Ion Binding by Metmyoglobin*

F. Friedberg and L. O. Emiola

ABSTRACT: The binding of K^+, Na^+, Cl^-, and SO_4^{2-} by deionized metmyoglobin from sperm whale skeletal muscle was investigated as a function of pH at 25° with the use of permselective membrane electrodes. Small but definite attachment of K^+ and Na^+ to myoglobin occurs.

A graph of $\bar{\nu}/c$ with respect to $\bar{\nu}$ gives a linear relationship for K^+; the apparent association constant and number of maximal binding sites for this ion deduced from the plot are 20 and 3.1, respectively. Whereas no binding of Cl^- by the native protein can be detected, SO_4^{2-} ions appear to be held even at the isoionic point. For the latter ion, the plot of $\bar{\nu}/c$ with respect to $\bar{\nu}$ does not result in a straight line and the existence of at least two types of sites with different association constants is suggested.

Since the structure of myoglobin is known almost in its entirety, this protein represents a desirable object for physicochemical studies. Here we report data on its affinity to potassium, sodium, chloride, and sulfate ions.

Experimental Procedure

Metmyoglobin (met Mb)[1] of sperm whale skeletal muscle purchased from Gallard-Schlesinger Chemical Manufacturing Corp. was stated to be salt free, 96% pure (Sephadex), and to contain 0.305% Fe. In our laboratory this lyophilized preparation was deionized as follows. Protein (500 mg) dissolved in 25 ml of cold, twice-distilled water was dialyzed against the same type of water in the cold room for 24 hr, then transferred onto a 40-cm column packed with well-washed ion-retardation resin AG 11A8 (Bio-Rad) and eluted at a flow rate of 0.3 ml/min with twice-distilled water.

A calculated amount of the solution of the salt under investigation was added to an aliquot of protein solution to give a concentration within the ionic strength desired. All solutions were prepared by weight. For K^+ binding measurements, pH was adjusted by KOH, for those of Na^+ by NaOH, for those of Cl^- by HCl, and for those of SO_4^{2-} by H_2SO_4 (also by weight). The concentration of protein was determined spectrophotometrically at 540 mμ after addition of solid potassium ferricyanide and sodium cyanide to convert the met Mb into the cyano form; E_{mol}^{540} 10.7×10^3 (Hanania *et al.*, 1966). The metmyoglobin showed a strong peak at 410 mμ and two smaller ones at 505 and 630 mμ.

Binding measurements were made with the aid of permselective membranes according to the method originally described by Saroff and Healy (1959) under conditions given in a previous paper (Floyd and Friedberg, 1966). All experiments were performed at 25° (±1.5°) and pH was measured on a Beckman Model GS. Sodium and potassium chloride served as sources of Na^+, K^+, and Cl^- ions, ammonium sulfate as that of SO_4^{2-} ions.

Results and Discussion

Table I and Figure 1 summarize the data for pH dependence of the binding of K^+ to met Mb. At the isoionic point (pH 7.57) no potassium is held by the protein. (It should be noted that our isoionic point differs slightly from the one reported by Breslow and Gurd (1962), 7.80–7.86.) At pH 9.57 about one and at pH 10.25 approximately two K^+ ions are bound per molecule of protein in the presence of approximately 0.03 M salt. The steep increase in binding capacity which starts at approximately pH 9 may be interpreted as reflecting the change in net protein charge with pH or the titration of side chains to which the cations bind. In myoglobin, little change in net charge occurs between pH 7 and 9. Above pH 9, the net charge is altered rapidly with pH due to ionization of tyrosine and lysine. The pH-dependence curve for the affinity of Na^+ ions to met Mb is similar to that for the affinity of K^+ ions (Table II and Figure 1).

The application of the law of mass action gives the following relationship (Scatchard, 1949) $\bar{\nu}/c = Kn - K\bar{\nu}$, where $\bar{\nu}$ is the moles of ion bound per mole of protein, c is the concentration of free ion, n is the number of binding sites, and K is the apparent association constant. This equation neglects correction for possible electrostatic effects which might be induced by the attachment of the ion to the protein. The value of the apparent maximum number of binding sites (n) (Figure 2, intercept

* From the Department of Biochemistry, Howard University College of Medicine, Washington, D. C. 20001. *Received January 29, 1968.* This work was aided by National Science Foundation Grant No. GB 6056.

The tables for this paper have been deposited as Document No. 9954 with the ADI Auxiliary Publications Project, Photoduplication Service, Library of Congress, Washington, D. C. 20540. A copy may be secured by citing the document number and by remitting $1.25 for photoprints or $1.25 for 35-mm microfilm. Advance payment is required. Make checks or money orders payable to: Chief, Photoduplication Service, Library of Congress.

[1] Abbreviation used that is not given in *Biochemistry 5*, 1445 (1966), is: met Mb, metmyoglobin.

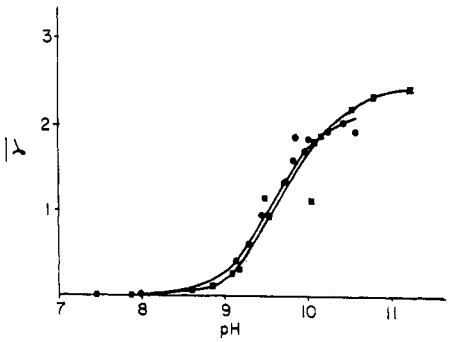

FIGURE 1: pH dependence of binding of Na^+ and K^+ to met Mb; salt concentration about 0.03 M. (●) Na^+ and (■) K^+.

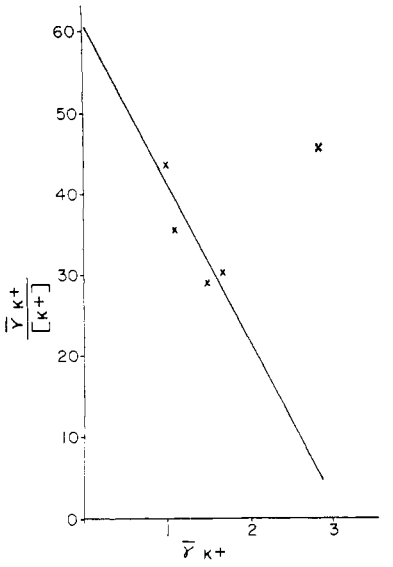

FIGURE 2: Plot of $\bar{\nu}_{K^+}/[K^+]$ with respect to $\bar{\nu}_{K^+}$ for met Mb; pH values are between 9.42 and 9.48.

on $\bar{\nu}$ axis) is 3.1 for the K^+ ions and the apparent association constant is 20 (Figure 2, intercept on $\bar{\nu}/c$ axis divided by n). From the plot of $\bar{\nu}/c$ with respect to $\bar{\nu}$ (Figure 2) one might assume that the binding sites are equivalent and independent. Nevertheless, the data seem inadequate to rule out the presence of one site which is significantly stronger than the other two sites.

Table III shows the experimental data for a study of the pH dependence of Cl^- ion binding by met Mb. Between the isoionic point and pH 4.65 there is no detectable affinity for this cation. Met Mb behaves "native" between approximately pH 4.5–11.5 (Breslow and Gurd, 1962). Breslow and Gurd (1962) have demonstrated that six imidazole groups are masked in native met Mb and and that these groups are released on acid denaturation (around pH 4.5) for the most part, in the basic form. The imidazole could account readily for the binding of approximately one Cl^- ion exhibited at and below the pH. An alternative explanation would be that binding is stronger to the denatured form of myoglobin because of its less compact conformation.

While met Mb in the native state does not bind Cl^- ions appreciably, it shows a stronger affinity for SO_4^{2-} ions. The data given in Table IV and Figure 3 indicate

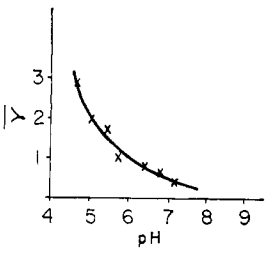

FIGURE 3: pH dependence of binding of SO_4^{2-} to met Mb; salt concentration about 0.014 M.

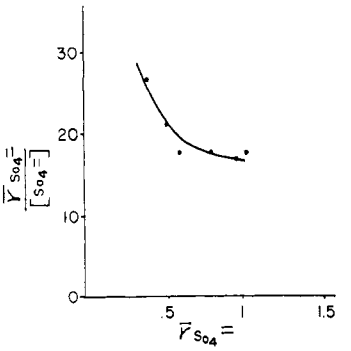

FIGURE 4: Plot of $\bar{\nu}_{SO_4^{2-}}/[SO_4^{2-}]$ with respect to $\bar{\nu}_{SO_4^{2-}}$ for met Mb; pH values are between 7.07 and 7.20.

that even at the isoionic point some bivalent anion attaches to the protein. At pH 5, in the presence of approximately 0.014 M $(NH_4)_2SO_4$, two ions per met Mb molecule are held and at pH 7, in the presence of approximately 0.05 M $(NH_4)_2SO_4$, one SO_4^{2-} ion per met Mb molecule is ligated. According to Kendrew (1964), when met Mb is crystallized from 4 M ammonium sulfate solution, 20–30 sulfate ions are present/protein molecule. Only two ions, however, are visible by X-ray. One has a definite tetrahedral shape,[2] the other sulfate ion is linked to one of the nitrogen (more electropositive) atoms of the distal histidine. The remaining sulfate ions, together with the water in which they are dissolved, are presumably moving randomly throughout the intermolecular space of the crystal. The difference in affinity of met Mb toward chloride and sulfate probably arises because sulfate, a divalent ion, has a higher electrostatic attraction and should be more strongly bound to the protein than the univalent chloride ion.

Figure 4 presents a plot of $\bar{\nu}/c$ with respect to $\bar{\nu}$ for SO_4^{2-} binding by met Mb. This relationship is not linear. Since assuming one kind of sites only and correcting for electrostatic effects by the exponential equation (Tanford, 1961), $\bar{\nu}e^{2wz\bar{z}}/a = K_{int}(n - \bar{\nu})$[3] does not annul this deviation, the loci of combination cannot belong to a single class with identical binding constants.

[2] It is bound interstitially between two protein molecules.

[3] In this equation a is the activity of the ion, z, its valence, $\bar{z}$, the average net charge per protein molecule, and K_{int}, the intrinsic association constant. The parameter, w, is calculated for each ionic strength from the relation $2w = 0.3746 - (2.343\sqrt{\mu}/1 + 7.076\sqrt{\mu})$. Myoglobin is assumed to be a sphere with a radius of 19.06 Å.

References

Breslow, E., and Gurd, F. R. N. (1962), *J. Biol. Chem. 237*, 371.

Floyd, B. F., and Friedberg, F. (1966), *J. Biol. Chem. 241*, 5533.

Hanania, G. I. H., Yeghiayan, A., and Cameron, B. F. (1966), *Biochem. J. 98*, 189.

Kendrew, J. (1964), New Prospective in Biology, Sela, M., Ed., New York, N. Y., Elsevier, p 23.

Saroff, H. A., and Healy, J. W. (1959), *J. Phys. Chem. 63*, 1178.

Scatchard, G. (1949), *Ann. N. Y. Acad. Sci. 51*, 550.

Tanford, C. (1961), Physical Chemistry of Macromolecules, New York, N. Y., Wiley, p 544.

The Structure of an Iron Core Analog of Ferritin*

G. W. Brady, C. R. Kurkjian, E. F. X. Lyden, M. B. Robin, P. Saltman, T. Spiro, and A. Terzis

ABSTRACT: The structure of a synthetic ferritin-like polymer of approximate composition $Fe_4O_3(OH)_4(NO_3)_2 \cdot 1.5H_2O$ has been investigated using X-ray scattering from aqueous solution, infrared and Mössbauer spectroscopy, and magnetic susceptibility measurements. The ferric ions are all tetrahedrally coordinated by O^{2-}, OH^-, and H_2O in a corner-sharing bridged structure, with Fe–O–Fe bond angles of 125 ± 30°. The nitrate ions are not coordinated directly to the iron, but act only as counterions to the positively charged polymer spheres. As with natural ferritin, the Mössbauer spectrum of the synthetic iron polymer has a paramagnetic doublet spectrum above 77°K and a magnetic hyperfine-split spectrum at 5°K, while in the range 40–70°K, both spectra appear simultaneously, as expected for superparamagnetic particles. The magnetic susceptibility demonstrates that the 70-Å spheres of the synthetic polymer are antiferrmagnetic with a Néel point above 300°K, and a blocking temperature of 8°K.

Ferritin is an iron-carrying protein known to consist of an inner micelle of hydrated ferric oxide–phosphate, having a maximum diameter of about 75 Å which is surrounded by an outer proteinaceous sheath (apoferritin) having an inner diameter of 75 Å and an outer diameter of approximately 120 Å (Harrison, 1964; Bielig *et al.*, 1966). Though the apoferritin shell may be filled with varying amounts of the iron hydroxide–phosphate, at maximum capacity, the core amounts to about 2000 iron atoms (Fischbach and Anderegg, 1965). The apoferritin is thought to consist of about 20 identical peptide chain subunits, and the iron core has a roughly spherical shape, but with many irregularities apparent in the electron micrographs (Haggis, 1965).

As part of an extensive study of the metabolism of iron in the human body, Saltman and coworkers succeeded in synthesizing a polymeric compound, $[Fe_4O_3(OH)_4(NO_3)_2]_n$, which appears to be a remarkable analog to the iron-containing core of ferritin (Spiro *et al.*, 1966). Its diameter of 70 Å is the same, and when the corresponding ferric citrate micelle is placed in a solution of noncrystalline apoferritin, the synthetic core is surrounded by the protein subunits to give a substance whose gross morphology is very similar to that of ferritin (Pape *et al.*, 1968). The micelles of the ferric citrate polymer have a diameter of 75 Å.

In this paper, we present a study of the structural and magnetic properties of the synthetic iron core polymer, and compare them briefly with the properties of the ferritin micelle. Since ferritin is so important in biological iron chemistry, and since little information is available on the structural properties of its iron core, it was hoped that a study of the synthetic compound might shed light on the structure of the natural material. Moreover, the synthetic iron core polymer is an unusual chemical species, and is of great interest in its own right. Our data includes the radial distribution function for the polymer micelles suspended in water, as determined from X-ray scattering, static magnetic susceptibility measurements on the powder from room temperature to 1.4°K, Mössbauer spectra in the range 5–298°K, and vibrational and electronic spectra.

Experimental Section

The synthetic iron core polymer was prepared by the addition of aqueous $KHCO_3$ to an aqueous $Fe(NO_3)_3$ solution, followed by gel filtration (Spiro *et al.*, 1966). In addition to standard analyses for iron, hydroxide

* From Bell Telephone Laboratories, Inc., Murray Hill, New Jersey 07971 (G. W. B., C. R. K., E. F. X. L., and M. B. R.), the Department of Biology, University of California, San Diego, La Jolla, California 92037 (P. S.), and the Department of Chemistry, Princeton University, Princeton, New Jersey 08540 (T. S. and A. T.). *Received January 29, 1968.* This research was supported in part by grants from the U. S. Public Health Service to P. S. (AM05484) and to Y. S. (GM13498).