhemerythrin solution which was between 4 and 5 millimolar in bound oxygen. Finally the oxyhemerythrin solution was clarified in a Servall SS-1 angle head centrifuge at full speed for one hour in a cold room. Such a stock solution of oxyhemerythrin was then analyzed for oxygen not more than 36 hours from the time when the worms were killed.

2. *Methemerythrin*: A lysate was prepared as above and was titrated with $K_3Fe(CN)_6$ until no further color change occurred. The resulting methemerythrin was crystallized, as above, by dialysis against cold 20% ethanol. The crystals were washed gently, dissolved in a minimum of 1.0 *M* $(NH_4)_2SO_4$ and the protein recrystallized by dialysis against cold ethanol. The protein was recrystallized four times to free it from unbound Fe. Finally, the last solution of methemerythrin in 1.0 *M* $(NH_4)_2SO_4$ was dialysed against 1.0 *M* NH_4Ac (acetate). After dialysis for two days in the cold with several renewals of the external acetate solution, SO_4^{--} could not be detected with Ba^{++}. After using the Servall SS-1 to remove any turbidity from the contents of the sac, the stock solution was ready for Fe analysis and for the determination of a specific extinction coefficient.

3. *Crystals*: Usually both met- and oxyhemerythrin crystals are long laths, rather thin for X-ray diffraction analysis; however, one batch of methemerythrin crystallized in a more suitable tabular form and these were used for both crystal volume and X-ray measurements.

Methods

1. *X-ray*: The large tabular crystals of methemerythrin were sealed into thin-walled pyrex capillaries in equilibrium with their mother-liquor. X-rays diffracted from them were photographed both with a modified oscillation camera[14] and with the Buerger precession camera[15] using Cu K_α radiation filtered with Ni foil.

2. The *volume of a single crystal* (V_c) was determined with an ocular micrometer in a dissecting microscope (total magnification 18 ×) by measuring the linear dimensions indicated in Fig. 1 and applying the formula:

$$V_c = H/6\,[A(2B + D) + C(B + 2D)]$$

which does not depend on the Miller indices of the faces of the crystal. Fig. 2 is a sketch of the cold stage which was used to minimize the evaporation of ethanol and thus to keep the crystals in equilibrium with mother-liquor.

3. *Specific extinction coefficient at 278 mμ*: An aliquot of the strong stock solution of methemerythrin in 1.0 *M* NH_4Ac was diluted with more 1.0 *M* NH_4Ac and the optical density at 278 mμ

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determined in quartz cuvettes with a Beckman model DU spectrophotometer using 1.0 *M* NH_4Ac as reference. Aliquots of the stock solution were also dried to constant weight at 105°C, as well as samples of the solution against which the final dialysis had been performed. In this way, solutions of the protein, a globulin, could be dried without contamination due to salt, the presence of which is necessary to bring the protein into solution. (NH_4Ac is completely volatile at 105°C.)

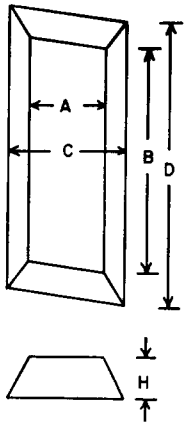

Fig. 1

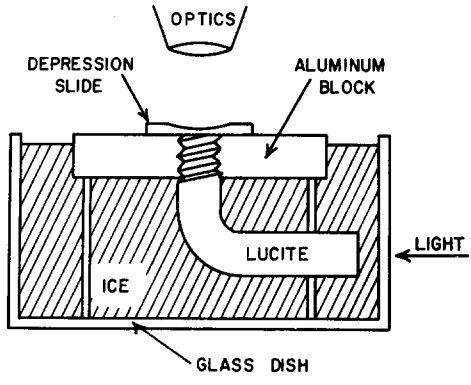

Fig. 2

Fig. 1. The dimensions of the crystals which were measured in order to compute their volumes.

Fig. 2. Cold stage used to minimize the evaporation of ethanol from mother liquor.

4. The *iron content* of suitable dilutions of the stock methemerythrin solution (in 1.0 *M* NH_4Ac was measured by PAUL's[16] modification of the sulfosalicylic acid method of LORBER. The digestion procedure of PAUL[16] was followed, using H_2O_2 in H_2SO_4. The 1.0 *M* NH_4Ac solution remaining outside the dialysis bag at the end of the preparation of the stock solution was also analyzed for iron, however without dilution, to show that all the iron inside the bag was non-diffusible, due to binding to protein. The content of iron was referred to the dry weight of the protein, via the specific extinction coefficient at 278 mμ.

5. The *oxygen content* of the oxyhemerythrin solutions obtained after clarification by centrifugation as above was determined by the microvolumetric method of ROUGHTON AND SCHOLANDER[17]. Three times the recommended volume of unknown solution was used. Otherwise the technique was unchanged, since ferricyanide in excess releases O_2 bound to hemerythrin[1,10]. The optical density of dilutions of these strong oxyhemerythrin solutions was determined at 500 mμ. Since the ratio OD_{500} mμ/OD_{278} mμ was found to be 1/17.6 for oxyhemerythrin, the oxygen content was also put on the same basis as the dry weight and iron measurements, *i.e.*, they were all referred to optical density of the protein at 278 mμ.

6. *Oxygen binding* was measured spectrophotometrically using the technique of ALLEN, GUTHE AND WYMAN[5]. The tonometer held 283.5 cc and a 1 cm cuvette of corex glass was attached to it via a graded glass seal*. To take readings of optical density, the tonometer was placed in the cell compartment of the Beckman model DU spectrophotometer and the former was covered with a black cloth to prevent light leakage. At times a spectrophotometer in the darkroom was used.

To insure that the initial deoxygenation of the pigment in the tonometer was complete, the air in the vessel was replaced repeatedly with H_2 which had been passed over hot platinized asbestos to remove oxygen. Both before and after each aspiration of the gases in the tonometer, it was rotated on a water bath at 0°C for twenty minutes, after which the optical density at 500 mμ was determined. When no further loss of color was seen after such a 30-fold change of the gas pressure inside the tonometer, it was concluded that deoxygenation was complete and only at this point was the injection of air into the tonometer begun. Such elaborate precautions are necessary with reactions in which the affinity for oxygen is as high as it is in this case.

* Made by Mr. JAMES D. GRAHAM, University Glassblowing Service, University of Pennsylvania, Philadelphia 4, Pa.

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RESULTS AND DISCUSSION

X-ray molecular weight

Oxyhemerythrin spontaneously becomes methemerythrin at room temperature in a few days and in a few weeks in the cold. (Deoxyhemerythrin stored under oxygen-free H_2 does not do this.) In the analytic ultracentrifuge both met- and oxyhemerythrin migrate as single peaks at the same rate[18], and therefore undoubtedly have the same molecular weight. Since methemerythrin is the stable form, the X-ray studies and iron analyses below were done on it.

On examination with X-rays the wet tabular crystals of methemerythrin mentioned above proved to be monoclinic with $a = 74.2$ A, $\pm$ 0.2 A; $b = 134.6$ A, $\pm$ 0.3 A; $c = 56.9$ A, $\pm$ 0.4 A and $\beta = 98°24'$, $\pm$ 2'*.

The precession photograph of the hkO net showed OkO to be absent when k was odd (to the 40th order), and photographs of other reciprocal nets revealed no further systematic absences. Since naturally occurring amino acids are usually of the laevo form, space groups possessing the symmetry element of inversion must be ruled out for proteins. Thus $P2_1$ is undoubtedly the space group because it is the only monoclinic space group with the observed absences which does not demand enantiomorphism[19]. The unit cell volume, V_u ($V_u = abc \sin\beta$), is 562,000 A^3, $\pm$ 4,400 A^3, and space group symmetry demands that there be two asymmetric units per unit cell. (See SCARBOROUGH[20] for a discussion of the propagation of errors.)

To determine the molecular weight of the asymmetric unit, measurements of crystal density and hydration are usually made. When the partial specific volumes of the components of the crystal are assumed to be the same in the crystal as in solution, a simple computation can be made of the asymmetric unit weight. (See LOW[21] for a discussion of this method.) The direct method used below has these advantages: No assumptions are made about the partial specific volumes of substances in the crystal (see MACMEEKIN *et al.*[22]). Irrespective of salt content, only the weight of the anhydrous protein per asymmetric unit is measured. The presence of salt or organic solvents in the mother liquor or in the crystals has no effect on the result, and need not be taken into account in the calculations at any point. A disadvantage of the method is that no measurement of the hydration of the protein crystal is obtained.

Single large crystals with well-formed faces and vertices were selected. The linear dimensions in Fig. 1 were measured with the crystals in equilibrium with their mother liquor and the crystal volumes (V_c) were calculated. Each crystal was then dissolved to make a small known volume of solution, V_s (in the present experiments $V_s = 2.08$ cc), and the optical density (OD_{278}) of the solution was then measured at 278 mμ. By use of the specific extinction coefficient (e) already determined by the method above (2.75 $\times$ mg^{-1} $\times$ cm^2, $\pm$ 0.01), the weight of protein alone per unit volume of crystal was calculated (0.71 mg $\times$ mm^{-3}, $\pm$ 0.004). This number, when multiplied by the volume of the unit cell, V_u, is the weight of dry protein per unit cell (3.98 $\times$ 10^{-19} g). This is the weight of protein in two asymmetric units and when it is divided by the number of asymmetric units per unit cell (n) and then multiplied by Avogadro's number (N), the molecular weight of the asymmetric unit is found to be 120,000, $\pm$ 1,000. In short:

$$\text{Mol. wt.} = \frac{V_s \times V_u \times OD_{278} \times N}{n \times V_c \times e}$$

* All errors given are standard errors of the mean.

In view of the value of 66,500 published for the molecular weight of hemerythrin from *Sipunculus nudus*[9] we must ask if the asymmetric unit is not possibly two molecules. Two considerations support the view that it is a single molecule of weight 120,000. First, a calculation of molecular weight can be made from the published values of the sedimentation velocity constant[23] ($6.2 \cdot 10^{-13}$ sec), and diffusion coefficient[24] ($5.8 \cdot 10^{-7}$ $cm^2 \times sec^{-1}$, $\pm 0.21 \cdot 10^{-7}$), provided a value is assumed for the partial specific volume of *Phascolosoma* hemerythrin. If the entirely ordinary value of 0.75 is taken for this as yet unmeasured constant, 105,000 $\pm$ 4,000 is computed (assuming no error in the sedimentation constant), which supports 120,000 rather than 60,000. This disagreement is doubtless due to the poor precision of the diffusion coefficient and a lack of knowledge of the actual partial specific volume of the protein. On the basis of the sedimentation velocity constant alone, one would expect a molecular weight a good deal higher than 60,000[25]. Second, the iron content of methemerythrin was found to be 0.88% by weight, $\pm$ 0.01%, on application of the above methods. Thus there are 19 (18.8, $\pm$ 0.3) iron atoms per asymmetric unit. Analyses of oxygen in oxyhemerythrin was referred to optical density at 278 mμ as above and gave 8 (7.91, $\pm$ 0.04) oxygen molecules per asymmetric unit. The ratio Fe/O_2 is thus 2.38, $\pm$ 0.05. If the asymmetric unit is truly two molecules, 9/4 or 10/4 must be the ratio Fe/O_2. The ratio found, 19/8 is statistically significantly different from these values. However, it must be remembered that two basic assumptions are beneath the statistical theory: (a) the estimates are drawn from a Gaussian distribution and (b) there are no systematic errors. Although systematic errors have been eliminated as far as possible, further work may well show that in spite of the apparent significance of the above results the asymmetric unit in the crystal is actually two molecules.

It might be supposed that the value of the ratio Fe/O_2 in *Phascolosoma* is high because the pigment was not fully saturated with oxygen. This hypothesis was tested by looking for an increase in red color on increasing the oxygen pressure. For this purpose the high pressure apparatus of SCHOLANDER AND VAN DAM[26] was used. It was found that the absorption of green light (oxyhemerythrin band) did not change when the oxygen pressure was changed from 0.2 to 140 atmospheres. Thus the peculiar ratio 19/8 does not represent incomplete saturation with oxygen, and the pigment is fully saturated with oxygen when in equilibrium with room air.

In view of the above results it is important that some other method, such as light scattering, be used to determine the molecular weight of *Phascolosoma* hemerythrin. Measurements ought also to be made of the molecular weights of some of the other hemerythrins. It is of course not at all necessary, *a priori*, that the hemerythrins which are found in different phyla, or even those from the same phylum or even genus, for that matter, should have the same molecular weight. It is of interest in this connection to note the report of BOERI AND GHIRETTI-MAGALDI[10] that the ratio O_2/Fe for *Sipunculus* hemerythrin is 1/2.

Oxygenation reaction

Using the technique of ALLEN *et al.*[5] as did KUBO[11], some preliminary observations have been made on the oxygenation reaction of *Phascolosoma* hemerythrin. The change of optical density of solutions of the pigment as a function of the pressure of oxygen with which they were in equilibrium has been observed at 500 mμ, 650 mμ and 800 mμ. It was noted, in confirmation of KUBO[11] and MARRIAN[12] that the affinity for oxygen

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is high. It was observed, in addition, that the facilitation of oxygen binding by the act of binding (heme–heme interaction in hemoglobin[6]) is itself a function of temperature. Hill's n decreases as temperature increases much more strongly here than in hemoglobin. In an attempt to obtain refined data at low O_2 pressures, and low degree of saturation, solutions of hemerythrin, as strong as possible, were used. On the three separate occasions when such experiments were done it was found that the partial pressure of oxygen inside the tonometer could not be calculated. The deoxyhemerythrin apparently took up more oxygen than in fact had been injected into the vessel. This calculation[5] rests on the assumption that the change in optical density on oxygenation is proportional to the degree of saturation. Since the above three experiments were done with considerable care, the validity of this fundamental assumption was tested by direct gasometric measurement. The results, shown in Fig. 3, indicate that the degree of saturation is not proportional to the change in optical density and deviates in exactly the correct way to account for the appearance of a negative pressure of oxygen inside the tonometer at low saturation, *i.e.*, there in fact existed a lower degree of saturation than that indicated by the change of optical density.

For the experiments illustrated in Fig. 3 various degrees of saturation were produced by injecting different amounts of air into the tonometer, which had been previously completely freed of oxygen. After each equilibration on the water bath, the optical density was measured in order to determine the degree of binding spectrophotometrically. For gasometric determination of saturation, a small volume of the partially saturated hemerythrin solution was withdrawn into a dry syringe which had been repeatedly flushed, just previously, with pure hydrogen. The oxygen content of the sample was measured with the ROUGHTON-SCHOLANDER technique[17]. More air was then injected into the vessel to proceed to higher saturation. In this fashion, the change in optical density was determined simultaneously with the degree of saturation.

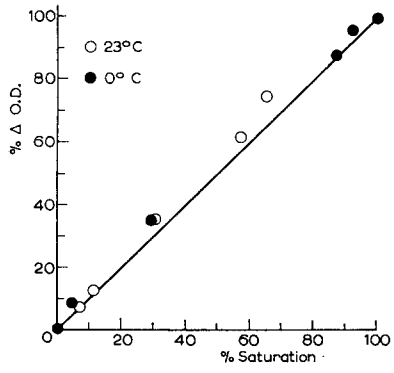

Fig. 3. The per cent change in optical density at 800 mμ as a function of the per cent saturation with oxygen. All experimental points fall above the theoretical strainght line of slope equal to 1. The temperatures at which the two experiments were performed are shown.

Great care was taken to insure complete deoxygenation at the start, as described above. All solutions of partially saturated hemerythrin were handled under mercury.

Unfortunately it is not possible to do this experiment with dilute protein solutions, so it is not known whether these findings are applicable to dilute solution. However, it is quite possible that this behavior occurs at all hemerythrin concentrations. In any case, the effect should be looked for, and if found, taken into account before thermodynamic conclusions are drawn from spectrophotometrically determined dissociation curves of this protein.

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SUMMARY

1. The molecular weight of hemerythrin from *Phascolosoma gouldi* is probably 120,000. There are 8 oxygen molecules and 19 iron atoms in each molecule of this size.

2. The change in optical density at 800 mμ which occurs on oxygenation of the protein is not proportional to the degree of saturation.

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