BOUND TRACE ELEMENT CONTENT OF BOVINE RETINAL DISK MEMBRANES AS DETERMINED BY PARTICLE-INDUCED X-RAY EMISSION

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ABSTRACT Particle-induced x-ray emission (PIXE) was used to determine the trace element content of bovine retinal disk membranes. PIXE is a multielemental analytical technique capable of the simultaneous detection and quantization of all elements from sodium and above in atomic number. The multielemental capability of PIXE allows the analysis time per element to be very low if a number of elements are detected in each sample. In addition, the multielemental capability of PIXE can be used to determine elemental content with respect to an internal reference. Here the content of detected trace element per rhodopsin was determined without recourse to an external rhodopsin assay. This was accomplished by using the sulfur content of rhodopsin as an internal reference. Detected trace element contents per rhodopsin were 1.58 ± 0.049 Ca, 0.081 ± 0.024 Fe, 0.393 ± 0.200 Cu, and 0.150 ± 0.031 Zn. Upper limits were placed on the amount of manganese, molybdenum, and nickel per rhodopsin as 0.019, 0.019, and 0.006, respectively. Two proteins known to be present in disk membranes, retinol dehydrogenase and a large protein, ~238,000 mol wt, are considered as potential metallo-proteins. No correlation was observed between the content of any detected element and bleaching levels.

INTRODUCTION

In 1970, Johannson et al. (1) demonstrated the usefulness of particle-induced x-ray emission (PIXE) as a multielemental analytical technique. Subsequently, significant research in varied fields has been performed using PIXE. Much of the PIXE analyses has been for environmental or materials research. Unfortunately, PIXE has not been widely used in biological research. This is probably due to the lack of commercial PIXE systems and the fact that most systems that exist were developed and are used by physical scientists. However, the studies that have been performed on biological materials have demonstrated PIXE's capabilities as a quantitative technique for certain types of biological samples, e.g., sera and tissues (2).

The disk membrane of vertebrate rod outer segments is unusual in that most of the protein is present as rhodopsin, which has been shown to constitute 85±5% of the membrane protein (3). This should result in a relatively simple membrane system whose functions can be studied and perhaps understood at the molecular level. One area of current interest is the composition of trace elements in disk membranes. This has been in part due to the calcium hypothesis of visual transduction proposed by Yoshikami and Hagins (4, 5) in 1971. This hypothesis assigns a messenger role in visual transduction to calcium and predicts that calcium is released from the disk membrane as a result of light absorption by rhodopsin. There have been numerous attempts to measure a decrease in the calcium content of retinal disk membrane (see reference 6 and references cited therein). Research has also indicated a possible function for zinc in disk membranes. Tam et al. (7) detected a greater than one-to-one stoichiometry of zinc to rhodopsin and also a change in zinc levels due to bleaching. In addition, studies have been performed detecting other trace elements in disk membranes, but not associating them with visual transduction (8, 9).

The purpose of this study was to apply PIXE to the determination of the trace element content of disk membranes and attempt to associate the detected levels with intrinsic disk membrane proteins. In addition, it will be demonstrated how trace elements can be used as an indicator and assay for the presence of metallo-proteins.

MATERIALS AND METHODS

Particle-induced x-ray emission requires a particle accelerator, a target to be analyzed, and a suitable x-ray detector. The accelerator furnishes a beam of heavy, charged particles (usually protons) that are then directed so that the beam is incident upon the target. The incident beam creates inner shell electron vacancies in the target atoms. These vacancies can be filled by an outer shell electron falling to a lower energy level and emitting a characteristic x ray.

All possible transitions are not used in PIXE analysis. This is because the intensities of possible x-ray emissions resulting from the filling of an electron vacancy vary widely. The most commonly used x-ray lines are the K_α lines (an electron filling a lowest shell vacancy [$n = 1$] from the next

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highest shell [$n = 2$]) and the K_β lines (an electron filling a lowest shell vacancy from any shell higher than $n = 2$). K_β transitions are always higher energy than K_α, and the intensities of the two lines have a constant ratio for a particular element. Thus, characteristic x-ray spectra are relatively simple to interpret as opposed to atomic transitions at other wavelengths.

Target preparation is an integral part of PIXE analysis. This is extensively discussed in reference 10. In general, all that is required is to stabilize the target so that it can be placed in a vacuum. However, this can result in the loss of volatile elements from the sample to be analyzed. Therefore care must be taken in the interpretation of data involving volatile elements. In addition, a target may require the use of a backing for mechanical strength. The selection of backings is also treated in reference 10.

Several aspects of beam-sample interaction should be considered before sample preparation. As the beam enters the sample it loses energy resulting in a change in the cross section for x-ray production. This effect must either be considered when data are fit or can be neglected if sufficiently thin targets are used. However, this results in lower sensitivity and/or longer analysis times. If the sample material is a poor conductor, sample charging can result in noisy spectra. This has been eliminated either by mixing the material with a conducting material, e.g., elemental carbon, or neutralizing the sample with an electron flood gun.

Most PIXE analyses are done with Si(Li) energy dispersive detectors. This enables the coupling of an accelerator and a Si(Li) detector to yield a rapid and sensitive analytical system capable of simultaneously determining most of the target's elemental composition. The spectra obtained are more complicated than those resulting from wavelength dispersive detectors and often require an accurate fitting program for data analysis. For more detailed discussions of the PIXE technique, the preceding references should be consulted, particularly reference 10, which is an excellent review of the technique.

Preparation of Cattle Retinal Disk Membranes

Bovine retinas were purchased from Hormel (Austin, MN). The whole eyes were removed within 20 min of the animals' death and immediately placed in wet ice. After 50 were collected, they were taken to a dark room where the retinas were removed under red light. Each retina was rapidly frozen by being placed in a vial on dry ice. The vials were filled with 50 retinas, wrapped with aluminum foil, and transferred to freezer storage until shipment. The vials were shipped in dry ice (J. Schmitz, personal communication). Each packaged contained dry ice upon receipt and was immediately transferred to a freezer maintained at −10°C until use. Intact disk membranes were obtained using the separation procedure of Smith et al. (11). In this procedure, rod outer segments are lysed by osmotic shock and osmotically intact disk membranes collected by flotation on a Ficoll solution. As a result of the extensive manipulation, the protein remaining with the disk membranes is probably integral membrane protein, and the trace metals remaining with these proteins are tightly bound.

During this procedure, a Kodak 1 filter was used to furnish dim red light (Eastman Kodak Co., Rochester, NY). Centrifugation was done at 4°C, and the preparations were maintained in ice during the remaining time except while being exposed to light.

Light Exposure of Disk Membranes and PIXE Target Preparation

After separation, the disk membrane solution from 50 retinas was divided into 10 aliquots, placed in small transparent vials, and stored in ice until used. The vials were equally divided between those to be exposed to light and controls. A vial to be exposed and its control were placed on a counter at a predetermined distance from a light source. The control vial was covered while the other vial was exposed to a predetermined light flux. Then both vials were immediately spun on a Beckman Microfuge (8874 xg; Beckman Instruments, Inc., Fullerton, CA) for 1 or 5 min to sediment the disk membranes. All 10 vials obtained from a preparation of 50 retinas were spun for the same time. Subsequently, the sediment was pipetted onto previously prepared Formvar films (2% Formvar; Electron Microscopy Sciences, Fort Washington, PA) held by a PIXE target holder. These targets were allowed to dry in closed containers in an inert atmosphere. As a control for possible contamination during the drying process, blank Formvar targets were placed in each container. PIXE targets were also made from solutions extracted at each step in the sample preparation procedure in order to determine the trace element contamination in the chemicals utilized during the separation procedure.

PIXE Analysis

PIXE analysis was done with a proton beam of 5 MeV obtained from the Florida State University (Tallahassee, FL) FN accelerator. The beam was made uniform by means of a beam sweep and subsequently collimated by a 4.5 mm diam carbon collimator located 1 m in front of the target chamber. The accumulated charge and beam current were monitored by a Faraday cup located past the target. Beam currents were kept low, ~5 nA, to prevent pulse pile-up in the x-ray detector. Each target was analyzed twice: once for an accumulated charge of 1 μC without the use of an external x-ray absorber, and then for an accumulated charge of 20 μC with a 0.356 mm external Mylar absorber.

Because the x-ray production cross section for light elements is large for the beam energy used and they are present in high concentrations, the x rays from these elements dominate the x-ray detector unless an external absorber is used to absorb lower energy x rays. For this reason two runs were made on each target. The first run (without external absorber) was to determine the sulfur content. Then an external absorber was placed in the x-ray path, and another run was made to determine the heavier element composition.

The resulting x rays were detected with a Si(Li) Kevex detector (Ortec, Oak Ridge, TN) with a measured full width at half maximum of 200 eV at 6.4 KeV. Count rates were between 3 and 5 thousand counts per second. The exit window from the PIXE chamber was 0.0064 mm thick Mylar. The total air gap between the exit window and detector window was 7 mm and the detector window was 0.013 mm thick Be. The vacuum in the PIXE chamber was below 10^{-5} torr. The target and detector were positioned so that the target was located in a vertical plane oriented so that the beam hit the target at an angle of 60° rather than perpendicular. The x-ray detector was located perpendicular to the target.

Determination of Elemental Content per Rhodopsin Molecule

One of the features of the PIXE technique is its multielemental nature that allows the determination of most elements simultaneously. (Low atomic number elements [sodium and lower] are not normally detected due to the low transmission and lower detector efficiency for x rays of these energies.) In this study, the multielemental aspect of PIXE was used to a further advantage. Since the number of sulfurs contained in rhodopsin has been studied (12), the disk membrane trace element content was determined per rhodopsin by referring it to the target's sulfur content. The trace element content per rhodopsin is the ratio of the amount of trace element detected to the amount of sulfur detected, modified for the number of sulfurs present per rhodopsin in the disk membrane. For these calculations, 26 sulfurs per rhodopsin were used as determined by Hargrave et al. (12). Since rhodopsin constitutes ~85% of the membrane protein, it would be expected to contain 85% of the membrane sulfur. This would result in 30.6 sulfurs per rhodopsin in disk membranes. Papermaster et al. (13) determined a slightly higher value of 90% of disk methionine contained in rhodopsin. Assuming the value for cystine to be similar implies a possible error of 6% in the assumption that the ratio of sulfur content of rhodopsin to sulfur content of disk membrane can be used as an internal reference. This enabled the trace element

content per rhodopsin to be determined for each individual sample. Therefore, separate rhodopsin assays were not required.

Other possible sources of sulfur would be free amino acids contained inside the disk lumen. Taurine is the most probable of these. Its presence in preparations of visual pigments has been studied by Pourcha (14). He used radioactive sulfur to localize taurine in the retina; however, none was detected in the disk membranes.

Mitochondria Assay

Since mitochondria contain large amounts of calcium, copper, and iron, an assay of succinate dehydrogenase was performed to determine the extent of mitochondrial contamination (15). The activity was determined for crude rod outer segments that were obtained after the first centrifugation and for the final disk membrane preparation. The succinate dehydrogenase activity in the disk preparation was lower by a factor greater than 20 compared with the crude rod outer segment preparation. Activity was expressed in terms of total protein as determined by the method of Bradford (16).

Another assay of potential mitochondria contamination is the iron content of the sample. Szuts (6) assumes that all iron present in his disk membranes preparations was due to mitochondria (17). He detected 0.04 ± 0.01 iron per rhodopsin. If this assumption is correct, the preparations used in this study are not as pure since the detected iron value was 0.081 per rhodopsin.

Spectral Data Fitting

All spectra were fit using the FORTRAN program HEX (18). HEX is a nonlinear least-squares fit using a Marquardt algorithm and incorporating the physical processes of x-ray production and detection. The nonlinear parameters model target, window, and detector x-ray absorption, detector efficiency, and bremstrahlung background. Parameters are used linearly to fit the intensity of each x-ray line and nonlinearly to model the adsorption process and beam energy loss. After the fit is optimized, these parameters are used to determine the amount of each element detected.

RESULTS AND DISCUSSION

The detected trace element content of dark-adapted disk membranes as determined by this study is given in Table I. None of the detected elements has a one-to-one stoichiometry with rhodopsin, using rhodopsin sulfur content as 26 sulfurs per rhodopsin. As previously mentioned, these values probably represent intrinsic membrane values instead of in vivo levels associated with disks.

The amount of calcium detected in disk membrane targets (1.58 ± 0.49 per rhodopsin) falls within the range determined by other researchers. Szuts and Cone (6) found 0.2 calciums per rhodopsin, Hess (8) reported 0.25 calciums per rhodopsin, Hendriks et al. (19) reported 11 calciums per rhodopsin, and Schnetkamp (20) detected 5.7 calciums per rhodopsin. As other researchers have determined, the calcium concentration is high enough to indicate it could function as the transmitter in visual transduction.

TABLE I
DETECTED TRACE ELEMENT CONTENT OF RETINAL DISK MEMBRANES

Element	Elemental content
Ca	1.58 ± 0.49
Fe	0.081 ± 0.024
Cu	0.393 ± 0.200
Zn	0.150 ± 0.031

The detected trace element content was calculated as a ratio of trace element to rhodopsin as described in the text. The data are the mean and standard deviation calculated from computer fits to x-ray spectra of 10 different PIXE targets.

Other roles for disk membrane calcium have been suggested. Hess determined that calcium content in in vivo light-adapted rod outer segments was higher than in in vivo dark-adapted rod outer segments, and indicated calcium may have a role in light-dark adaptation (9). In addition, it has been proposed that calcium may function as a regulator of various enzymes and nucleotides that may be involved in the visual transduction process (21).

The level of copper in disk membranes as determined in this study is 0.393 ± 0.200 per rhodopsin. From PIXE analysis for trace elements in the chemicals used during separation, it was found that sucrose contained copper as a contaminant. For a 5% sucrose solution, the copper level was 0.08 mM. If copper binds to disk membranes, it is possible this contamination contributed a significant amount to the copper content. Therefore, the copper levels detected may not be indicative of the true disk levels. However, Hess had previously detected copper in rod outer segments and suggested it may be due to superoxide dismutase, a cytoplasmic enzyme containing two zinc and two copper ions per molecule (9).

The case for a zinc binding protein appears much better. As Table I shows, zinc levels are relatively consistent and indicate that zinc is closely associated with the amount of disk membrane. Detected levels were 0.150 ± 0.031 zinc per rhodopsin. One possibility for a zinc binding protein is retinol dehydrogenase. It has been detected as a minor protein in bovine rod outer segments by Blaner and Churchich (22). It is a membrane bound enzyme that catalyzes the interconversion of retinol and retinal. They were not able to completely purify the enzyme, and therefore, only an upper limit, of 120,000, for the molecular weight is available. The trace element content of this enzyme is not known. However, liver alcohol dehydrogenase has been thoroughly studied. It contains 2-g atoms of zinc per mole of protein and has a molecular weight of 73,000 (23). If retinol dehydrogenase is assumed to contain two zinc ions, the zinc value in Table I yields 0.075 retinol dehydrogenase per rhodopsin. For a rhodopsin molecular weight of 38,000 and a retinol dehydrogenase molecular weight of 73,000, 12.2% by weight, of the disk membrane would be retinol dehydrogenase. This would include most of the nonrhodopsin disk membrane protein.

Tam et al. (7) found a one-to-one stoichiometry between zinc and rhodopsin in preparations that were obtained from previously unfrozen retinas, but they were unable to detect zinc in preparations from retinas that had been previously frozen. Since the retinas used in this study had been frozen

TABLE II
DETECTED TRACE ELEMENT CONTENT OF DISK MEMBRANES AS A FUNCTION OF RHODOPSIN BLEACHING

Bleaching	Number of targets	Ca	Fe	Cu	Zn
None	10	1.59 ± 0.49	0.079 ± 0.024	0.425 ± 0.193	0.219 ± 0.112
0.1%	2	1.65 ± 0.006	0.079 ± 0.006	0.261 ± 0.025	0.157 ± 0.006
1%	6	1.70 ± 0.17	0.100 ± 0.037	0.447 ± 0.275	0.199 ± 0.031
10%	2	1.55 ± 0.268	0.113 ± 0.031	0.420 ± 0.137	0.203 ± 0.081
100%	3	1.39 ± 0.094	0.072 ± 0.025	0.454 ± 0.212	0.192 ± 0.019

The detected trace element content was calculated as a ratio of trace element to rhodopsin as described in the text. The data are the mean and standard deviation calculated from computer fits to x-ray spectra for the number of targets indicated at each bleaching level.

and zinc was easily detected, comparison of their results with this study is difficult.

The concentration of iron is 0.081 ± 0.024 per rhodopsin and is the lowest of the elements listed in Table I. In biological systems iron is bound to high molecular weight substances that could be either water soluble or membrane bound proteins. There are many known iron containing membrane-bound proteins, some of which are present in mitochondria (21). Szuts (17) assumed all detected iron was due to mitochondria contamination. However, Hess (9) has also detected iron in outer segment preparations. At present there is no suggested role for iron in disk membranes.

In addition to the trace elements in Table I, the amount of chlorine (which would be present as the chloride anion) was determined by PIXE to be 2.92 ± 1.47 per rhodopsin. As the standard deviation of the data indicates, the chlorine content varies widely among samples and perhaps indicates it is not tightly bound by disk membranes.

In addition, upper limits were placed on the presence of manganese, molybdenum, and nickel, which are commonly occurring biological trace metals. The upper limits are 0.019, 0.019, and 0.006 atoms per rhodopsin, respectively. These are still high enough to allow the presence of small amounts of proteins containing these elements as a part of the 15% nonrhodopsin disk membrane protein.

Another known integral disk membrane protein is the large protein studied by Papermaster et al. (13). This protein has a molecular weight of ~238,000 in cattle rod outer segments and is present as 1–3% of the outer segment membrane protein. If each molecule contains one atom of a trace metal, it would be present at a concentration between 0.0019 and 0.0056 per rhodopsin. Therefore, the detected zinc, copper, and iron values are high enough for this protein to contain one or more of these ions. It is also possible this molecule could contain manganese, molybdenum, or nickel.

As Table II indicates, there were no discernible differences between those samples that were kept in the dark and those that were exposed to known amounts of light. This agrees with most other attempts to directly detect changes in disk membrane calcium (24). However, due to the probable loss of lightly bound calcium, this study cannot be considered a critical test of the calcium hypothesis.

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