Journal of
Synchrotron
Radiation

ISSN 0909-0495

Received 8 February 2005
Accepted 28 April 2005

X-ray fluorescence methods for investigations of lipid/protein membrane models

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The protective effect of the bisphosphonate drug xydiphone (K,Na-ethidronate) on membrane-bound enzyme damaged by lead ions has been studied. A protein/lipid film of Ca-ATPase/phosphatedylethanolamine deposited on a silicon substrate was used as a model system. The position of lead ions within the molecular film before and after the xydiphone treatment was determined using the total-reflection X-ray fluorescence method. This technique is based on the simultaneous measurement of the X-ray reflection and the yield of the fluorescence radiation excited by X-ray inelastic scattering. The possibility of directly locating lead ions is the main advantage of this approach. Xydiphone has been found to effectively eliminate lead ions that have been incorporated into Ca-ATPase molecules during a preliminary incubation in lead acetate solution. The lead ions that were bound at the sites of the Ca-ATPase attachment to the phospholipid monolayer have proved to be inaccessible for xydiphone. A preliminary incubation of Ca-ATPase in the xydiphone solution precluded the incorporation of lead ions into the protein.

Keywords: X-ray fluorescence; total external reflection; membrane-bound enzymes; Langmuir–Blodgett layers; Ca-ATPase; lead ions.

1. Introduction

The development of structure-sensitive physical techniques for studying biological systems nowadays offers new possibilities for biologists and medical scientists. The use of ordered protein/lipid films on solid substrates, which are adequate models of biological membranes, has given rise to a new approach for studying the structural organization and damage mechanisms of cell membranes. This approach provides a unique opportunity to model various biophysical and biochemical processes in biological membranes and obtain information on the structural and functional states of the protein/lipid layer at the molecular level. Here we demonstrate the possibilities of investigation of ordered protein/lipid films by the total reflection X-ray fluorescence method, one of the most promising modern techniques for analyzing thin films and near-surface layers. The molecular mechanisms of the protective effect of chelating agents on membrane proteins damaged by lead ions have been studied. A molecular film of the membrane enzyme Ca-ATPase and the phospholipid phosphatedylethanolamine deposited on a silicon substrate was used as a model system.

2. Chelators as the most effective agent for increasing the excretion of toxic metals

The mechanisms of the toxic effects of heavy metals on cell membranes, as well as the development of protective measures in the cases of acute and chronic intoxication with heavy metals, are currently paid much attention because of the large-scale environmental pollution of various industrial wastes (Meldrum & Ko, 2003; Perazella, 1996; Pande *et al.*, 2001; Flora *et al.*, 2003).

Lead is one of the most common heavy metals in the environment. It enters animal and human bodies *via* the respiratory and digestive systems and is very slowly removed. All lead compounds exert toxic effects, including the persistent damage of membrane proteins and the disturbance of the transport and barrier functions of cell membranes. Lead ions readily bind with functional polar groups of amino acids

(sulphhydryl and carboxyl groups) and lipids (Pande *et al.*, 2001; Flora *et al.*, 2003; Smith *et al.*, 1998).

Chelation therapy has proved to be very effective in metal intoxication. The main property of chelating agents is their ability to form stable low-toxic water-soluble compounds (chelates) with ions of most metals. This allows the intoxication to be reduced through the excretion of incorporated toxic metals from the body. The most extensively used chelating agents are, for example, sodium calcium EDTA (Tetacinum-calcium), D-penicillamine (Cuprenil), BAL and Succimerum (Meldrum & Ko, 2003; Flora *et al.*, 2003; Smith *et al.*, 1998).

In vivo studies on the excretion of incorporated toxic metals from the body with the use of chelating agents have demonstrated the following characteristic pattern of this process. Large amounts of heavy metals are eliminated at the initial stage of the treatment. During subsequent treatment, however, the excretion drastically decreases or even completely ceases, although some metal still remains in the body, which is confirmed by the experimental and clinical signs of intoxication (Pande *et al.*, 2001; Flora *et al.*, 2003; Smith *et al.*, 1998). The excretion of 'residual' heavy metals is difficult; it involves additional administration of chelating agents, which is often accompanied by undesirable effects on the patient (Meldrum & Ko, 2003).

Notwithstanding numerous laboratory tests and clinical research, the aforementioned decrease in the rate of heavy-metal excretion remains unexplained. The explanation should probably be searched for at the molecular level rather than at the level of heavy-metal accumulation in individual organs (Meldrum & Ko, 2003; Pande *et al.*, 2001; Flora *et al.*, 2003).

Another problem is that almost all known chelating agents have undesirable side effects, especially when administered for a long time. Therefore the search for chelating agents with pre-determined characteristics that effectively eliminate metals at doses harmless for the patient is an important task.

In this study we have used xydiphone (K,Na-ethidronate), a bisphosphonate chelating agent. Bisphosphonates have a P—C—P chemical bond and are analogs of pyrophosphates, natural chelators of biological origin (Matkovskaya *et al.*, 2001; Fleisch, 1998; Gulson *et al.*, 2003). Clinical trials have demonstrated that the bisphosphonate xydiphone effectively enhances the excretion of incorporated metals and has no toxic effect in the case of long-term treatment.

3. Materials and methods

We used 1,2-dipalmitoyl-sn-glycero-3-phospho-*O*-ethanolamine DPPE (Sigma, USA), the chelating agent xydiphone (Moskhimpreparaty, Russia) and $Pb(CH,COO)_2$ (Reakhim, Russia). The salts and the components for protein solubilization were $CaCl_2$ and $MgCl_2$, imidazole (Sigma, USA) and triton X-100 (Merck, Germany).

The membrane preparations of Ca-ATPase were singled out from skeletal muscles of rabbits by the method of differential centrifuging at the Department of Biochemistry of the Biological Faculty of Moscow State University (Ritov *et al.*, 1977). Ca-ATPase or a calcium pump of sarco(endo)plasmic reticulum is one of the most important energy-dependent calcium-regulating components of a cell. The protein has a molecular mass of 110–115 kDa and dimensions 100 Å × 80 Å × 140 Å (Toyoshima *et al.*, 2000). Various iso-forms of Ca-ATPase consist of 994–1042 amino acid residues among which there are 24 SH-containing cysteinic molecules, which can bind lead ions. The protein was solubilized using a solution of the following composition: imidazole 25 m*M*, triton X-100 0.5%, CaCl 1 m*M* and MgCl 1 m*M*. Protein concentration in the solution was 5.25 mg ml^{-1}.

3.1. Immobilization of Ca-ATPase molecules on the solid substrate

Protein adsorption on a preliminarily formed lipid monolayer carrying a specified electric charge is one of the most efficient methods for deposition of ordered protein/lipid films in which the functional activity of the proteins is preserved (Tiede, 1985). We immobilized Ca-ATPase on a monolayer of DPPE lipid, which had been transferred onto a solid substrate by the Langmuir–Blodgett method. Thus the combination of the Langmuir–Blodgett method with molecular self-assembling has been used.

3.1.1. Control sample. A solution of phospholipid DPPE at a concentration of 0.3 mg ml^{-1} in the mixed solvent (6/1 chloroform/methanol) was spread onto a subphase (triply distilled water, pH 6.5). The monolayer was compressed at a rate of 0.01 m^2 min^{-1} up to a surface pressure of 40 mN rn^{-1}. Phospholipid monolayers were transferred to hydrophobic silicon substrates by the vertical-lift (Langmuir–Blodgett) method at a rate of 3 mm min^{-1}. The substrate with a DPPE monolayer was placed into a vessel on the bottom of a Langmuir trough. Upon cleaning the subphase surface from the Langmuir DPPE monolayer, the substrate in the vessel filled with water was taken away from the trough.

Adsorption immobilization of protein molecules on the transferred phospholipid monolayer was performed for 24 h from the working solution with a protein concentration of 5.25×10^{-2} mg ml^{-1} at room temperature. Upon protein adsorption, the sample in the vessel was washed with distilled water and the vessel was placed on the bottom of the Langmuir trough filled with water (subphase without monolayer). Then the phospholipid monolayer was formed on the water subphase at a surface pressure of 40 mN m^{-1}. Finally the substrate was taken away through a newly formed DPPE monolayer (the velocity of the substrate motion was 1.5 mm min^{-1}). As a result, the protein layer was sandwiched between two phospholipid monolayers. A schematic view of the sample is shown in Fig. 1.

3.1.2. Sample 1. During solubilization of Ca-ATPase we added the solution of lead acetate; the lead concentration in the solubilized solution was 3×10^{-4} *M*. The mixture was incubated for 1 h and then added to the vessel filled with water; the lead concentration in the vessel was 3×10^{-6} *M*. Protein was immobilized in the same way as for the control sample. The substrate was taken away through a newly formed DPPE monolayer.

Table 1
The investigated samples.

Sample	Description	Aims of investigations
Control sample	Pure Ca-ATPase was immobilized on the silicon substrate	To examine the efficiency of the used immobilization method
Sample 1	Ca-ATPase incubated in the solution of lead acetate was immobilized on the silicon substrate	To examine the adsorption efficiency of the protein molecules altered by lead ions; to determine the lead ions distribution inside the protein/lipid film
Sample 2	Ca-ATPase incubated in the solution of lead acetate was immobilized on the silicon substrate and then washed with a xydiphone solution	To determine the lead ions distribution inside the protein/lipid film after treatment by xydiphone solution; to study the effect of xydiphone on protein molecules damaged by lead ions
Sample 3	Ca-ATPase preliminarily incubated in the xydiphone solution was immobilized on the silicon substrate and then washed with a solution of lead acetate	To examine the preventive protective effect of xydiphone
Sample 4	Pure Ca-ATPase was immobilized on the silicon substrate and then washed with a solution of lead acetate	To test the capacity of protein molecules immobilized on the solid substrate for binding lead ions

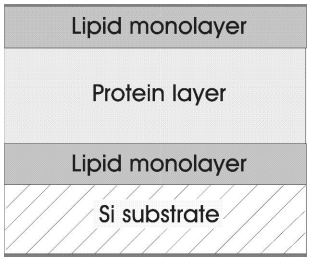

Figure 1
Schematic view of a protein/lipid film on a solid substrate.

3.1.3. Sample 2. During solubilization of Ca-ATPase we added the solution of lead acetate; the lead concentration in the solubilized solution was 3×10^{-4} *M*. The mixture was incubated for 1 h and then added to the vessel filled with water; the lead concentration in the vessel was 3×10^{-6} *M*. Protein was immobilized in the same way as for the control sample. However, upon incubation in the working protein solution the sample in the vessel was first washed with distilled water and then kept for 3 h in 0.07 *M* xydiphone solution. Then the sample was washed again with water and the vessel with the sample was placed at the bottom of the Langmuir trough filled with water (subphase without a monolayer). The substrate was taken away through a newly formed DPPE monolayer.

3.1.4. Sample 3. During solubilization of Ca-ATPase we added xydiphone; the xydiphone concentration in the solubilized solution was 0.07 *M*. The mixture was incubated for 5 h. Protein was immobilized in the same way as for the control sample. However, upon incubation in the working protein solution the sample in the vessel was first washed with distilled water and then kept for 3 h in a 3×10^{-6} *M* solution of lead acetate. Then the sample was washed again with water and the vessel with the sample was placed at the bottom of the Langmuir trough filled with water (subphase without a monolayer). The substrate was taken away through a newly formed DPPE monolayer.

3.1.5. Sample 4. Protein was immobilized in the same way as for the control sample. However, upon incubation in the working protein solution the sample in the vessel was first washed with distilled water and then kept for 3 h in the 3×10^{-6} *M* solution of lead acetate. Then, the sample was washed again with water and the vessel with the sample was placed at the bottom of the Langmuir trough filled with water (subphase without a monolayer). The substrate was taken away through a newly formed DPPE monolayer.

The philosophy of the samples preparation is summarized in Table 1.

3.2. Investigation of thin films by the total-reflection X-ray fluorescence method

The total-reflection X-ray fluorescence (TRXF) technique is based on simultaneous measurements of the angular dependence of X-ray reflection and fluorescence yield excited by the primary X-ray beam as the incident angle is scanned through the total external reflection region. A detailed description of the TRXF technique can be found elsewhere (see, for example, Bedzyk *et al.*, 1989; Yun & Bloch, 1990; Zheludeva *et al.*, 1997). In TRXF experiments, energy spectra of characteristic fluorescence emitted from the sample are recorded at each point in the reflectivity curve, in contrast with conventional X-ray fluorescence spectroscopy where the angle of the incident X-ray beam is held constant. The integrated intensity under a specific peak from a selected element is plotted as a function of the incident angle. As the incident angle is increased from zero to the critical angle for total external reflection, θ_C, the intensity of the complicated X-ray field (the so-called X-ray standing wave/evanescent wave) changes and in turn essentially induces dramatic changes in the fluorescence yield. As a result the angular dependence of the fluorescence yield will exhibit large modulations in the total external reflection angular region. The key point of the TRXF method is that the shape of these modulations is strongly dependent on the mean position of the atoms in the direction normal to the surface and the thickness of the random distribution about this position. These two parameters can be determined from TRXF data with an accuracy of a few Å. In addition, owing to the element selectivity of TRXF measurements, the behaviour of several types of atoms inside the film may be studied individually. Thus the TRXF method offers the opportunity to locate the atom position directly from the analysis of the angular dependence of the fluores-

cence yield. The possibility of using the TRXF technique for the characterization of biomembrane models has been demonstrated in a set of experiments investigating protein films (Wang *et al.*, 1994; Zheludeva *et al.*, 2001).

TRXF experiments should be performed at a fixed energy in the hard X-ray range (5000–20000 eV) and at small grazing angles (0–1°). The main difficulty with TRXF measurements on protein films is the very poor counting of the fluorescence signal, because the density of ions emitting fluorescence is low. For this reason, high brilliant synchrotron X-ray radiation is required for experiments of this kind. All X-ray reflectivity and fluorescence measurements presented in this paper were performed at two synchrotron radiation facilities, BESSY II station KMC2, Berlin, Germany (Erko *et al.*, 2000) and ESRF beamline ID10B, Grenoble, France (Konovalov *et al.*, 2002).

In both experiments the energy of the X-ray beam was chosen to be 13500 eV, providing the optimal condition to excite Pb $L\alpha$ fluorescence. The reflected X-ray beam was measured by a scintillation (NaI) detector. The fluorescent signal was recorded using a Peltie cooled detector (ROENTEC). In experimental measurements at BESSY (control sample and samples 1–3) the synchrotron X-ray beam from the bending magnet was monochromated by a double-crystal monochromator (SiGe graded crystals), and the incident and reflected beams lay in the horizontal plane. In experimental measurements at ESRF (sample 4) the synchrotron X-ray beam from the undulator was monochromated by two diamond (111) crystals, the incident and reflected beams lay in the vertical plane, and the primary X-ray beam was deflected from the horizontal plane and sent to the sample surface at a desired angle of incidence by means of the deflecting crystal.

4. Results and discussion

4.1. Control sample

Fig. 2 shows a typical fluorescence spectrum of the control sample. All spectra were recorded for angles smaller than the critical angle for total external reflection for silicon substrate, which allowed us to reduce the background radiation. The peaks of argon (Ar K_α) from the atmosphere and silicon (Si K_α) from the substrate are clearly seen in the spectra. These control measurements allowed us to exclude the effect of artifacts, such as admixtures in the protein/lipid films and impurities in the incident radiation, during subsequent studies.

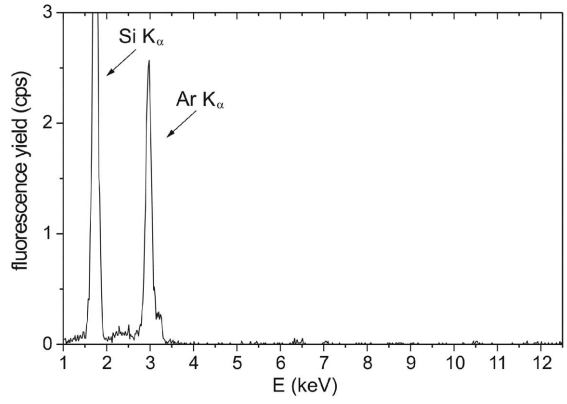

Figure 2
X-ray fluorescence spectrum from the control sample (cps = counts per second).

To test the quality of the protein/lipid film, we performed additional studies using atomic force microscopy (AFM). The measurements were performed in air with the atomic force microscope P47-SPM-MDT (Russia, NT-MDT) in tapping mode. We used NSC11 silicon cantilevers (Mikromasch, Estonia) with a point radius $R \leq 10$ nm.

Fig. 3(*a*) shows a topographic image of the surface of the control protein/lipid film. As can be seen, the film was formed by microscopic particles uniformly distributed over the surface. The shape of the particles (globules) was close to spherical; their size was 18–19 nm, which corresponded to the size of the Ca-ATPase molecules (Toyoshima *et al.*, 2000). Analysis of surface profiles allowed us to estimate the micro-relief height difference at 1.5 nm.

4.2. Samples 1 and 2

Figs. 4(*a*) and 4(*b*) present the characteristic spectra of the fluorescence radiation from protein/lipid films 1 and 2. The

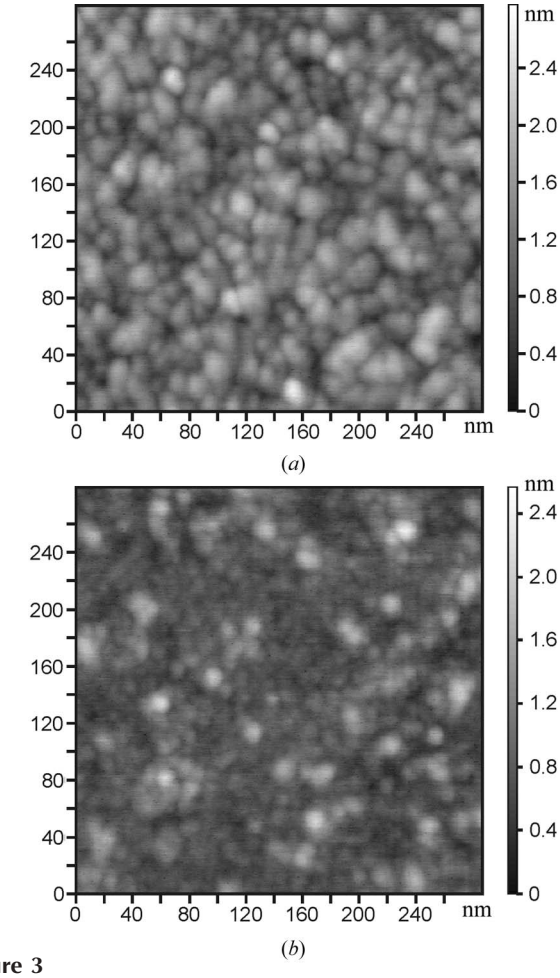

Figure 3
Typical AFM images of the protein/lipid films deposited on the silicon substrate: (*a*) control sample, (*b*) sample 3.

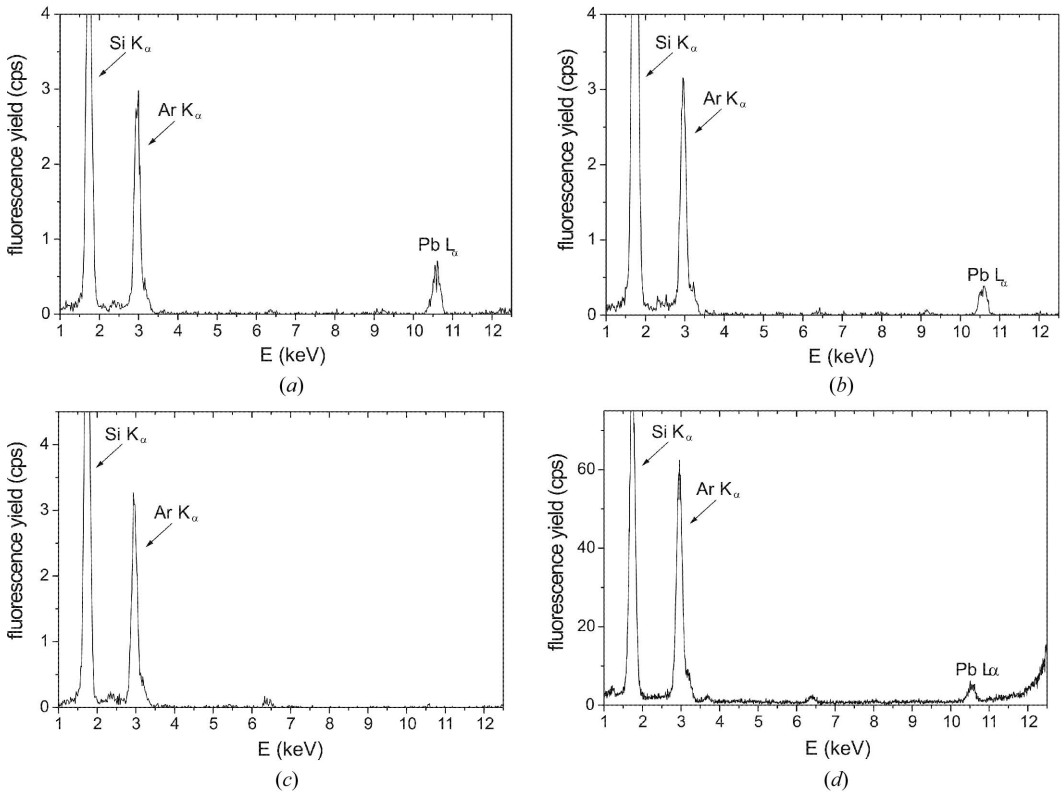

Figure 4
X-ray fluorescence spectrum from the protein/lipid films: (*a*) sample 1, (*b*) sample 2, (*c*) sample 3 and (*d*) sample 4 (cps = counts per second).

integrated intensity of Pb L_α fluorescence as a function of angle is plotted in Fig. 5. Even a simple inspection of the fluorescence curves for samples 1 and 2 shows the considerable differences in the shape of these curves. The fluorescence curve from sample 2 is sharply peaked at $\theta = \theta_C$, which is characteristic of the situation when ions, that emit fluorescence radiation, are located in a thin layer near the bottom boundary of the film (at the film/substrate interface). In this case the maximum of the fluorescence yield is observed in the vicinity of the critical angle for total external reflection for the silicon substrate, where the node of the X-ray standing wave coincides with the substrate surface. The maximum of the fluorescence yield from sample 1 is noticeably displaced towards small angles and the fluorescence curve is much broader. This is typical of the situation when lead ions are distributed over a thicker layer whose thickness is comparable with or exceeds the period of the standing wave in the vicinity of the critical angle of total external reflection of the substrate, θ_C. Quantitative estimation of the thickness of the layer in which lead ions are distributed can be obtained by fitting model calculations to the Pb fluorescence data. For calculating the reflectivity and the intensity of the fluorescence yield from protein/lipid film the recursive method developed by Parratt (Wainfan *et al.*, 1959) has been used. The best fit of the Pb fluorescence data corresponds to a thickness of the Pb layer of 13 nm for sample 1 and 1 nm for sample 2. Thus analysis of the TRXF data shows that, in the case when Ca-ATPase was incubated in the lead acetate solution and then immobilized

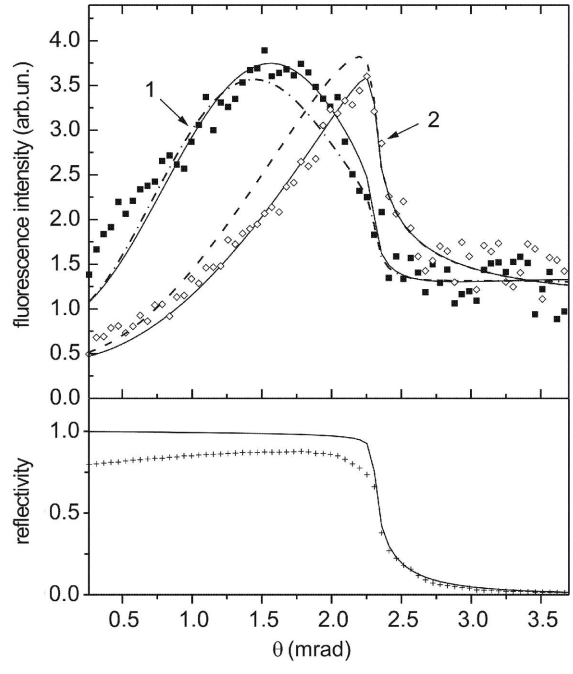

Figure 5
Experimental and calculated fluorescence angular dependencies from the protein/lipid films. Curve 1: Pb fluorescence angular dependence for sample 1. Solid line, best fit (lead layer thickness 13 nm); dashed line, calculations for lead layer thickness of 15 nm. Curve 2: Pb fluorescence angular dependence for sample 2. Solid line, best fit (lead layer thickness 1 nm); dashed line, calculations for lead layer thickness of 3 nm. Bottom curves, X-ray reflectivity.

on the solid substrate, lead ions are distributed in the 13 nm-thick layer. When the protein/lipid film was washed after immobilization in the xydiphone solution the lead ions were found to remain in the thin layer at the bottom of the protein/lipid film.

In order to demonstrate the sensitivity of the TRXF measurements to the thickness of the lead layer, the fluorescence curves calculated for 15 nm and 3 nm are also plotted in Fig. 5.

4.3. Samples 3 and 4

As in the control sample, the characteristic fluorescence spectra for samples 3 and 4 were recorded for several angles in the region below the critical angle for total external reflection for silicon substrate. Measurements on sample 3 were performed to examine the preventive protective effect of xydiphone. The protein was preliminarily incubated in the xydiphone solution and then immobilized on the solid substrate and treated with a lead acetate solution. Fig. 4(*c*) shows the characteristic fluorescence spectrum of sample 3. In this spectrum the peaks of argon and silicon, but not the lead peak, are seen.

In order to confirm the presence of a protein/lipid film on the substrate we carried out the APM measurements on sample 3 (Fig. 3*b*). Comparing APM images for the control sample and sample 3 one can see that the surface density of the globules is considerably smaller in sample 3, where the protein was incubated in the xydiphone solution before immobilization. This might be accounted for by incorporation of xydiphone molecules into Ca-ATPase that resulted in the reduction in binding of Ca-ATPase molecules with the phospholipid monolayer.

To test the capacity of Ca-ATPase immobilized on the solid substrate for binding lead ions, we performed additional TRXF measurements on sample 4. The characteristic fluorescence spectrum of sample 4 is presented in Fig. 4(*d*). Here the Pb L_α fluorescence peak is clearly distinguished. This experimental result indicates that lead ions are present in this film, *i.e.* the protein molecules immobilized on the solid substrate may bind lead ions from a lead acetate solution.

5. Conclusions

The ordered protein/lipid films damaged by lead ions and treated by a bisphosphonate drug xydiphone have been studied by the spectroscopic-selective X-ray method of total external X-ray fluorescence. It was demonstrated that this technique permits Ca-ATPase molecules to be scanned in the direction normal to the surface and the position of lead ions incorporated into the protein molecules to be determined before and after the xydiphone treatment. We found that even for the case of separate isolated protein/lipid film the chelating agent xydiphone did not ensure the removal of all lead ions. At easily accessible sites, xydiphone chelated lead ions bound to Ca-ATPase molecules; these water-soluble chelates were subsequently removed by washing. Lead ions incorporated into Ca-ATPase at the bottom of the protein layer where the protein globules attached to the phospholipid layer were inaccessible for the chelating effect of bisphosphonate. It was also shown that xydiphone is effective in preventing lead incorporation into Ca-ATPase: protein preliminarily incubated in the xydiphone solution and then immobilized on the phospholipid monolayer did not bind lead ions; this can probably be attributed to the shielding of potential binding sites by xydiphone molecules incorporated in the protein globules.

The authors are grateful to Professor Yu. A. Vladimirov for many valuable comments which were used to improve the paper. This work was performed under the financial support of the Russian Foundation for Basic Research (grants 05-02-17391-A and S-1404.2003.2).

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