Structural Basis for the Anticoagulant Activity of Heparin. 2. Relationship of Anticoagulant Activity to the Thermodynamics and Fluorescence Fading Kinetics of Acridine Orange–Heparin Complexes†

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ABSTRACT: Complexing heparin or dermatan sulfate with the fluorescent probe acridine orange provides a means of studying electrostatic as well as static and dynamic conformational aspects of these glycosaminoglycans via the thermodynamic and photochemical (fluorescence fading) properties of these complexes. The cooperative binding constants (K_q), fluorescence fading rate parameters (r''), and anticoagulant activities of heparins fractionated according to anionic density all showed qualitatively the same dependence upon anionic density. When K_q and r'' were plotted against anticoagulant activity, empirical relationships were observed. Interestingly, the corresponding values for unfractionated dermatan sulfate fell on the lines defined by the heparin fractions. Temperature-dependence studies demonstrated that differences in fading rate observed for heparins of different anionic densities are entropic in origin and reflect differences in the ability to assume a special configuration. Differences in activation entropy for fluorescence fading can be empirically correlated with anticoagulant activity. The latter correlation suggests a physical similarity in the roles played by anionic density in both fluorescence fading and anticoagulant activity.

One of the main structural differences between heparins, which generally show a high anticoagulant activity, and other glycosaminoglycans, which do not, is the high anionic charge of the heparin. Thus, it was suggested that the highly anionic nature of heparin was somehow important to its anticoagulant effect (Jorpes & Bergstrom, 1937). In the previous paper (Hurst et al., 1979) we clearly demonstrated that anticoagulant activity varies with the anionic density of the heparin; however, the data did not lend themselves to ready interpretation in physical terms.

Several investigators have developed models to account for the anticoagulant activity in terms of charge properties [e.g., Davis (1975) and Stivala (1977)]. These models have usually sought to develop an explanation in terms of an effect of charge upon the static, or equilibrium, properties of the heparin. Yet recent work with specific blood proteins (e.g., antithrombin) has been interpreted in terms of a conformational change in the protein which is induced by the heparin (Rosenberg & Damus, 1973; Machovich et al. 1975; Feinman & Li, 1977; Sturzebecher & Markwardt, 1977; Machovich & Aranyi, 1978). If such conformational changes in the protein are indeed involved, then dynamic conformational factors of the heparin may also be involved.

The work herein reported was undertaken to determine how some static and dynamic physical properties of heparin and dermatan sulfate vary with the anionic density and whether these physical properties can be correlated with anticoagulant activity. Two properties of AO–GAG[1] complexes have previously been demonstrated to depend on either electrostatic or dynamic factors. Firstly, among the various GAGs, differences in the value of the cooperative binding constant, K_q, primarily reflect differences in the electrostatic properties of the polymer (Menter et al., 1977, 1979a). Secondly, the photochemical fading of AO–GAG fluorescence under continuous irradiation with exciting light involves a second-order photooxidation of AO in which two adjacently bound AO molecules react in the rate-limiting step (Menter et al., 1978, 1979b). Because of the involvement of two dye molecules in the slow step, the rate of fluorescence fading (r'') depends critically on the geometry and conformation of the binding sites. Measurements of cooperative binding constants and fluorescence fading rate constants yield different information. The first yields information concerning the electrostatic equilibrium aspects, whereas fluorescence fading yields information on the dynamic conformational aspects.

Recently, heparin has been separated into fractions of essentially constant molecular weight but which vary systematically in the number of charged groups (Hurst et al., 1979). The isolation of these fractions has made it possible to systematically investigate the behavior of r'' and K_q of the AO complexes of these fractions in relation to anionic density and to compare the results with the anticoagulant activities of each fraction. The variations in these physical properties which occur with changes in anionic density can be ascribed to electrostatic and/or dynamic conformational aspects. In this manner, it is possible to begin to understand the physical mechanisms by which charge affects the dynamics of the heparin polyanion.

Materials and Methods

The heparin samples, methods of analysis, and fractionation procedures have been described in the previous paper (Hurst

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[1] Abbreviations used: AO, acridine orange; DS, dermatan sulfate; GAG, glycosaminoglycan; HA, high affinity; LA, low affinity; NA, no affinity.

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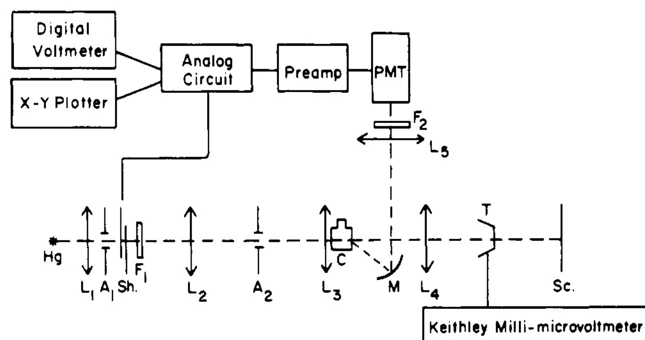

FIGURE 1: Block diagram of fluorescence fading fluorometer. Symbols are explained in the text.

et al., 1979). As before, the fractionated samples are referred to by the molarity of NaCl at which they were extracted, and the high, low, and very low antithrombin affinity samples are referred to as HA, LA, and NA, respectively. Hog mucosal dermatan sulfate (DS) was a generous gift of Dr. Lennart Rodên. Its anticoagulant activity was assumed to be 57 units/mg, as determined on a similar sample by Stivala (1977). Purification of acridine orange and preparation of AO complexes have been previously described (West et al., 1977). Binding constants were evaluated at 25 °C at a total dye concentration, $[AO]_0$, of 5 μM, as described by Menter et al. (1977).

Photolysis of AO complexes was carried out in thermostated 1-cm cuvettes on an instrument constructed in our laboratories (see Figure 1). The optics are similar to those found in Koehler illumination and are designed to provide a uniform field of illumination in the plane of the cuvette. The 546-nm Hg line is isolated from a 100-W Hg lamp (Illumination Industries, Sunnyvale, CA) by means of a three-cavity interference filter (Ditric Optics, Marlborough, MA). The arc is imaged in the aperture plane A_2 by lenses L_1 and L_2, collimated by lens L_3, and passed through sample cuvette C. Aperature A_1, which controls the field size, is imaged in the plane of the cuvette by L_2 and L_3. Lens L_4 reimages the arc entirely within the light-sensitive area of a removable Eppley Thermopile, T, which provides for measurement of the excitation fluence rate. The fluorescence emitted by the sample (λ_{max} = 660 nm) is collected off-axis by concave mirror M. The mirror and lens L_5 serve to focus the fluorescence on an EMI 9558 QA photomultiplier tube (PMT) after it passes through a Leitz 580-nm barrier filter–Oriel 660-nm interference filter combination (F_2). The fluorescence intensity I_f can be read either directly (in volts) or as $1/I_f$ on the digital voltmeter. The X–Y recorder (Houston Instruments, Model 2000) is triggered simultaneously with the opening of shutter Sh, and $1/I_f$ is plotted as a function of time.

Evaluations of the fading parameter r'' (Menter et al., 1978) were carried out at $[AO]_0$ = 8 μM for the heparin samples and at 10 μM for the dermatan sulfate sample. The polymer-binding site to dye ratio was 6.6 in all cases. These concentrations ensured that the photochemical fading, and not AO–AMPS complex formation, was rate determining [see Menter et al. (1978)]. Thus, the fading constant, r'', is independent of AO binding. Excitation at 546 nm provided optically dilute conditions of irradiation using 1.0-cm path length. This choice of excitation wavelength resulted in r'' values which were lower by a factor of 14 than those previously reported for excitation at 436 nm, 0.025-cm path length (Menter et al., 1978). Equation 4 in Menter et al. (1978) predicts that r'' should vary directly with the molar extinction coefficient at the excitation wavelength, and the factor of 14 is in agreement with the 436 nm/546 nm ratio of complex extinction coefficients.

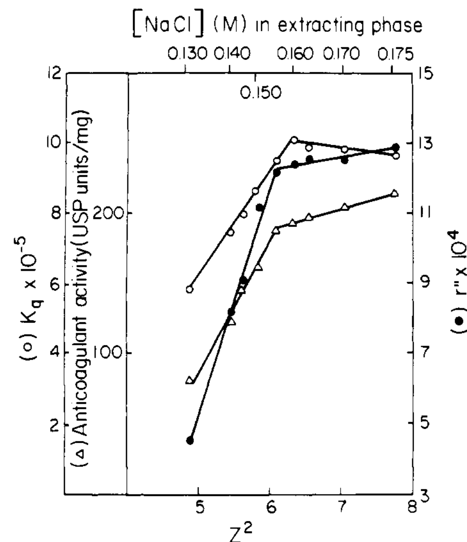

FIGURE 2: Cooperative binding constant K_q (O), fluorescence fading rate parameter r'' (●), and anticoagulant activity (Δ) (USP units/mg) as functions of Z^2 (where Z is the anionic density as defined in the text) for acridine orange–heparin complexes at 25 °C. Other experimental conditions are as described in the text.

The kinetics of fluorescence fading are empirically described by the following equation (Menter et al., 1978):

$$(1/E_0)[I_f(0)/I_f(t)] = r''t + 1/E_0 \quad (1)$$

where $I_f(t)$ and I_f are the fluorescence intensities at photolysis times t and zero, respectively, E_0 is the fluence rate of the exciting light, and r'' is a second-order parameter which is directly related to a molecular rate constant for fading (Menter et al., 1978).

Since the fading parameter r'' is proportional to a molecular rate constant, the temperature dependence of r'' can be interpreted in terms of transition-state theory:

$$r'' = c(kT/h)e^{\Delta S^{\ddagger}/R}e^{-\Delta H^{\ddagger}/RT} \quad (2)$$

where R, k, T, and h have their usual significance and $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ are the respective enthalpy and entropy of activation for the fading reaction. Since the numerical value of the constant c in eq 2 is not known, one cannot arrive at absolute preexponential factors from ln r'' vs. $1/T$ (Arrhenius) plots; i.e., we cannot determine the absolute magnitude of $\Delta S^{\ddagger}$. However, taking *ratios* of r'' with respect to an arbitrarily chosen "reference sample" automatically results in elimination of the unknown constant and makes it possible to express our results in terms of differences in activation entropies, $\Delta\Delta S_x^{\ddagger} \equiv \Delta S_x^{\ddagger} - \Delta S_{ref}^{\ddagger}$, which are readily interpretable in an absolute physical sense. We have arbitrarily chosen the 0.150 M sample as the reference sample and have calculated $\Delta\Delta S_x^{\ddagger}$ for the other samples from the Arrhenius plots and from appropriate manipulation of eq 2.

Results

Heparin Anionic Density Fractions and Dermatan Sulfate. In Figure 2, binding constant K_q, fading constant r'', and anticoagulant activity of each sample are plotted as a function of Z^2, where Z is the chemically measured number of anionic groups per uronate moiety.

The anticoagulant data (with the exception of the DS sample) have been presented in the previous paper; the data are included here for the sake of comparison. Each plot is seen to represent a complex function which we have chosen to

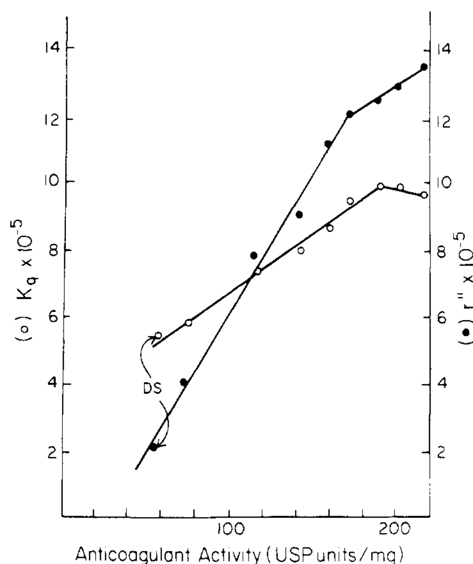

FIGURE 3: K_q (○) and r'' (●) as functions of anticoagulant activity (USP units/mg) for acridine orange–heparin and acridine orange–dermatan sulfate complexes at 25 °C. Other experimental conditions are as described in the text.

represent as two straight lines which intersect at $Z^2 \approx 6$ [see Hurst et al. (1979)]. All three quantities appear to exhibit a qualitatively similar sensitivity to anionic density, which suggests a possible similarity in the physical mechanism by which anionic density affects AO binding, fluorescence fading, and anticoagulant activity. If this is so, then both r'' and K_q will show a definite empirical relationship to anticoagulant activity. Such a relationship is shown in Figure 3. As in Figure 2, both plots of r'' and K_q vs. anticoagulant activity are represented by two intersecting straight lines. Interestingly, the results for DS lie on these lines, even though DS is not a "heparin-like" polymer.

The existence of the relationships shown in Figure 2 suggests that the study of the phenomena associated with the physical parameters K_q and r'' is capable of providing insight into the physicochemical forces by which the statics and dynamics of the heparin polyion are related to anionic density. With this in mind, we investigated the temperature dependence of r'', which provides qualitative and quantitative information on the reaction pathway involved in fading. Arrhenius plots of ln r'' vs. $1/T$ yielded straight lines in all cases. These lines were vertically displaced with respect to each other, but their slopes were identical with each other within the experimental uncertainty of ±10%. The average slope corresponds to an apparent activation energy (E_a) of 12 ± 1 kcal mol^{-1}, and since $\Delta H^{\ddagger} = E_a - RT$, one obtains $\Delta H^{\ddagger} = 11$ kcal mol^{-1}. From these results, it is evident that $\Delta\Delta H_x^{\ddagger} = 0$ and that the observed differences in r'' therefore are due only to preexponential (i.e., entropic) factors. This finding, in conjunction with eq 2 and Figure 3, suggests that a relationship exists between $\Delta\Delta S_x^{\ddagger}$ and the logarithm of anticoagulant activity. That this is so is shown in Figure 4. As expected, the plot is similar in shape to that of Figure 3. The salient points of Figure 4 are that (a) the magnitude of anticoagulant activity of this heparin can be related to entropy changes in the heparin, (b) the anticoagulant effect of dermatan sulfate is also described to the extent that dermatan sulfate follows the trend set by the heparin fractions, and (c) a small change of 4 eu in entropy results in large differences in anticoagulant activity, namely, 180 USP units.

Affinity-Fractionated Heparins. Previous work has shown that heparin binding to antithrombin is necessary, but not sufficient, for high anticoagulant activity (Laurent et al., 1978). Moreover, as was shown in the previous paper, the ability to bind antithrombin was shown to be at least approximately independent of anionic density (Hurst et al., 1979). Thus, it was of interest to determine values of K_q and r'' on the HA, LA, and NA samples. The results are listed in Table I. The HA heparin has at least 10 times the anticoagulant activity of the LA and NA heparins, yet the differences in K_q and r'' are minimal in comparison to the differences shown in Figure 3. Therefore, the presence or absence of the dodecasaccharide responsible for antithrombin binding (Hopwood et al., 1976) does not appear to markedly affect the overall electrostatic and conformational properties of the heparin polyanion.

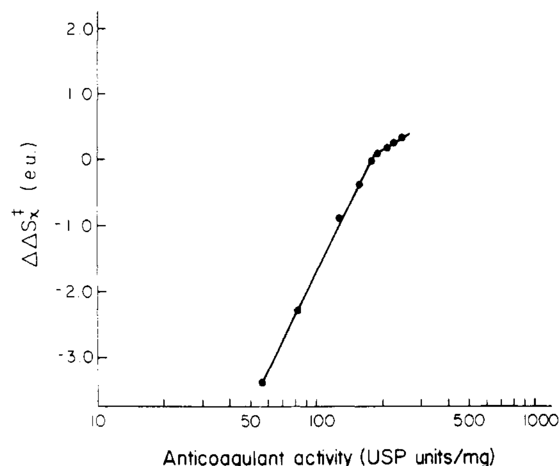

FIGURE 4: Relationship between $\Delta\Delta S_x^{\ddagger}$ (in eu) and log anticoagulant activity for acridine orange–heparin and acridine orange–dermatan sulfate complexes. $\Delta\Delta S_x^{\ddagger}$ is the difference between the activation entropy of a given sample and that of the 0.150 M fraction as defined in the text.

Table I: Biophysical Properties of Affinity-Fractionated Heparins

heparin	K_q ($\times 10^{-5}$) (M^{-1})	r'' ($\times 10^5$) (m^2 J^{-1})
HA	9.3	12.5
LA	8.7	11.0
NA	7.9	10.8

Discussion

As was shown in the previous paper (Hurst et al., 1979), the anticoagulant activity is a complex function of anionic density. In this paper we have explored the dependence upon anionic density of K_q and r'', which are related to the electrostatic and dynamic conformational aspects of the heparin. The observation that the anticoagulant activity, K_q, and r'' all show qualitatively the same kind of dependence upon anionic density (Figure 2) suggests that some similarities may exist in the physical mechanisms by which each of these three properties are affected by the charge of the heparin. These studies have aided in describing some aspects of the electrostatic and conformational properties of heparins and how these are related to the charge of the heparin. By better understanding the nature of these factors, it may be that clues may arise concerning the physical origins of the dependence of anticoagulant activity upon anionic density.

The cooperative binding constant, K_q, of AO–heparin reflects both dye–polymer and dye–dye interactions (Menter et al., 1979). The dye–polymer interactions are electrostatic in origin, but the dye–dye interactions are not. In this case, one might expect a complex relationship between K_q and Z^2, which is similar to that actually observed in Figure 2. It is most likely

that these dye–dye interactions, which reflect van der Waals stabilization and steric and conformational factors, are responsible for the nonlinear behavior of K_q vs. Z^2. Unfortunately, these data do not permit these factors to be separated from each other.

Quite different information is obtained from the studies of fluorescence fading kinetics. The value of r'' reflects the dynamic conformational properties of the heparin chain. At AO concentrations used for these experiments the values of r'' are independent of the binding constant (Menter et al., 1978). Thus, it does not necessarily follow that the r'' vs. Z^2 plot and the K_q vs. Z^2 plot should have similar shapes.

The photochemical fading of AO–heparin fluorescence involves a second-order photooxidation whereby two molecules of AO adjacently bound to the heparin framework react in the rate-determining step (Menter et al., 1978, 1979b). The role of the heparin, or other GAGs, appears to be mainly to bring the AO molecules together for the photooxidation reaction without itself undergoing detectable chemical alteration in a manner similar to a heterogeneous catalyst. The similarity of the activation energies for all heparin fractions as well as for the dermatan sulfate sample shows that the basic chemical mechanism of fading is the same for different GAGs but that the large differences in the rate of fading for different AO–GAG complexes arise from entropic factors.

The activation entropy is a measure of the dynamic conformational properties of the polymer; it reflects the conformational change that the polymer must undergo to accommodate a transition state between two reacting dye molecules. In comparing differences in activation entropy, more positive values of $\Delta\Delta S_x^\ddagger$ imply a greater driving force toward achieving a transition state, which in turn implies that the transition state is easier to reach. The increase in $\Delta\Delta S_x^\ddagger$ with anionic density (Figures 2 and 4) therefore probably reflects a less drastic rearrangement of the polymer in cases where adjacent dye molecules are closer to each other to begin with.

The correlation between anticoagulant activity and $\Delta\Delta S_x^\ddagger$ (Figure 4) is simply an empirical relation. Nevertheless, there is a physical basis for proposing that such a correlation might reflect a basic similarity in the role that heparin, or at least the physical manifestation of its charge, plays in both fluorescence fading and anticoagulant behavior. Laurent et al. (1978), in an expansion of an idea proposed by Markwardt & Walsmann (1959), have proposed that heparin promotes the interaction between thrombin and antithrombin which are adjacently bound to the same heparin chain in a manner similar to that of a heterogeneous catalyst. The physical and chemical role of heparin in such a process would probably be very similar to its role in promoting the second-order photooxidation of adjacent bound AO. If the ideas of Laurent et al. are correct, one would *expect* a correlation between $\Delta\Delta S_x^\ddagger$ and anticoagulant activity. Conversely, the observation of such a correlation provides evidence in favor of the above mechanism.

Following the same line of reasoning, the correlation between anticoagulant activity and $\Delta\Delta S_x^\ddagger$ suggests that the dynamic conformational properties of the heparin (or dermatan sulfate) polymer play a major role in determining the magnitude of anticoagulant activity. Previous authors (Rosenberg & Damus, 1973; Machovich et al., 1975; Feinman & Li, 1977; Struzebecher & Markwardt, 1977) have indicated that heparin induces conformational changes either in thrombin, in other proteins in the coagulation cascade, or in antithrombin prior to the interaction between antithrombin and the other protein. Our studies suggest that the ability of heparin to induce such changes may be linked to its own ability to assume a special configuration. These results are in general agreement with those of Machovich & Aranyi (1978), who reported that heparin increases the activation entropy of the antithrombin–thrombin reaction. Although Teien et al. (1976) have observed that antithrombin interactions may not play a major role in the case of DS, the result that it follows the trend set by the heparins (Figures 2, 3, and 5) suggests that DS also acts in a manner similar to heparin, even though the substrate proteins may be different.

The existence of an empirical relationship between anticoagulant activity and r'' suggests the possibility of using this absolute, readily measured physical property to characterize heparins or as a means of quality control to partially or completely replace the biological assays of anticoagulant activity. Were it possible to substitute physical measurements for bioassays, greatly improved standardization and comparability among different heparins could result (Brozovic & Bangham, 1975). At present, variations in the fraction capable of binding to antithrombin among different heparin preparations cannot be accounted for by physical measurements. Thus, either a means must be developed to take such variation into account or the physical measurements can be applied only to samples wherein variation in this factor would not ordinarily be a factor. For example, such measurements could, under such conditions, provide a basis for comparing hog mucosal preparations with each other but possibly not with beef lung preparations.

Acknowledgments

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Copper(II) Protoporphyrin IX as a Reporter Group for the Heme Environment in Myoglobin†

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ABSTRACT: Copper(II) protoporphyrin IX has been introduced into apomyoglobin, and its utility as a reporter group of the heme environment has been examined. The Soret and visible absorption bands and electron spin resonance spectrum show that the Cu(II) is five coordinate, probably through coordination to the F-8 proximal histidine. The resonance Raman spectrum does not indicate any appreciable distortion from the solution conformation of copper(II) protoporphyrin IX dimethyl ester in CS_2. The ultraviolet circular dichroism shows no alteration of the helical content of the globin from that of metmyoglobin. The circular dichroism of the porphyrin transitions suggests that the packing of the amino acid side chains around the porphyrin is different than that in the native metmyoglobin.

A variety of metalloporphyrins have been used as reporter groups for the immediate porphyrin environment in heme proteins. In myoglobin substituted with various metalloprotoporphyrin IX derivatives, the most thoroughly studied are those in which cobalt (Hoffman & Petering, 1970; Hoffman et al., 1971; Chien & Dickinson, 1972; Yonetani et al., 1974; Yamamoto et al., 1974; Hoffman & Gibson, 1978) and zinc (Andres & Atassi, 1970; Hoffman, 1975) have been substituted for iron. To a lesser degree silver, copper, manganese, nickel, chromium, ruthenium, rhodium, and ytterbium (Atassi, 1967; Andres & Atassi, 1970; Yonetani & Scrivastaua, 1974; Hoffmann & Gibson, 1978; Horrocks et al., 1975) have been studied.

The ideal reporter group with which to study the native structure and function of a protein is an isotopic substitution, i.e., ^{13}C for ^{12}C (Jones et al., 1976). For studies relating structure to function, small, well-defined changes in structure accompanied by useful spectroscopic probes are desirable in reporter groups. For example, nitroxide spin-labeled protoheme has been used to study heme–protein interactions in several hemoproteins (Asakura et al., 1971).

A wide variety of copper porphyrins have been studied and characterized by a variety of physical and spectroscopic techniques. Both their coordination chemistry and stereochemistry are different from the iron(II) and iron(III) porphyrins. The copper(II) porphyrins provide several useful spectroscopic probes for the study of the immediate heme environment by UV–vis, ESR, CD, and resonance Raman methods.

We report here a study of the utility of copper(II) protoporphyrin IX as a reporter group for the heme environment in myoglobin.

Materials and Methods

Sperm whale metmyoglobin was obtained from Sigma Chemical Co. A 1% (w/v) solution of $Fe^{III}Mb$[1] was prepared in distilled water, and the small percentage of $Fe^{II}Mb$ present was oxidized by the addition of solid $CuCl_2$ (Breslow & Gurd, 1963). The copper ions were removed by exaustive dialysis against 0.01 M NaOAc, 0.002 M EDTA, and 0.07 M KCl, and the pH was adjusted to 5.6 with 1 M HOAc. Dialysis was continued with three to four changes of glass-distilled water and finally against 0.01 M phosphate buffer, pH 6.1. Any insoluble material was removed by centrifuging or filtering. The $Fe^{III}Mb$ solution was absorbed on a 5 × 16 cm carboxymethylcellulose column equilibrated with 0.01 M phosphate buffer, pH 6.1, and operated under the conditions described by Hardman et al. (1966). The main component, peak IV, was then concentrated to 100–200 mL by using a Millipore molecular separator and applied to a 2.5 × 90 cm Sephadex G-25 (coarse) column equilibrated with distilled water. The purified $Fe^{III}Mb$ was concentrated to a smaller volume and used within 7 days or lyophylized and stored at 0–5 °C.

The heme group was removed from $Fe^{III}Mb$ by the procedure of Teale (1959) using 2-butanone, and apoMb was further prepared by the method of Breslow (1964), with the following modification. The apoMb was dialyzed against several changes of glass-distilled water to remove excess ketone, dialyzed twice against $NaHCO_3$ (50 mg/L), dialyzed again against several changes of distilled water, centrifuged, and used within several days. The apoMb concentration was determined by the extinction coefficient of 15.8 mM cm^{-1} at 280 nm as reported by Stryer (1965).

PPDME was prepared from whole blood according to Grinstein (1947), and the purity was checked by the extinction coefficients reported by Falk (1964).

^{63}Cu isotope (99.89% ^{63}Cu, 0.11% ^{65}Cu) was purchased from the Union Carbide Corp., Oak Ridge National Laboratories, in the form of cupric oxide. The cupric oxide was converted to cupric acetate monohydrate by heating the oxide (200 mg)

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[1] Abbreviations used: MMb, a metalloprotoporphyrin IX myoglobin; PP, protoporphyrin IX; PPDME, protoporphyrin IX dimethyl ester; TPP, *meso*-tetraphenylporphin; MesoDME, mesoporphyrin IX dimethyl ester; TNB, trinitrobenzene; ESR, electron spin resonance; CD, circular dichroism; Mes, 2-(*N*-morpholino)ethanesulfonic acid; IHP, inositol hexaphosphate; OEP, octaethylporphyrin; DBM, Debye-Bohr magnetons.

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