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CONFERENCE CONTRIBUTION

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Speciation of metal ions in proteins by combining PIXE and thin layer electrophoresis

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Abstract Particle induced X-ray emission (PIXE) spectroscopy is a simple and convenient method of quantitative multielemental analysis with sensitivities in the μg/g range, that can be successfully used for trace analysis of metal ions in proteins or enzymes. However, due to its elemental character the technique alone is not a priori suitable for speciation. Keeping track of the metal ions of interest throughout a proper biochemical separation technique, on the other hand, could be a useful strategy for speciation. Different versions of thin layer electrophoresis (polyacrylamide gel, agarose or cellulose acetate electrophoresis) are very effective and sensitive methods to separate proteins or protein fragments. Due to the high absolute sensitivity of PIXE the metal ions concentrated in the narrow bands of an electropherogram can be in situ successfully detected. The present paper describes this unique combination of biochemical separation and ion beam analysis which significantly extends the information obtained from electrophoresis. Illustrative applications are given and the advantages and limitations of the method are discussed. Possible extensions of the technique are also outlined.

Introduction

The importance of trace element analysis in the life sciences has been widely accepted by now. Very low concentrations of the so-called essential trace elements could cause deficiency illnesses, while, on the other hand, unwanted accumulation of harmful (or toxic) heavy metals in different organs may also lead to serious pathological consequences. Depending on its concentration the same trace element could be either essential or toxic. These trace elements, in the majority of cases metal ions, are bound to certain proteins or enzymes and exert their effect as an inherent part of the active center of the protein in question. In order to understand the structure and function of these biological macromolecules as well as the role played by the particular metal of interest, it is necessary to identify, quantify and locate these metal ions. The first two processes – the identification and quantification – belong to the basic tasks of any quantitative chemical analysis. Location of the metal ions, i.e. the determination of the particular protein (or its subunits), where these ions are bound, on the other hand could be, in general, much more difficult. In addition to the analytical technique sensitive to the trace element of interest, utilization of protein specific biochemical methods are also necessary. For proteins polyacrylamide gel electrophoresis is the most widely used separation technique. Applying carefully dedicated cleavage of composite macromolecule the subunits can be also separated. By subsequent trace elemental analysis of the separated macromolecules keeping track of the metal ions is also possible. Taking into account that in the usual practice of electrophoresis the absolute amounts of the separated proteins are in the range of 10–50 μg and the area of the protein containing regions are only a few mm^2, an analytical technique of high sensitivity and good spatial resolution is required for the detection of the metal ions. Recently the utilization of an energetic ion beam analytical technique, the so called PIXE spectrometry was suggested and also successfully applied in the case of polyacrylamide gel electropherograms (PAGE). In the present work the basic features of this PIXE-PAGE method are described, illustrative applications are given and the advantages and limitations of the method are discussed. Possible extensions of the technique are also outlined.

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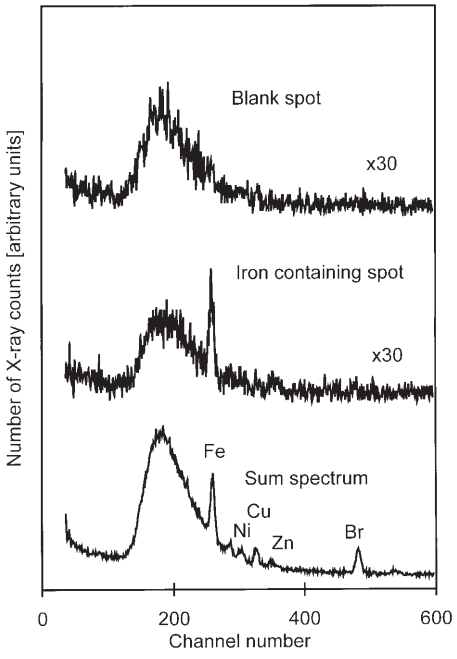

Fig. 1 PIXE spectra taken at a metalloprotein-free region (upper curve), at a Fe containing protein band (middle curve) of a polyacrylamide gel electropherogram, and the sum of all spectra taken along the gel (bottom curve)

Basics of the PIXE-PAGE method

PIXE spectrometry

PIXE spectroscopy is an analogue of the more familiar energy dispersive X-ray fluorescence analysis. A comprehensive description of the method can be found, e.g. in a recent monograph [1]. In PIXE a properly focused ion beam, in most cases of 2–4 MeV protons, is used to produce the characteristic X-rays instead of γ- or X-rays, as done in XRF. The lowest curve in Fig. 1. displays a typical PIXE spectrum. The well-resolved characteristic X-ray peaks of Fe, Ni, Cu, Zn and Br are sitting on a smoothly curved, continuous background originating mainly from the bremsstrahlung of the secondary electrons ejected from the target. The low energy cut-off of the spectrum is due to the rapidly increasing absorption by the materials shielding the radiation (external absorbers, Be window of the detector, etc.). Taking into account that in a PIXE spectrum the bremsstrahlung is lower by several orders of magnitude compared to those observed in electron beam excitation in an electron microscope equipped with EDAX system, the detection limits of medium elements are also lower by several orders of magnitude. In a thick organic matrix this sensitivity is in the μg/g range for the most important trace elements (Mn, Fe, Cu, Zn,...).

Thin layer electrophoresis

Electrophoresis is defined as the migration of charged molecules in electric field gradient. All theoretical aspects of electrophoresis together with a wide range of applications are presented in the monograph by A.T. Andrews [2]. In the overwhelming majority of cases the migration takes place in a thin layer of an appropriate supporting matrix instead of a free solution. For the separation of proteins polyacrylamide gels have proved to be the optimal supporting medium. Different molecules will migrate at different rate depending on their shape and charge-to-mass ratio. If the sample is initially present as a narrow band, proteins or protein fractions of different mobilities will migrate as discrete narrow bands. After switching off the electric potential difference the migration stops and the bands remain captured in the gel. These bands can be visualized by various staining procedures. From the measured distances the molecular masses of the particular macromolecules can be determined after proper calibration. The molecular mass determination is more straightforward when a sodium dodecyl sulfate (SDS) gel system is used. In this special case the separation is dependent on the molecular masses, only.

The PIXE-PAGE method

Electrophoresis alone does not provide any direct information on the identity or functional properties of the separated proteins. Similarly nothing can be learned about their possible metal content. In order to answer such questions, e.g. to speciate, in situ elemental analysis of the bands is needed. The comparison of the sensitivity of the PIXE technique to the expected absolute amounts of metal ions in a metalloprotein band has suggested the direct use of PIXE for this elemental analysis. The first successful experiments were carried out on polyacrylamide gels [3], therefore the name "PIXE-PAGE" was given to the method. The basic scheme of the experiment is shown in Fig. 2. The properly dried gel section is inserted into the measuring chamber, and its parts are bombarded by the collimated proton beam. X-ray spectra are taken with an energy dispersive Si(Li) detector. If at some place the gel contains metalloprotein and the amount of the bound metal ions exceeds the

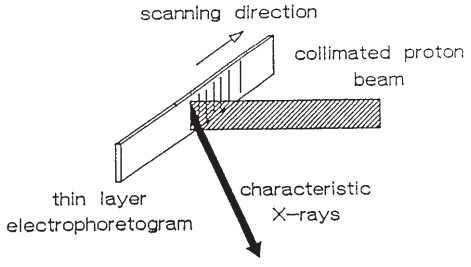

Fig. 2 Basic scheme of the PIXE analysis of thin layer electropherograms

limit of detection among the actual measuring conditions the characteristic X-ray peak has to appear in the spectrum. By fine step-by-step movement of the gel perpendicular to the proton beam the regions containing metals can be scanned and the lateral intensity distribution of the characteristic X-ray peak can be recorded. After the analysis the bands can easily be identified by help of standard staining procedures. Staining procedures generally wash out the bound metal ions in a rather uncontrollable way, therefore the most straightforward strategy of staining first and analyze afterwards, can not follow in this experiment, the gels have to be analyzed "blindly". The general practice in electrophoresis is, however, that in order to monitor the progress of electrophoretic process bromphenol stain is added to the sample solution, which allows one at least to notice the front of the migration: The appearance of the Br peak in the X-ray spectrum makes certain that the interesting gel section has ended. Specific agents can cleave multimeric proteins in two or more subunits and these subunits also form in general well-separated bands. The PIXE-PAGE analysis of these bands can provide experimental evidence about the possible binding sites of the metal ions. Therefore this analysis certainly meets the requirements of speciation. Measurements on gels containing well-known amounts of a very stable Fe containing protein (HiPIP), on the other hand, have also confirmed the quantitative sensitivity of the technique: the integrated Fe X-ray counts over the protein bands were found to be proportional to the amount of proteins supplied for electrophoresis [4].

Experimental setup

In order to make lateral scanning possible our standard PIXE chamber was equipped with a special target moving device. The gel section is placed on an Al target holder frame and is fixed to the inside end of the moving central rod of this scanner. The proton beam of 2–3 MeV energy is generated by the 5 MV Van de Graaff accelerator of our institute. The final shape of the beam spot on the sample is given by the carbon end-collimator of 6×0.5 mm^2 in size. This narrow bombarding area fits well the similar shape of the protein bands to analyze. Although the Canberra Si(Li) X-ray detector itself is placed outside the vacuum chamber at an angle of 135° to the beam direction, an Al sheath dipping into the chamber allows for a very close approach the sample. The X-rays produced pass through the 7 μm thick vacuum tight Kapton end-window of this sheath and enter the detector. The signals of the detector are amplified and processed by standard nuclear electronics and spectra are stored in the memory of a PC equipped with a custom designed multichannel analyzer card. Avoiding the high count rate the unconcerned low energy part of the X-ray spectrum is reduced by a 1 mm thick Plexiglas absorber. The beam current was kept below 10 nA to reduce the thermal deformation of the gel. X-ray spectra were taken and stored at pre-selected gel positions, the positioning of the target and the collection of X-rays are controlled by the dedicated PIXE-PAGE PC program package. The integrated X-ray counts from of up to five pre-selected characteristic peaks (Fe, Ni, Cu Zn and Br in most cases) are on-line displayed as a function of the gel position. Instead of one long lasting serial scan of the sample fast scans are repeated several times. This strategy allows to observe the metal containing regions in good time, and by proper readjustment of the scanning positions more detailed analysis of the relevant regions and the skip of blank or metal-free ones are possible. The uppermost spectrum of Fig. 1 was taken at a gel position where no metalloprotein was present. The absence of any characteristic peak demonstrates the purity of the chemicals used. The spectrum in the middle was collected at a part of a band of iron containing protein. (Among the actual experimental conditions the protein bands were wider than the width of the proton beam.) As a consequence of the strategy of fast but repeated scanning the recognition of the elements cropping up requires several scans. To overcome this difficulty the sum of all spectra is calculated at the end of each scan. Such a sum spectrum is displayed on the bottom of Fig. 1. The characteristic peaks indicate that in addition to Fe attention should be paid to Ni, Cu and Zn, too. (The Br peak is coming from the front indicator.) The more accurate final elemental distributions are obtained by off-line evaluation of the spectra by the commercially available AXIL program package [5].

Illustrative applications

The whole development of the PIXE-PAGE technique was initiated by the interest in identification and location of the indispensable metal ions in hydrogenase enzymes prepared from the membrane of different photosynthetic bacteria [6]. Hydrogenases are metalloenzymes that cat-

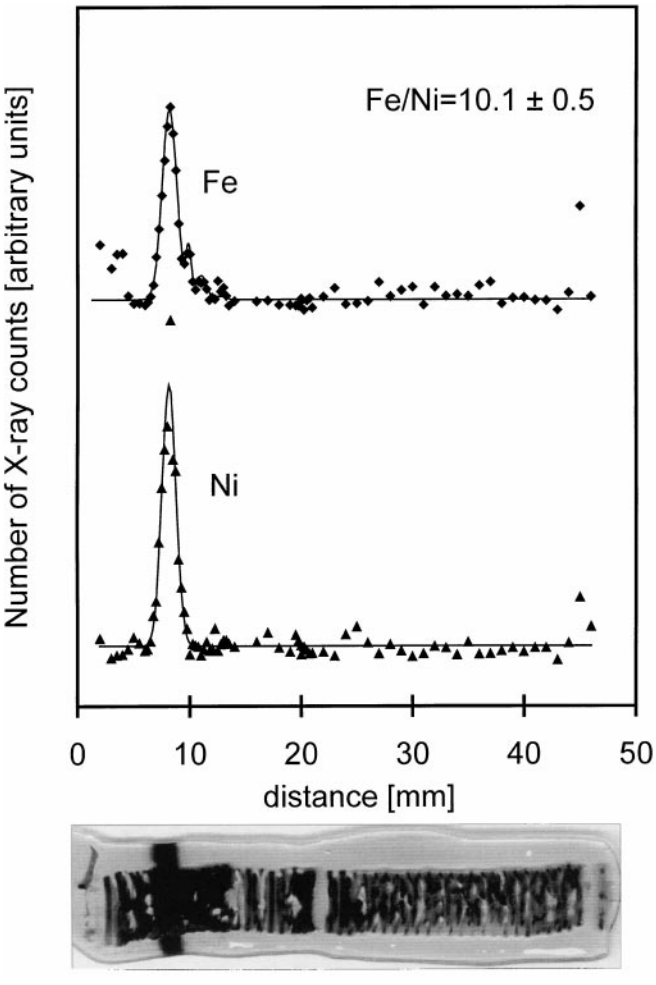

Fig. 3 Fe and Ni distributions along a native PAGE electropherogram containing *Desulfovibrio gigas* hydrogenase and the photograph of the post-stained gel

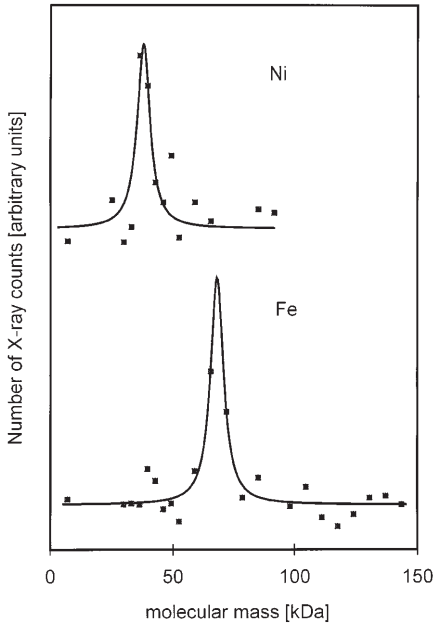

Fig. 4 Location of the Fe and Ni ions on different subunits of *Chromatium vinosum* hydrogenase separated by SDS-PAGE

alyze the formation and decomposition of molecular hydrogen. There exist at least two distinct groups of hydrogenases: the Fe-only enzymes have several Fe-S clusters while the Ni-Fe hydrogenases contain at least one Fe-S cluster and a Ni atom which is covalently bound to the protein. The knowledge of presence and relative number of Ni ions, as well as the binding sites of Fe and Ni is essential in understanding the structure and function of these enzymes. In Fig. 3 the result of the 3 MeV proton PIXE-PAGE experiment on *Desulfovibrio gigas* hydrogenase is shown. The separation was performed on a 1 mm thick 11% BRC (Biological Research Center, Szeged, Hungary) minigel. Each spot was exposed to 1 μC incoming proton charge. The sample solution was supplied for gel elctrophoresis in native conditions, therefore no decomposition was expected. Accordingly the photograph of the bombarded and post-stained gel on the bottom of the figure shows only one protein band. This band contains both iron and nickel. The iron/nickel ratio was calculated from the peak areas taking into account the relative X-ray production cross sections and absorption corrections. An example for the location of these ions is given in Fig. 4. In this case the hydrogenase was prepared from *Chromatium vinosum*, an other photosynthetic micro-organism and SDS-PAGE was performed among similar experimental conditions. The enzyme in this case was expected to decompose into two subunits of different molecular weights. In order to minimize the possible loss of metal ions the sample cocktail solution did not contain the otherwise usual EDTA and mercaptoethanol. The sample was boiled for 10 min before application on top of the gel. The results clearly show that Fe ions are bound to the larger subunit while the Ni ions are carried by the smaller subunit. The solid curves on the figure are the results of a Gaussian computer fit to the measured points. The explanation of the large Fe/Ni ratio and the different location required a revision of the former models of these hydrogenases [7]. The discussion of these problems is, however, out of the scope of the present work.

Discussion

PIXE spectroscopy was shown to be suitable to detect the metal content of the tiny bands of gel electropherograms where metalloproteins were concentrated. Thus a routine technique is available to identify, locate and to a certain extent quantify – or shortly speciate – these metal ions. The information obtained from electrophoresis was significantly widened. The method is best suited for elements where the sensitivity of the PIXE technique is the highest, that is for elements around iron. Fortunately in metalloenzymes mostly these elements are present. In the case of certain important heavier elements, such as Se or Cd, the applicability of the technique, on the other hand, is rather dubious because with increasing target atomic number the X-ray production cross section rapidly decreases at a given proton bombarding energy. Due to the danger of more serious thermal deformation this decrease in sensitivity can not be fully compensated by the increase of the incoming charge. The use of higher bombarding energy is somewhat more promising: the Fe/Se X-ray intensity ratio of about 16 at 2.5 MeV proton energy decreases to about 11 when 4 MeV protons are used, but there is no experience about the thermal deformation caused by the higher energy bombardment. The damage can certainly be reduced by the use of the so-called “external” proton beam, where the bombarding particles exit through a proper window foil (Kapton, Al, etc.) to the external atmosphere where the sample cools down more effectively. For heavier or volatile elements the generalization of the method may be useful, e.g. the use of properly tuned monochromatic synchrotron radiation for X-ray fluorescence (SXRF). The more difficult access to synchrotron radiation, however, will certainly reserve this technique to the analysis of a few very important samples. Undoubtedly the method is particularly suitable for identification and location of the ions. As far as the quantitative aspects are concerned, important questions have remained open. The use of high purity chemicals and surroundings in sample preparation is a basic requirement, of course. The main problem has nothing to do with PIXE analysis but with the behaviour of metalloproteins during electrophoresis. Many examples exist in the literature of metalloproteins losing all their metal ions bound by physical forces (electrostatic ionic bonds or other polar bonds such as dipolar, induced dipolar, van der Waals forces, etc.) during separation by electrophoresis under the influence of the electric field. On the other hand, the PIXE technique itself presents an opportunity to obtain information even in this respect. A standard PIXE measurement on an aliquot of the initial protein solution can provide the total

amount of metal ions of interest. The comparison of this result to those obtained by systematic PIXE-PAGE measurements on electropherograms prepared using different conditions can reveal the secrets of metal ion losses.

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