

White lines in L-edge x-ray absorption spectra and their implications for anomalous diffraction studies of biological materials

(anomalous scattering/crystallography/biological structure)

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ABSTRACT We have measured high-resolution x-ray absorption spectra of lanthanide (Ln) and heavy transition metal complexes that display prominent narrow absorption peaks near the L_2 and L_3 absorption edges. The anomalous scattering factors (f' and f''), which are mathematically related to the absorption cross section, have correspondingly sharp changes in their magnitude within 5–10 eV of the absorption edge. Calculations of the magnitude of the change in f' and f'' demonstrate that significant changes (on the order of 20 electrons in f') can be expected for these materials. These substantial changes in the anomalous scattering factors have applications to deriving structural information for macromolecules from x-ray diffraction studies. The magnitude of the changes indicate that the anomalous scattering technique is a powerful means of obtaining structural characteristics for macromolecules in single crystals, in solution, and in biological membranes.

The earliest uses of anomalous scattering were in single-crystal diffraction measurements in which the consequences of the effect were used in the determination of absolute configuration (handedness) of chiral molecules in crystals. More recently, the effects have been used extensively in macromolecular crystallography for locating anomalously scattering atoms, in refining their positions, and in assisting in phase determination. Other applications, such as unique phase determination by using multiple wavelengths and extraction of elemental specific radial pair correlation functions in polycrystalline or disordered systems have been suggested. These applications were reviewed at the Madrid symposium on anomalous scattering (1).

Before synchrotron radiation became available, measurement of anomalous scattering terms was impractical except at specific wavelengths because the emission from standard anode x-ray sources supplies photons predominantly at discrete wavelengths. This discreteness limits the measurement of f' and f'' to specific photon wavelengths not necessarily close to an absorption edge. Because the largest changes in the anomalous scattering are within a few electronvolts of the edge, the large magnitude of the effects could not be accurately measured (or used experimentally) with characteristic lines from these anode sources. Calculations of the anomalous scattering terms based on Hartree-Fock atomic methods have been tabulated (2) but are accurate only in regions that do not include absorption edges. Because the potential usefulness of anomalous scattering is dependent on the maximum change in f' and f'' that can be attained by varying the wavelength, previous methods of determining the anomalous scattering factors do not illuminate the most interesting regions of the anomalous scattering spectra.

Synchrotron emission is a source of radiation in which the photon energy can be varied continuously over a wide range

limited only by the monochromator and operating conditions of the electron storage ring used to produce the radiation (3). Our x-ray absorption studies using synchrotron radiation indicate that large anomalous effects are present as the incident photon energy approaches an L (p -state ionization) absorption edge. The failure of the Hartree-Fock atomic calculations in predicting these features implies that the effects are not describable solely in terms of atomic states and that transitions into excited molecular electronic states must be included. Transitions to highly excited continuum states give rise to the extended absorption fine structure called EXAFS, and these modulations are reflected in the anomalous dispersion terms. With the availability of an x-ray diffractometer on a synchrotron radiation source (3), anomalous scattering terms could be accurately measured at arbitrary energies for a crystal of known structure. The anomalous scattering terms for cesium in cesium hydrogen tartrate have been determined near the L_1 , L_2 , and L_3 edges (4, 5). These studies revealed values of f' of -26.7 electrons at the L_3 edge (5016 eV) and of -13.5 electrons 50 eV below and 5 eV above the L_3 edge.

We performed a survey (unpublished results) of high-energy resolution absorption spectra of various Ln and heavy metal complexes by using synchrotron radiation. The survey revealed even larger changes in absorption at L_3 and L_2 edges than was found in the cesium study. Similar sharp changes were dubbed "white lines" by Coster (6) because of their characteristic trace left on photographic negatives in absorption measurements. Because f'' is related to the absorption cross section by the optical theorem and f' can be derived from f'' by using the Kramers-Kronig dispersion relationship (see below), we have carried out a systematic study of the absorption spectra of a large number of L edges for the lanthanide (Ln) and other heavy metal complexes in order to assess their anomalous scattering terms. This paper reports this new high-energy resolution data on L absorption edge cross sections, evaluates the implications of the data for anomalous scattering terms, and discusses the use of these effects for structural applications to single crystal, solution, and membrane scattering.

MATERIALS AND METHODS

The absorption measurements were carried out at the Stanford Synchrotron Radiation Laboratory. The data were recorded by transmission using the high-energy resolution x-ray beam line [EXAFS beam line I; resolution, 1–2 eV at 8 keV with Si (220) channel cut crystal]. The samples, commercially available reagent grade chemicals, were pressed into neat pellets and placed in the beam. Gaseous ionization chambers were used to monitor x-ray intensities in front of and behind the sample. The initial and final intensity readings were recorded at each photon

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Abbreviations: Ln, lanthanide; EXAFS, extended x-ray absorption fine structure.

energy by using a PDP 11/10 computer and programs developed initially for EXAFS analysis (3). The absolute energy was calibrated by using the copper foil K absorption edge, the maximum in the first derivative of the pre-edge transition being equated to 8980.3 eV. Subsequent analysis of the absorption data (Fig. 1) was carried out by using more of the EXAFS programs (7) and other programs developed specifically for anomalous scattering calculations on a PDP 11/55 computer.

PHYSICAL INTERPRETATION OF WHITE LINES

The physical origin of white lines is suggested from Fig. 2. The L_3 and L_2 transitions both have white lines, and the L_1 transition does not. This same behavior has been observed for ≈ 15 complexes of Ln and other heavier transition metals including Re, Os, and Ir. The white lines are clearly associated with a $2p \rightarrow$ final d -state transition of the L_3 and L_2 edges as opposed to a $2s \rightarrow$ final p state for the L_1 edge. On state-counting grounds alone, one expects an intensity ratio of $4 \times 10:2 \times 6$ ($p_{3/2} \rightarrow d: s \rightarrow p$)—i.e., 10/3 for an L_3 edge relative to an L_1 edge (5/3 for an L_2 edge).

In practice, this ratio is much enhanced by the localization of final d -state electron density due to molecular orbital effects (Rydberg type exciton states) or by d -band effects in the case of metals, as first pointed out by Mott (8, 9). [For a recent review, including a summary of the historical development of white line phenomenology, see Brown *et al.* (10)].

Recent self-consistent field calculations in our laboratories (11, 12) emphasize the importance of molecular environment on the amplitude of the final-state wave function near the core-level region of the absorbing atom and hence on the oscillator strength for the transition. In the case of the Ge K-edge in tetrahedral GeCl_4 , the calculations showed that the oscillator

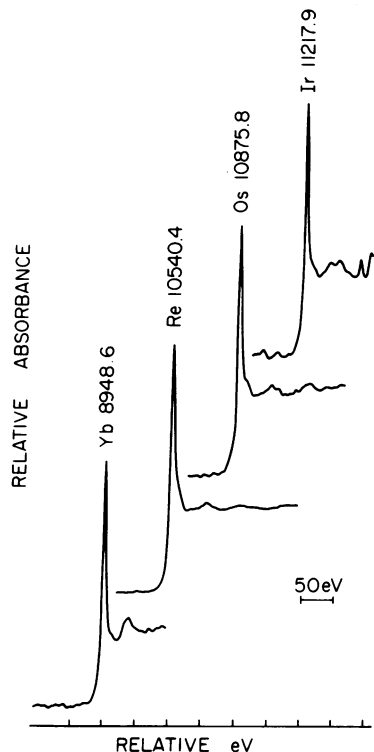


FIG. 1. The L_3 absorption edges for Yb and several heavier transition metals. The peak heights are normalized to unity, and the energy scale is relative to facilitate comparison. The absolute energy values are given over the maximum of each absorption peak. The complexes are $\text{Yb}(\text{AcAc})_3$, ReCl_3 , $\text{OsCl}_6(\text{Bu}_4\text{N})_2$, and NaIrCl_6 .

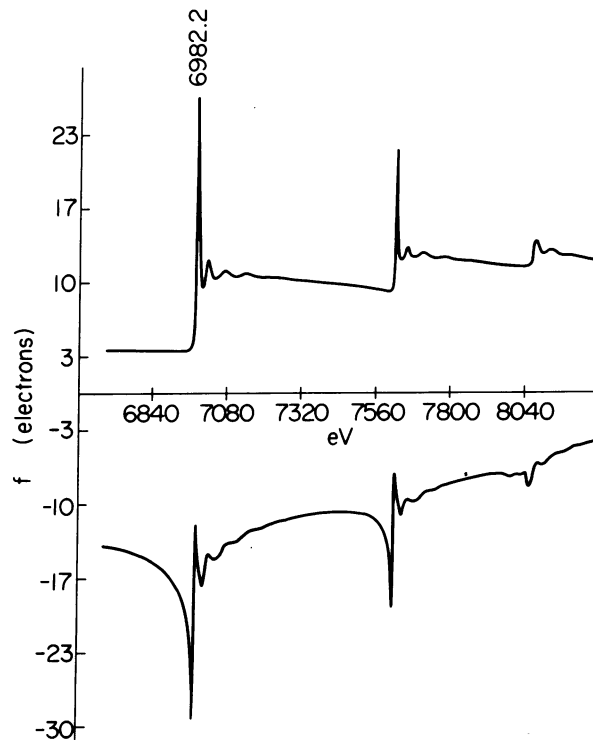


FIG. 2. Values of f'' and f' for $\text{Eu}(\text{PhAcAc})_3$. The values of f' were obtained by using a Kramers–Kronig transform as discussed in the text. The vertical scale is calibrated from tabulated values of f' and f'' at $\text{CrK}\alpha$ and $\text{CuK}\alpha$. The L_3 edge is the lowest in energy (to the left) and L_1 is the highest. The similarity between the L_3 and L_2 edges is apparent; the L_1 lacks the white line. These effects are discussed in the text.

strength for the $1s \rightarrow$ final T_2 symmetry bound-state transition was increased by a factor of 5 on going from the weak molecular potential of GeH_4 to the stronger molecular potential of GeCl_4 . This may be thought of as an “inner well” confining effect of the cage of Cl ligands on the wave function of the T_2 molecular orbital state. In a similar way one expects that the intensity of white lines in Ln complexes may be dependent on molecular environment, so that the values of the anomalous scattering factors near the $L_{2,3}$ edges will not be quantitatively transferable from complex to complex, although the presence of a white line would be expected [except, perhaps, in the metallic state (10)].

It should be noted that the existence of L-shell white lines for a given chemical element appears to be generally correlated to the existence of unfilled atomic valence states of d character. Thus, the absence of white lines at the L edges of iodine may be explained by the fact that the $4d$ shell is filled.

CALCULATION OF f' AND f'' BASED ON ABSORPTION MEASUREMENTS

The usefulness of anomalous scattering techniques for problems in structural studies by diffraction methods is dependent on the magnitude of the changes in the scattering that can be induced by approaching an absorption edge. Because angular dependence of the anomalous part of the x-ray scattering amplitude is weak, the imaginary part f'' is directly proportional to ω times the total absorption cross section through the use of the optical theorem (13). Changes in the imaginary term, f'' , are easily measured from absorption experiments, but f' is not so easily measured directly. In practice, variations in f' lead to more

significant changes in the magnitude of the scattering factor. Templeton *et al.* (4, 5, 14) have measured values of f' and f'' by application of fitting procedures to crystallographic data for several wavelengths near the $L_{1,2,3}$ absorption edges. The measurements can only be made on crystalline samples of known structure, but they provide strong experimental evidence that dispersion equations accurately relate f' to f'' . Kawamura and Fukamachi (15) have demonstrated that the standard Kramers–Kronig dispersion relationships validly represent the relationship between f' and f'' near an x-ray absorption edge where excited state lifetimes are included. Thus, it appears that accurate values of f' can be calculated, obviating the need to measure f' directly in cases in which such measurement is difficult.

With these justifications and starting with our high-resolution data for f'' (absorption measurements), a typical high-resolution spectrum of f' can be calculated by using the Kramers–Kronig relationship (16):

$$f'(\omega) = \frac{2}{\pi} \int \frac{\omega' f''(\omega') d\omega'}{\omega^2 - \omega'^2} \quad [1]$$

in which $f''(\omega) \propto \omega\mu(\omega)$, μ being the absorbance of the Ln.

The imaginary term f'' is first extracted from the absorption scan by multiplying by ω , subtracting the background, and scaling to fit tabulated values of f'' at specific wavelengths. These tabulated values are from calculations (17) made at standard energies away from the sharp changes at the edge. The real part of the anomalous term, f' , was calculated with a digital computer using Eq. 1. Because values of f' are tabulated for the standard wavelengths, the calculated f' can be shifted by a constant to provide a quantitative interpolation for the absolute scattering corrections, although the relative changes are most accurately calculated.

The particular Ln complex used in the calculation reported herein, $\text{Eu}(\text{PhAcAc})_3$, was chosen for its ability to be scaled most reliably due to the placement of the standard wavelengths ($\text{FeK}\alpha$ and $\text{CuK}\alpha$) relative to the edge and for the quality of data in the absorption scan. The fine structure in f'' at the edge is typical of many Lns (Figs. 1 and 2), so the changes in f' are presumably representative. It should be noted that experimental energy resolution appears to be limiting the height of the whiteline in that the white line continues to grow as the resolution is increased to the observed limit determined by a combination of monochromator resolution and natural lifetime of the $2-p$ core state (here, about 2 eV). Thus, the calculated changes in f' are also probably resolution limited. This will be reflected in a corresponding resolution limitation on the changes usable in an experiment.

The Kramers–Kronig transform indicates that substantial changes in f' can be induced by the white line—on the order of 17 electrons given this energy resolution (see Fig. 2). This is consistent with the magnitude of changes reported elsewhere (14) and bodes well for the usefulness of anomalous diffraction techniques in compounds that exhibit white lines.

APPLICATION OF ANOMALOUS SCATTERING

Protein Crystallography. Bijvoet *et al.* (18) realized that anomalous scattering can be used to solve the crystallographic phase problem. By varying the wavelength across an absorption edge of an atom in the crystal structure, changes can be induced in the diffraction pattern which can be used first to locate the atom in the unit cell and then to phase the Bragg reflections. Raman (19) has discussed the principles of this approach. Changes in the real part, f' , of the anomalous scattering with wavelength and Bijvoet differences due to the imaginary part, f'' , mimic isomorphous replacement changes. Moreover, be-

cause the real and imaginary parts are 90° out of phase with respect to each other, they act like two isomorphous derivatives in which the phase ambiguity left by one is resolved by the other for all Bragg reflections. Experiments demonstrating the usefulness of this technique in protein crystallography have been performed using conventional (20) and synchrotron radiation (21, 22) sources.

It is interesting to show how the large anomalous scattering effects described in our studies of anomalous scattering from Cs tartrate (4, 5) and the even larger effects described herein have altered our concept of the possible power of this phasing technique. It is well known that the contribution to a Bragg reflection from various scatterers within the unit cell can be described as a sum of vectors in the complex plane where the total scattering vector F is given by:

$$F = F_p + f_h + f' + f'' \quad [2]$$

in which F_p is the sum of scattering from light atoms, f_h is the normal scattering from the heavy atom, and f' and f'' are the real and imaginary components of anomalous scattering of the heavy atom.

The magnitude of F is the experimental quantity normally measured. When the wavelength of the incident x-ray beam is varied through an absorption edge of the heavy atom, it is clear from the earlier discussion that f' and f'' for the heavy atom will vary while F_p and f_h stay essentially constant. Thus, F is altered slightly in magnitude as f' and f'' vary. The magnitudes of any three sets of F s obtained at different wavelengths in principle are sufficient to yield unambiguously the phase of a Bragg reflection. This can be shown clearly by using the Harker construction (23). From such a construction for the three wavelength phasing case, it can be seen that the common intersection of the circles representing the three measurements determines the phase. Analogously, it could also be shown that measurement of the reflection at two wavelengths plus the Friedel related reflection at a third wavelength would also lead to an unambiguous value for the phase. In practice, of course, there are statistical errors in the determination of the magnitude of the F s, which necessitate that the various intersections are not points but rather are volume elements. It is common to treat the "lack of closure" by using the statistics derived from the intensity measurements (24). In order to address the question of the utility of anomalous scattering effects in phase determination, one must resort to some quantitative means for estimating the magnitude of the changes induced in the diffraction pattern or, more appropriately, given that the data are measured to a certain precision, to estimate the mean phase error that will result from certain anomalous scattering-induced intensity changes.

The relative scattering amplitudes of the protein and the anomalous scattering can be estimated by using a formula analogous to that derived by Crick and Magdoff (25) but modified to include anomalous scattering effects as discussed in ref. 26. Using these estimates, computer simulations were done to evaluate the accuracy with which the phase can be determined for several data collection schemes (26). Unlike isomorphous replacement, in which a specific number of derivatives may be available, anomalous scattering allows a continuous variation of the intensity changes. Consequently, the decision must be made whether to collect data at many wavelengths to low accuracy (due to time limitations) or at a few well-chosen wavelengths to higher accuracy or to make some compromise.

By the method based on the extension of the Crick–Magdoff formulation described in detail in ref. 26, calculations were carried out with the assumptions of one anomalously scattering

Cs atom per asymmetric unit in P2₁2₁2₁ and that 3% and 5% statistics were accumulated. The results were calculated with data measured at only three wavelengths: the first pair of a given reflection was chosen to maximize f'' [the measured value of f'' being 16 electrons and that of f' , -17 electrons (5)] and the third was selected for the largest change in f' given the first condition (f' and f'' of -27 and 9 electrons, respectively). For proteins of M_r 12,000, 25,000, 50,000, and 100,000, the assumption of 5% statistics resulted in a mean phase error of 30°, 35°, 43°, and 50°, respectively. A σ of 3% gave mean phase errors of 19°, 27°, 30°, and 42° for the same proteins. Even with data having a statistical error of 5%, anomalous scattering effects from the single Cs atom give a mean phase error of only 50° for a M_r of 100,000. Because maps should still be interpretable with 35°–40° mean phase errors, such results could lead to a structure solution. Additional measurements at different wavelengths or applications to a lower M_r protein would lead to reduced mean phase errors. Furthermore, if the data were measured to 3% (which is clearly possible by using electronic detection) one should certainly be able to phase a M_r 100,000 protein with the single derivative. The values used for f' and f'' are conservative in light of the earlier discussion, and the even larger effects observed for the Lns with white lines show substantial promise in multiple wavelength phasing experiments.

Solution Scattering. The scattering of x-rays from solution is commonly used to measure the general shape and size of solvated macromolecules. In this section we show that, through the use of anomalous scattering effects, it is possible in principle to increase the amount of structural information that may be extracted from a solution-scattering experiment. We discuss in particular the determination of specific intramolecular distances by using anomalous scattering.

Sufficiently dilute solutions contain independent solute molecules that are free to assume any angular orientation. In a solution scattering experiment, the scattering from many such randomly oriented molecules is observed simultaneously. Consequently, the measured scattering pattern is the unweighted average over all molecular orientations and can be represented as a sum of contributions from individual atoms and pairs of atoms within one molecule (27):

$$|A|^2 \sim \sum_{p=1}^n |f_p|^2 + \sum_{p,q=1}^n (f_p^r f_q^r + f_p^i f_q^i) \frac{\sin \mu r_{pq}}{\mu r_{pq}} \quad [3]$$

in which f_p (the complex scattering factor for atom p) is equal to $f_p^r + if_p^i$, r_{pq} is the distance between atoms p and q ; μ is $4\pi \sin \theta / \lambda$; and n is the number of atoms in the molecule.

The distance between a given pair of atoms can be extracted from the scattered intensity if the oscillatory term associated with the pair can be identified. There are several features of the above equation that make it possible to identify the $\sin \mu r / \mu r$ term for heavy atom pairs, specifically those with accessible absorption edges characterized by white lines. First, the scattering factor of an atom is constant as a function of wavelength except in a small range about its absorption edge (that is, except for the variations caused by the edge itself and the fine structure or EXAFS found above the edge). This effect permits the specific alteration of only those terms to which an anomalous scatterer contributes.

Furthermore, those atoms with white lines at their absorption edges clearly exhibit large changes in their scattering factors near the edge. This gives rise to correspondingly large changes in the coefficients of the oscillatory terms associated with the anomalous scatterer. These changes can be measured by the difference between on-edge and off-edge scattering intensities. Because the change in wavelength between these two cases is

a fraction of a percent, all of the variables other than the anomalous scattering factors can be considered constant and only the anomalous terms contribute to the difference.

For large changes in the scattering factor, the most dramatically changed terms are those in which both atoms of a pair are anomalous scatterers. For these pairs, the anomalous terms affect both scattering factors (of the pair), making the change in the coefficient of the oscillatory term more pronounced than with the anomalous/normal scatterer pairs.

The difference between the scattering on and off the absorption edge is dominated by the heavy atom pair oscillatory term(s). From computer simulations, the difference should amount to a few percent of the total scattered intensity for a small protein labeled with a single pair of anomalous scattering atoms. The oscillation of the difference can be analyzed to extract the anomalous scatterer pair spacing within the molecule. For larger molecules, anomalous/normal scattering changes will become increasingly important and will tend to obscure the term of interest. This will necessitate more sophisticated data acquisition and analysis procedures.

In order to estimate the applicability of this technique, x-ray solution scattering has been simulated by computer using Eq. 3. As an example, gramicidin A, a small ionophoric protein, was chosen because it is known to bind two Cs ions (28) in its dimeric form. From the crystallographic data, the Cs—Cs distance is 4.5 Å. Solution anomalous scattering data were simulated for this case, using the known values of the Cs anomalous scattering terms near the absorption edge. The difference between on-edge and off-edge scattered intensity (a change of about 2%) was estimated by measuring the period of the most pronounced oscillation graphically. In an actual experiment this would be difficult due to statistical fluctuations, and a more sophisticated analysis using a numerical Fourier transform of the difference spectrum would be more appropriate.

The interatomic spacing calculated from this period was observed to agree well with the value originally assumed. From this result, it appears that appropriate data analysis methods may be developed to extract interatomic distances from experimental data for macromolecules in solution.

Conceptually similar studies in which small-angle scattering was measured with and without heavy marker atoms have been done by Vainshtein *et al.* (34).

Biological Membranes. One class of biological systems in which structural information at the molecular level is difficult to obtain is that of biological membranes. Ln ions are known to bind quite strongly to natural and to artificial membrane systems. In view of the large x-ray anomalous scattering effects reported in this paper, it is of interest to discuss how these effects might be used to help in structural investigations of membrane systems.

Despite the application of direct methods to assign phases for x-ray diffracted intensities from membrane samples, inherent ambiguities remain in the resulting electron density profiles (29, 30). As in the x-ray crystallography applications discussed above, the anomalous scattering for membrane samples containing adsorbed Ln ions is expected to show sizable changes in the scattered intensity as the incident x-ray photon energy is tuned through an L₂ or L₃ edge. Provided the adsorbed Ln ions are well localized relative to the bilayer, these changes may be used to provide a unique phase assignment for the scattered intensity and hence to yield a unique electron density profile for the bilayer. This may be shown as follows. If the structure factor for a membrane of thickness d and electron density profile $\rho(z)$, in the absence of the Ln ions, is denoted by

$$F(k) = f_0 \int_0^d dz \rho(z) \exp(ikz) \quad [4]$$

in which f_0 is the mean scattering amplitude of the membrane material, and the complex scattering factor of the Ln ions located at z_0 is $f_{Ln}(\lambda)$ for x-rays of wavelength λ , then the structure factor of the Ln-doped bilayer is

$$F_{Ln}(k) = F(k) + f_{Ln}(\lambda)\rho_{Ln} \exp(ikz_0) \quad [5]$$

in which ρ_{Ln} is the change in electron density due to the Ln ions. Hence, for two different wavelengths λ_1, λ_2 in the vicinity of the L_2 or L_3 edge, the change in the scattered intensity is given by

$$\Delta I = (|f_{Ln}(\lambda_1)|^2 - |f_{Ln}(\lambda_2)|^2)\rho_{Ln}^2 + 2\rho_{Ln} R_e [(f_{Ln}(\lambda_1) - f_{Ln}(\lambda_2)) F^*(k) \exp(ikz_0)] \quad [6]$$

in which R_e denotes real part. So if z_0 is determined (by a Patterson transform), the angular dependent part of ΔI measures the phase of $F(k)$ directly.

To see how this might be applied, we consider the case of egg phosphatidylcholine for which a number of studies of Ln ion binding have been performed based on the effects of paramagnetic ions on the positions and widths of NMR lines (31).

The binding constant of La^{3+} to egg phosphatidylcholine is found to be on the order of 10^4 (≈ 10 times stronger than that of Ca^{2+}). The binding constant is yet an order of magnitude greater for acidic phospholipids such as phosphatidylglycerol. The binding site appears to be the phosphate region of the phosphatidylcholine head group, and two molecules appear to bind to each La^{3+} ion. We have performed computer simulation studies based on the known scattering of oriented phospholipid bilayers. Using the changes in f' of -20 electrons described above, we find changes of $>20\%$ in the small-angle diffraction intensities as the wavelength is varied from off to directly on the strong white-line absorption of the Ln ion.

In addition to providing phasing information, studies of this type should help confirm conclusions drawn from NMR work, that the Ln binding to the phosphatidylcholine head group causes a conformational change in which the head group moves from lying along the surface of the bilayer to an orientation normal to the bilayer (32).

Application to natural membranes or to artificial membranes containing incorporated proteins should also be useful in providing phasing information in these systems. Work on erythrocyte membranes (33) has suggested that Tb^{3+} binds preferentially to the protein component of the membrane. Because it is likely that the binding constant of Ln ions will be a strong function of the ionic character of surface groups on the membrane-bound protein, it may be expected that further studies will allow the geometry of specific surface binding sites to be elucidated.

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