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METAL COMPOSITION ANALYSIS OF HYDROGENASE FROM THIOCAPSA ROSEOPERSICINA BY PROTON INDUCED X-ray EMISSION SPECTROSCOPY

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SUMMARY: Polyacrylamide gel electrophoresis combined with proton induced X-ray emission spectroscopy is suitable to identify and to determine the relative amounts of protein bound metals in situ. An analysis of the hydrogenase from Thiocapsa roseopersicina has shown the feasibility of the technique and provides new insight into the relative amount as well as the intramolecular location of Fe and Ni metal atoms in this enzyme.

A number of key metabolic processes involve enzymes containing metal atoms; among these are electron transport chains, generation of membrane potential, photosynthesis, and nitrogen fixation. Wherever metals are found as constitutive parts of biopolymers they play determining role in the biological activity of the macromolecules. It is therefore imperative that methods are available to detect and study the protein bound metals.

Direct measurement of metals in proteins is generally difficult by chemical methods since the relative concentration of the metal atoms in a biological

Abbreviations used: PIXE, proton induced X-ray emission spectroscopy; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; R_f, retention factor = migration distance of protein over migaration distance of buffer front.

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sample is very low. PIXE offers an outstanding sensitivity in this respect.

Metal determinations in biological samples are often erroneous because metals not attached to the macromolecule *in vivo* may adsorb onto it during sample purification.

The combination of PIXE with PAGE or SDS-PAGE eliminates or at least decreases this type of error by separating metals confined to the protein from the free or loosely held metal ions. PIXE analysis following PAGE and SDS-PAGE provides information both on the metal content of a particular protein and on the distribution of the metal(s) among the composite subunits. Similar information can be gathered only by X-ray diffraction, however, that method is considerably more difficult and requires much more of the precious protein sample that is cumbersome to crystallize.

Here we report on the first PIXE measurements on hydrogenase and on its subunits. The biochemical significance of the new information on the location of Fe and Ni in this metalloprotein is briefly discussed.

MATERIALS AND METHODS

Bacterial strain

The purple sulfur photosynthetic bacterium *Thiocapsa roseopersicina* strain BBS was a kind gift from Prof. E. N. Kondratieva (Moscow State University, USSR). The bacterium was cultivated as described earlier (1).

Purification of hydrogenase

The enzyme was purified from the frozen cell paste as in (2,3). The purity of the final preparation was checked by SDS-PAGE, the functional integrity of the purified hydrogenase was monitored by enzyme activity measurement (4).

Electrophoresis

The discontinuous buffer system developed by Neville (5) was used. Acrylamide concentration was 10 %, crosslinking 1 %, 0.1 x 5 x 5 cm gel slabs were cast. Samples were incubated with equal volume of a sample buffer at 100 ^{o}C for 10 min or at room temperature. Electrophoresis following heat denaturation of the samples is called SDS-PAGE as opposed to loading the samples without boiling which is considered as native PAGE. 100 μg of the pure protein was applied in a single slot along the gel width. After separation, a portion of the gel was stained for enzyme activity (6) and/or for protein with Coomassie Brilliant Blue R-250. The

remaining part of the gel was covered with a 1 mg/cm^2 Nuclepore membrane of 12 μm pore size and dried on a gel slab drier. The Nuclepore membrane was easily removed from the dried gel leaving the gel surface directly accessible for proton irradiation.

PIXE measurements

A detailed description of the particle induced X-ray emission technique has been given elsewhere (7,8). The experimental set up is shown in Figure 1. Sections of the dried gel were placed on an Al holder frame and were placed into the vacuum chamber of 5 MV Van de Graaf accelerator. The X-ray spectra induced by irradiation of the sample with a proton beam of 3 MeV energy were analyzed with a Canberra X-ray spectrometer. The proton beam was focused on a 0.6 x 7 mm area at the gel surface. The target sample was moved in 1 mm steps perpendicularly to the proton beam in order to scan the entire gel length. At each position an X-ray spectrum was recorded, 1 μC incoming proton charge was collected.

After proton irradiation the sample was rehydrated and stained with Coomassie Brilliant Blue R-250. The areas hit by the proton beam were clearly deteriorated and the impact marks of the bombardment were easily visualized as sharp traces on top of the protein bands. This phenomenon made the accurate assignment of the X-ray spectra to the protein bands possible.

RESULTS AND DISCUSSION

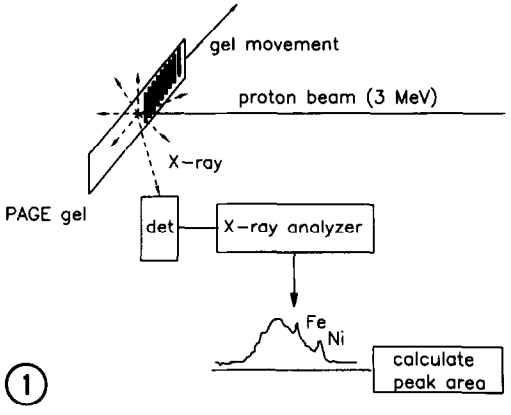
It has been demonstrated earlier (8) that metalloproteins can be identified on the basis of their X-ray spectra in a dried PAGE sample.

Hydrogenase represents a challenge for the PAGE-PIXE technique. Metals are indispensable for the enzymic function of this protein (9), yet the intramolecular location of the metal atoms is unknown.

Hydrogenase of Thiocapsa roseopersicina is composed of two nonidentical subunits of 64 and 34 kDa molecular mass, respectively. One 4Fe-4S cluster and one Ni per native enzyme molecule has been found by chemical analysis (2). Most hydrogenases are unstable, however, the membrane bound enzyme isolated from T. roseopersicina retains its activity even under PAGE conditions unless the sample is boiled for several minutes before the electrophoresis (6).

PAGE-PIXE of native hydrogenase

Scanning the protein components separated in PAGE by the proton beam along the gel gives direct information about the distribution of elements in the gel. In the



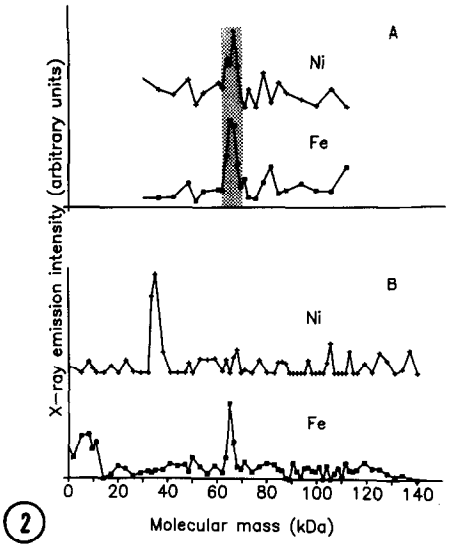


Figure 1. Schematic diagram of the PIXE measurement.

Figure 2. The distribution of Fe and Ni in the native (A) and denatured (B) hydrogenase from Thiocapsa roseopersicina after PAGE-PIXE. The hydrogenase activity band is indicated by the dotted area.

native hydrogenase sample the Fe and Ni peaks are at the same position, at R_f=0.35, which corresponds to the migration of the hydrogenase activity in PAGE (Figure 2A). The mobility of the native hydrogenase band is not proportional to its apparent molecular mass. Nevertheless, one can conclude that the native enzyme contains Fe and Ni as expected from previous chemical analysis and electron spin resonance measurements. It is remarkable that we have found a Fe to Ni peak area ratio of 7(±1):1 by PAGE-PIXE. This result is not compatible with the one 4Fe-4S cluster and one Ni atom containing hydrogenase model proposed earlier (2). A new hydrogenase structure involving two Fe-S clusters and a Ni atom per molecule is to be invoked. Recent spin-echo studies (R. Cammack et al., Eur. J. Biochem. 1989, in press) corroborate this model.

SDS-PAGE-PIXE of hydrogenase subunits

The subunit polypeptides are separated according to their molecular mass in SDS-PAGE (6). The results of SDS-PAGE-PIXE clearly demonstrate (Figure 2B) that the Fe atoms migrate together with the large (64 kDA) subunit

while the Ni is bound to the small (34 kDa) subunit. On the one hand, it is to be noted that the Fe and Ni metal centers are in close spatial proximity according to a number of spectroscopic observations (10). On the other hand, the SDS-PAGE-PIXE results presented here indicate that the metals are bound to separate polypeptides. Consequently, the Fe and Ni centers should be at or close to the surface of their respective subunits at a domain where the subunits are held together.

It is also attractive to speculate about the possible role of the metals in maintaining the three dimensional structure of hydrogenase in addition to their well established functional role. Since hydrogenases from various microorganisms show striking structural similarities (11) the location of metal centers may be similar in other hydrogenases as well.

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