PHOTOELECTRON QUANTUM YIELDS OF HEMIN, HEMOGLOBIN, AND APOHEMOGLOBIN

POSSIBLE APPLICATIONS TO PHOTOELECTRON MICROSCOPY OF HEME PROTEINS IN BIOLOGICAL MEMBRANES

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ABSTRACT Hemoglobin is examined as a model system for intrinsic photoelectron labeling experiments. The absolute photoelectron quantum yields of hemin, hemoglobin, and apohemoglobin thin films were measured in the 180–230 nm wavelength region. Hemin exhibits a quantum yield of approximately 6×10^{-4} electrons per incident photon at 180 nm, 9×10^{-5} electrons per incident photon at 210 nm, and 2×10^{-6} electrons per incident photon at 230 nm. At all wavelengths the hemin curve lies approximately a factor of 20 above that of hemoglobin and two orders of magnitude above that of apohemoglobin. High image contrast is observed between hemin and apohemoglobin in low magnification photoelectron micrographs, suggesting the feasibility of intrinsic labeling studies involving heme proteins. The quantum yield of hemoglobin is discussed in terms of linear contributions from heme groups and protein weighted by their relative surface areas. The fractional surface areas based on the known structure of hemoglobin are consistent with values derived from the quantum yields of hemin and apohemoglobin.

INTRODUCTION

Photoelectron microscopy is a promising approach to biological surface studies (Griffith et al., 1972; Burke et al., 1974). The feasibility of *extrinsic* labeling experiments, in which reporter groups will be selectively bound to the sample surface, has been discussed by Birrell et al. (1973). An alternate approach is to rely on *intrinsic* photoelectron labels. These naturally-occurring reporter groups must have sufficiently high electron quantum yields to produce contrast against the background of proteins and other biological surface components. One interesting possibility is the heme group. With hemes it might prove possible, for example, to eventually map the distribution of the cytochromes in the inner mitochondrial membrane or the heme proteins of the endoplasmic reticulum. The excitation source required for these experiments is not yet available, nor is there any published quantum yield data on hemes or heme proteins on which to determine the soundness of this approach. The excitation source is under development (Massey, 1974), however, and in order to obtain quantum

yield data we have chosen hemoglobin as a model system. The purpose of this paper is to report the absolute photoelectron quantum yields of hemin, apohemoglobin, and intact hemoglobin in the 180–230 nm wavelength region. Besides reporting photoelectric properties of this important protein, these measurements allow an assessment of the feasibility of mapping distributions of heme proteins using photoelectron microscopy.

EXPERIMENTAL

Hemin (ferriheme chloride) (bovine, Type I, Sigma Chemical Co., St. Louis, Mo.) was recrystallized by the method of Fischer (1941). Expected elemental analysis: C, 62.43%; H, 4.95%; N, 8.5%. Found: C, 62.06%; H, 4.94%; N, 8.44%. Bovine hemoglobin (Sigma, Type I, lot 63c-8000) was dissolved in water (140 mg/ml) and purified by fractional precipitation with ammonium sulfate (Rossi-Fanelli and Antonini, 1959). The crystals were recovered after centrifugation for 15 min at 4300 *g* and 5°C and redissolved in water. The ammonium sulfate was removed by dialysis against distilled water for 18 h at 5°C with several changes of the dialysis bath solution. Apohemoglobin was prepared by acidic acetone splitting of the purified hemoglobin (Rossi-Fanelli and Antonini, 1958).

Absolute quantum yield measurements were performed on fresh samples by the method described previously (Dam et al., 1974). Metal-free phthalocyanine was used as a photoelectron quantum yield standard. Solutions of recrystallized hemin (0.1 mg/ml) were prepared in methanol, acetone, acidic acetone (3 ml of 2 N HCl per liter acetone) and dilute aqueous ammonium hydroxide (1 ml 30% NH_4OH in 50 ml water). Thin films of hemin on stainless steel sample rods were prepared from these solutions by a spraying technique described previously (Dam et al., 1974). The resulting surfaces contained microcrystals and some structure was visible in the low magnification (approximately × 50) photoelectron micrographs. More uniform samples resulted from allowing several drops of 5 mg/ml solution in pyridine to dry in air at room temperature on the sample rod. The measured quantum yields of hemin prepared by these two methods were the same within experimental error. Samples of hemin prepared from the hemoglobin splitting gave results identical to those obtained from the recrystallized Sigma product. Hemoglobin and apohemoglobin films were prepared by allowing several drops of fresh 10 mg/ml aqueous solution to dry on the sample rod at room temperature.

RESULTS

Low magnification photoelectron images of hemin, hemoglobin, and apohemoglobin thin films are shown in the composite micrographs of Fig. 1. These are identical exposures taken at an excitation wavelength of 190 nm. In Fig. 2 the absolute quantum yields (photoelectrons emitted per incident photon) of hemin, hemoglobin, and apohemoglobin thin films are plotted as a function of wavelength. Also shown are the yield curve for the reference compound, metal-free phthalocyanine (Pc) (Schechtman,

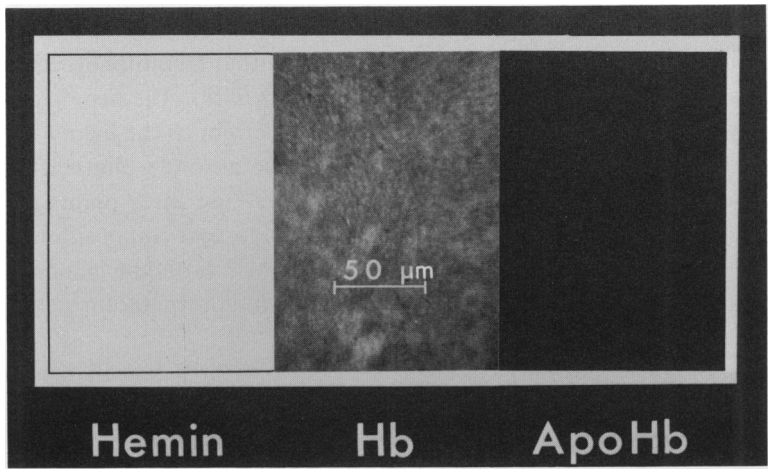

FIGURE 1 Low magnification photoelectron micrographs of hemin, hemoglobin, (Hb), and apohemoglobin (ApoHb) surfaces. Exposure times, print development, and magnification are identical for all three samples and the incident wavelength is 190 nm (bandwidth approximately 10 nm).

1968), and the band defining the quantum yields of the amino acids (Dam et al., 1974). The photoelectron quantum yields of 19 common amino acids fall between the upper and lower dashed lines of the shaded area. At wavelengths longer than 230 nm the quantum yields of all compounds in Fig. 2 decrease and rapidly fall below the limit of detection (5×10^{-8} electrons per incident photon).

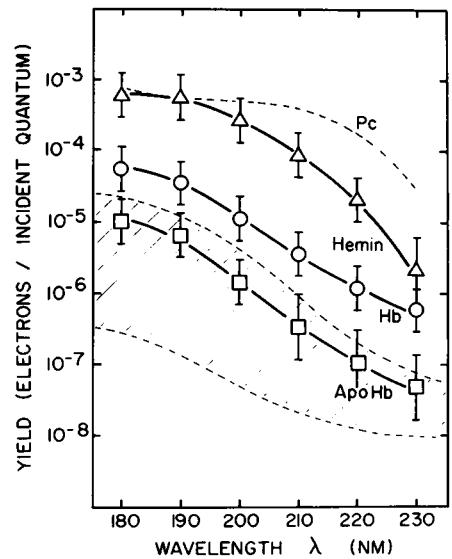

FIGURE 2 Spectral quantum yield curves for hemin, hemoglobin (Hb), and apohemoglobin (ApoHb). The dashed curve (Pc) is the yield of phthalocyanine reported by Schechtman (1968). The shaded band contains the quantum yield data for the amino acids (Dam, 1974).

DISCUSSION

The photoelectron micrographs of Fig. 1 clearly show that hemin photoemits strongly at 190 nm, whereas the apohemoglobin surface appears dark. The hemoglobin surface appears grey under the same conditions. It is not as bright as the hemin but is easily distinguishable from the apohemoglobin surface. The absolute photoelectron quantum yields of Fig. 2 quantify these observations. There are three points to consider: (*a*) Is the apohemoglobin curve consistent with the amino acid composition? (*b*) Is the contrast of hemin sufficient for its use as an intrinsic label? (*c*) Is the hemoglobin curve predictable from the known structure of the molecule and the quantum yields of hemin and apohemoglobin?

Regarding the first question, the quantum yield of a protein will depend on the amino acid composition and orientation of the polypeptides within the photoelectron sampling depth of the surface. Another significant point is whether photoemission from the amino acid residues in a protein is simply additive, or whether the environment imposed by the polypeptide structure significantly alters the quantum yield. These factors are not known with sufficient confidence to predict the photoelectron quantum yield values of a protein accurately. However, the present data provide new information relevant to this general problem. Previous work has shown that of the 21 amino acids whose quantum yields have been measured, 19 exhibited yield curves which fall within the shaded band of Fig. 2. Only tryptophan and tyrosine have quantum yields significantly above the band at wavelengths below 200 nm (Dam et al., 1974).

Assuming simple additivity and barring an abnormally high surface concentration of the more photoemissive amino acids, the quantum yield curve of a protein such as apohemoglobin (which has no composition anomalies) should lie within the limits found for the majority of the amino acids. Additivity is suggested by experiments showing that amino acid polymer films photoemit very much like the amino acid monomers (Dam et al., 1974). The apohemoglobin yield curve of Fig. 2 falls within the band defined by the amino acid monomers, indicating that photoemission from the amino acid residues in a protein thin film is additive and that all proteins (except perhaps those with composition anomalies) should exhibit similar photoelectron quantum yields. We have subsequently measured the quantum yield curve of one other protein, bovine serum albumin, with similar results, in agreement with this conclusion.

Fig. 2 shows that the photoelectron quantum yield curve of hemin lies approximately two orders of magnitude above that of apohemoglobin over the wavelength range 180–230 nm. The corresponding photoelectron image contrast between hemin and apohemoglobin (see Fig. 1) is clearly sufficient to allow use of the heme group as an intrinsic label. Thus, heme groups should be visible against a protein background in photoelectron microscopy. Fig. 2 also indicates that hemin is a photoemitter comparable in strength to phthalocyanine between 180 and 200 nm, but decreases somewhat more rapidly than the dye between 200 and 230 nm. The similarity in quantum

yields can be attributed to structural similarity, insofar as hemin and phthalocyanine both contain large conjugated π-electron ring systems.

Thirdly, we consider the photoemission observed from a thin film of hemoglobin. The quantum yield curve and photoelectron image brightness lie between those of hemin and apohemoglobin at all observed wavelengths. The simplest interpretation of the reduced brightness of hemoglobin relative to hemin is that the protein dilutes the photoemission by decreasing the relative amount of hemin exposed on the sample surface. The magnitude of this effect can be estimated as follows. Hemoglobin is a roughly spherical molecule consisting of two pairs of polypeptide chains in a tetrahedral arrangement (Perutz, 1964). Each polypeptide subunit contains one heme group, embedded approximately edgewise, into a cleft exposed to the surface. Thus, we take as a model of the hemoglobin molecule a protein sphere of radius r containing four embedded discs (the heme groups) of radius r' and thickness w' at the vertices of a tetrahedron circumstribed by the sphere. The radius $r \approx 30$ Å but this number does not affect the calculation directly since only the ratio r/r' is involved. Consider a thin film of hemoglobin molecules with one surface exposed to UV light in a photoelectron microscope experiment. It is known from previous work on phthalocyanine thin films that the depth resolution (escape depth) is on the order of 10–15 Å (Burke et al., 1974; Pong and Smith, 1973). The ultraviolet light is absorbed in successive molecular layers, but only the electrons photoionized within the top 10–15 Å actually escape from the surface and are detected. The corresponding depth resolution for hemoglobin is not known, but it is almost certainly less than the diameter of one hemoglobin molecule. We define a unit surface area A_{HB} to be one hemoglobin molecule in projection. The average number of exposed heme groups per unit surface area will then lie between one and four, depending on the actual depth resolution figure and the orientation of the proteins at the surface of the thin film. Thus, the fractional surface area of the heme groups, f_H, will range between A_H/A_{HB} and $4(A_H/A_{HB})$, where A_H is the exposed surface area of one heme group. The exposed surface area of one heme group is roughly $2r'w'$ (considering an edge-on projection) where r' and w' are the radius and thickness of the heme group. Conversely, $A_{HB} = \pi r^2$ where r is the radius of the hemoglobin molecule. The lower limit to the fractional surface area of the heme groups is therefore

$$f_H = 2r'w'/\pi r^2. \tag{1}$$

The molecular weights of the heme group (M_H) and of apohemoglobin (M_B) are approximately proportional to their volumes. Thus $M_H/M_B \simeq \pi r'^2 w'/(4/3)\pi r^3$, and combining this relation with Eq. 1 yields

$$f_H = A_H/A_{HB} = (8/3\pi)(M_H r/M_B r'). \tag{2}$$

The molecular weight of heme is 616 and that of apohemoglobin is approximately 62,000 depending on the source. For r/r', we estimate a value of 3 based on visual

examination of models of hemoglobin. Inserting these values into Eq. 2 gives as a lower limit, $f_H = 0.025$. From these simple considerations of molecular geometries f_H should lie in the range of $0.02 \leq f_H \leq 0.10$.

The quantity f_H can also be calculated from the observed image brightnesses (or quantum yields) assuming a dilution model. Let the surface consist of heme groups H and apohemoglobin B, with photoelectron quantum yields and fractional surface areas Y_H, Y_B, f_H, and $f_B = 1 - f_H$, respectively. We assume the image brightness of pure H (I_H) and pure B (I_B) are proportional to the quantum yields, so that $I_H = kY_H$ and $I_B = kY_B$ where k is a constant. At low magnification the image brightness of a surface of hemoglobin molecules (I_{HB}) will be their sum, weighted by fractional surface areas. Thus

$$I_{HB} = k(f_H Y_H + f_B Y_B). \tag{3}$$

Solving for f_H and simplifying gives

$$f_H = ([Y_{HB}/Y_H] - [Y_B/Y_H])/(1 - [Y_B/Y_H]). \tag{4}$$

If the model is accurate, f_H calculated by this formula should be independent of wavelength. When the observed quantum yield data of Fig. 2 are substituted, we find that f_H varies by a factor of about two, from $f_H = 0.03$ to 0.07, over the wavelength range 180–220 nm. This is well accounted for by the combined experimental uncertainties in the data. These values for f_H lie in the range predicted by the simple model using Eqs. 1 and 2, indicating that the dilution model is essentially correct.

The ultimate resolution of the photoelectron microscope is 25–40 Å (Griffith et al., 1972). A fundamentally important question is whether heme proteins can be viewed in a field of non-heme proteins under optimal conditions. Eqs. 3 and 4 were derived by considering the image brightness of a very large number of heme protein molecules when the resolution area element was much larger than the molecular dimensions. The same equations can be used to determine the image contrast ratio between a single heme protein molecule and a non-heme protein background at high resolution, provided that an appropriate value of f_H is used in the expressions. The fractional surface area of the heme group must be calculated with respect to a resolution area element rather than a molecular surface area as in the above derivations. We denote the effective fractional surface area of the heme group located within a resolution area element by $f_H(\text{eff})$. The brightness ratio between such an area element and one which contains no heme group is given by dividing Eq. 3 by $I_B = kY_B$ and substituting $f_H(\text{eff})$ for f_H. Simplification gives

$$I_{HB}/I_B = 1 + f_H(\text{eff})([Y_H/Y_B] - 1). \tag{5}$$

Inserting an average photoelectron quantum yield ratio of 100 for hemin and apohemoglobin gives a brightness ratio of $I_{HB}/I_B \simeq 1 + 100 f_H(\text{eff})$. If the resolution of

the photoelectron microscope were of atomic dimensions, f_H(eff) would approach unity at the site of a heme group and the contrast ratio would be approximately two orders of magnitude. The resolution limit of 25–40 Å is substantially larger than atomic dimensions and thus a dilution effect will be important. This resolution figure concides roughly with the dimensions of one of the hemoglobin molecule subunits and thus $f_H(\text{eff}) \simeq f_H$. Since f_H is in the range $0.02 \leq f_H \leq 0.10$, the image brightness or contrast ratio between hemoglobin and non-heme proteins (Eq. 5) should lie in the range $3 \leq I_{HB}/I_B \leq 11$. These values are well in the range of detectability. Furthermore, preliminary data suggest that the quantum yields of other surface components (e.g., phospholipids and polysaccharides) are no greater and perhaps less than the quantum yields of non-heme proteins (Birrell et al., 1973). Based on these data and the above order of magnitude calculations, we conclude that membrane proteins with exposed heme groups exhibit sufficient contrast to be visible when instrumentation capable of 25–40 Å resolution is available. We must point out, however, that there are challenging technical problems attending high resolution surface imaging (e.g., possible photochemical damage and specimen preparation procedures).

In summary, the photoelectron quantum yield of hemoglobin can be treated as a superposition of strongly emitting heme groups, covering 2–10% of the sample surface, against a relatively nonemissive protein background. The large separation of quantum yields between hemin and apohemoglobin, when correlated with the low magnification photoelectron micrographs, suggests that heme proteins should be detectable against a non-heme protein surface in high magnification photoelectron microscopy experiments.

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