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X-RAY SCATTERING STUDIES OF HEAVY ATOM BOUND ERYTHROCYTE MEMBRANE SUSPENSIONS

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Summary

We have observed a periodic intensity variation of scattered x-rays from uranyl labeled erythrocyte membranes. Using unique x-ray scattering methods, we have made these measurements from membrane suspensions in which the vesicles appear, by phase contrast microscopy, to be normal in shape. The periodic intensity variation is not present for membranes labeled on one side only. The frequency of the variation permits calculation of membrane width, which we find to be 55Å.

INTRODUCTION

Biophysical and biochemical studies of erythrocyte membranes have contributed immensely to our general knowledge of membrane structure and function. Studies of particular membrane proteins have produced fascinating results concerning specific functions and mutual relationships. While the complexity of the membrane makes structural studies difficult, the lack of crystallographic ordering within the plane of the isolated membrane makes structural analysis on the level of modern protein crystallography impossible. Although such studies are possible for certain membranes which are naturally crystalline, it would be difficult to accurately describe plasma membranes based on these unusual membranes. Thus studies of the erythrocyte membrane as a model for plasma membranes continue.

In this study we ask a simple question of the complex erythrocyte membrane; what is its' width as an isolated vesicle? The experiment consists of unique x-ray scattering techniques combined with heavy atom labeling methods. By labeling both sides of the membrane with UO_2^{++} ions, we can detect a periodic intensity variation in which the frequency is directly related to membrane width. The enhancement of scattered intensity by the use of heavy atoms combined with unique scattering techniques make possible recording this intensity variation from vesicles which are readily observable by phase contrast optical microscopy. Thus this measurement may be made in the same environment where many biochemical studies are performed.

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MATERIAL & METHODS

Whole human blood was collected in heparinized vacuutainers. Membranes were prepared by the procedure of Dodge, et. al.[1] at 4°C. Erythrocytes were washed four times in equal volumes of isotonic (310 mOsm) sodium phosphate buffer at pH7.4-7.6. The wash ratio was 14 volumes buffer per 1 volume of 50% cell suspension. A button of aggregated material was removed between the third and fourth washes. The final membrane suspension was hemoglobin free. Gel electrophoresis showed a normal banding pattern.

Membranes and whole erythrocytes were labeled with UO_2^{++} ions using uranyl acetate. Although the uranyl salt is highly soluble in distilled water, it precipitates above pH5 even at low ionic strength. In addition it forms a precipitate with phosphate buffer. Therefore, to label whole cells the following procedure is followed. Erthrocytes are washed into isotonic NaCl. 20mM uranyl acetate is made isotonic with NaCl and added dropwise to the 50% cell suspension with constant stirring at 4°C. The pH is maintained between 6.5 and 8. After labeling, the cells are washed free of excess uranyl acetate with isotonic NaCl. Membranes are then prepared by the standard procedure described above with 15 mOsm phosphate buffer at pH7.4. Unlike unlabeled membranes, these ghosts retain some hemoglobin.

For labeling hemoglobin free membranes, the ghosts are washed twice more into 7.5mM NaCl (14:1 wash ratio, neutral pH). The pH is then adjusted to 6.3 by the addition of dilute HCl. We have found that uranyl acetate-sucrose solutions do not precipitate at high pH. For binding UO_2^{++}, an equal volume of 20mM uranyl acetate in 310mM sucrose at pH6.3 is added dropwise with stirring to an equal volume of ghosts in 7.5mM NaCl at 4°C. No drift of pH is observed during binding. The membranes are then washed free of excess uranyl acetate-sucrose and into 7.5mM NaCl at pH6.3.

Protein was estimated by Lowry assays[2] in 2% sodium dodecyl sulfate. UO_2^{++} concentration was determined by absorption at 430nm.

X-rays were obtained from a copper rotating anode with a projected source size of $(0.2mm)^2$ operating at 50 kV and 80 mA. $CuK\alpha_1$, x-rays ($\lambda = 1.54Å$) were selected and focused by a large doubly curved quartz crystal placed at 500 mm from the source. The crystal has been previously described.[3] Approximately 10^8 photons/sec. passed through the sample. Diffracted x-rays were collected at 280 mm from the specimen using a stable position sensitive proportional detector. The detector is filled with Ar-CH_4 at 100 bars and is $\approx$90% efficient. A damage resistant nichrome wire was used with charge division encoding. The position resolution of the detector corresponds to an angular resolution (for the scattering angle) of 2×10^{-3} radians. Detailed tests of the detector showed that it is extremely stable, resistant to radiation damage, and linear.

The apparatus produces an enhancement factor between 10^2 and 10^3 over conventional methods (film detection, singly curved crystal). This enhancement makes possible recording weak scattering from a true suspension of membranes. The central features of the pattern could be recorded in five minutes. However, to obtain better statistics longer exposures were used. Patterns recorded at the beginning and end of the exposure did not differ.

All patterns were corrected as follows. Camera background was removed by scaling background patterns to the attenuated central beam and then subtracting. Scattering due to buffer was removed by subtracting buffer patterns (background removed) after scaling at large angles. All patterns were corrected for spherical averaging by applying a Lorentz factor of S^2 where $S = \frac{2\sin\theta}{\lambda}$ and 2θ is the scattering angle.

RESULTS

Fig. 1 shows a phase contrast micrograph of UO_2^{++} labeled ghosts. The concentration corresponds to that used for x-ray scattering. Therefore the membranes are in a true suspension for x-ray scattering and appear normal in shape.

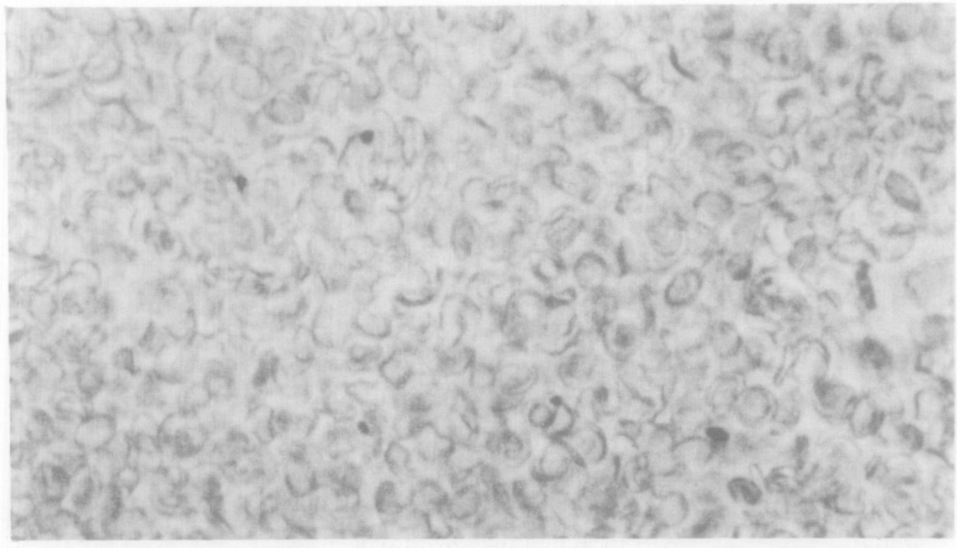

Figure 1 A phase contrast micrograph of a UO_2^{++} labeled erythrocyte membrane suspension. The heavy atoms are on both sides of the membrane. The concentration corresponds to that used for x-ray scattering.

We determined that the labeled suspensions contained ~4mg protein /ml. For ghosts labeled on both sides we found ~1.8 mg UO_2^{++} /mg protein. UO_2^{++} determinations were not made for ghosts prepared from labeled whole cells due to interference from increased bound hemoglobin. Unlabeled ghosts contain ~2.5mg/ml protein when x-rayed.

Results of our x-ray investigation are shown in Figs. 2-4. The pattern from membranes labeled on both sides is shown in Fig. 2. A periodic intensity variation is observed. Mem-

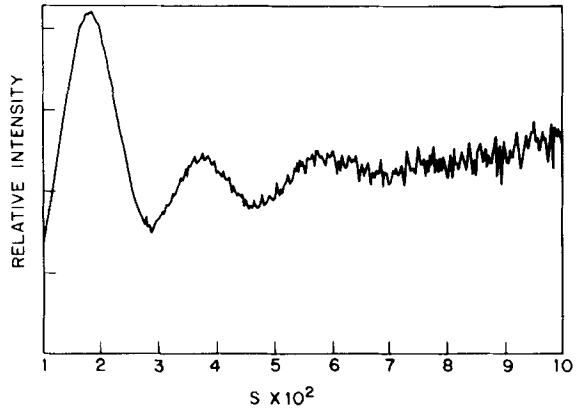

Figure 2 The x-ray scattering pattern of erythrocyte membranes labeled on both sides of the membrane with UO_2^{++} ions. the pattern has been corrected for camera background, buffer scattering, and sperical averaging (see text). $S = \frac{2\sin\theta}{\lambda}$ and is in units of $Å^{-1}$.

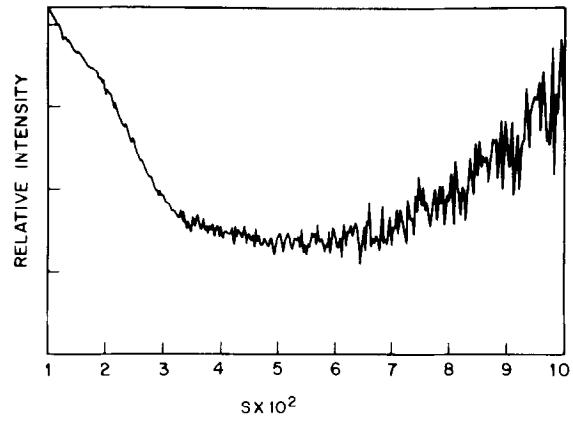

Figure 3 The corrected scattering pattern of erythrocyte membranes labeled on the extracellular surface with UO_2^{++} ions. S is the same as in Figure 2.

branes prepared from UO_2^{++} labeled whole cells produce a pattern (Fig. 3) which has no periodic variation. For reference we show the scattering from a suspension of unlabeled ghosts in Fig. 4.

DISCUSSION

Our previous work[4] provides a theoretical basis for these scattering studies and an experimental test using a synthetic lipid bilayer system below the hydrocarbon chain melting transition.

By comparing the total small angle scattering of an erythrocyte membrane suspension with and without UO_2^{++}, it is apparent that the total scattering is dominated by the heavy atom distri-

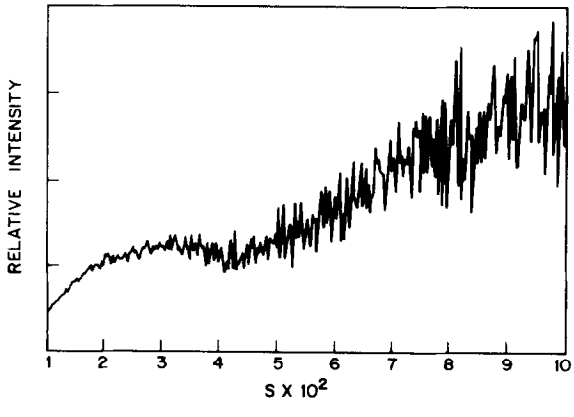

Figure 4 The scattering from unlabeled erythrocyte membranes. S is the same as in Figure 2. The scattering is extremely weak (compared to labeled membranes) and shows that the heavy atom distribution dominates the pattern for labeled membranes.

bution. For example, at S = 0.02 $Å^{-1}$, the intensity for membranes labeled on both sides (per mg protein and corrected for absorption) is approximately an order of magnitude greater than for unlabeled membranes. This is not true for synthetic bilayers due to the more structured nature of the lipid and limited binding.[4] Under these conditions, we assume that the angular distribution of the scattering is dominated by the heavy atoms.

As shown in our previous analysis,[4] if the projection of the heavy atom distribution on an axis orthogonal to the membrane plane can be approximated by a Gaussian, then the intensity is given by:

$$I(s) = (NG_n - (G_n + G_\alpha)\alpha)^2 + 4\alpha(N - \alpha)G_nG_\alpha\cos^2\pi sd$$

where

$$G_{n/\alpha} = e^{-\pi w_{n/\alpha}^2 S^2}$$

and

S = $\frac{2\sin\theta}{\lambda}$ (2 θ is the scattering angle)

N = the total number of units of heavy atoms on the membrane

α = the number of units of heavy atoms on one side of the membrane

$W_{n/\alpha}$ = the width of the Gaussian on either side of the membrane (N or α side)

d = the separation between Gaussian layers.

Thus, in principle, it is possible to determine the width of the membrane (characterized by d) and the width of the label (characterized by the widths W_n and W_α). The intensity distribution for membranes labeled on both sides, as shown in Fig. 2, has three maxima centered at 0.0182$Å^{-1}$, 0.0369$Å^{-1}$ and 0.0571$Å^{-1}$. The peaks are on top of a progressively increasing background. As previously shown and from the equation for I(s), asymmetric binding (i.e., $N/2 \neq \alpha$) produces a decaying background term. Thus the increasing background indicates that in addition to asymmetric binding, the UO_2^{++} ions have some correlations in the plane of the membrane which are not described by the above equation.

By subtracting a constant background passing through the minima shown in Fig. 2, the heights of the three maxima are 6.3, 1.65, and 1.0 respectively. These heights and the periodic

ripple frequency may then be described with respect to the last term in I(s). From the ripple frequency we find $d = (0.0182)^{-1}\text{Å} \cong 55\text{Å}$. From the maxima we estimate that $W \cong 12\text{Å}$ (more precisely $W_N W_\alpha \cong 160\text{Å}^2$).

We interpret these measurements of W and d in terms of the heavy atom distribution. It is known the UO_2^{++} ionically binds tightly to both protein and lipid although specific binding constants have not been determined for the major membrane protein bands or lipids. Thus, the measurements strongly suggest that in a suspension, erythrocyte membranes contain large regions which are ~55Å wide. Previous studies with partially dehydrated oriented membrane stacks and totally hydrated poly-L-lysine agglutinated dispersions also suggest that the membrane contains extensive 55Å wide regions.[5] More recent studies of glutaraldehyde fixed erythrocyte membranes also indicate that there exists a basic 55Å structural element.[6]

Under certain conditions we observe a pattern similar to that determined for dispersions.[7] This pattern has the second lobe diminished relative to the first and third. These conditions correspond to preparations at higher pH. This may correspond to a sample in which the scattering of heavy atoms is significantly perturbed by the rest of the membrane. In other words, the scattering cross term between heavy atoms and membranes is significant, as it is for lipid bilayers.[4] Although the pattern is similar to that found for concentrated dispersions,[7] the sample still corresponds to a suspension in which individual membranes may be viewed by phase contrast microscopy.

Our pattern recorded from a suspension of unlabeled membranes differs from that reported for a dispersion.[7] However, the signal to background ratio for unlabeled membrane suspensions is extremely small. Thus, this background removed pattern is subject to error. The important point is that unlabeled membrane suspension scattering is very weak compared to that found for labeled membranes.

This work shows that extensive 55Å wide regions exist in the membrane vesicles. It is realistic to expect that some proteins extend far beyond this width. Nevertheless, the 55Å ion barrier is sufficiently well defined so that it dominates the scattering in the angular range which was explored.

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