ANALYSIS OF THE CRYSTALLINITY OF CALCIUM PHOSPHATE DEPOSITS IN RAT LIVER MITOCHONDRIA BY ELECTRON SPIN RESONANCE SPECTROSCOPY

Kazimierz OSTROWSKI, Anna DZIEDZIC-GOCŁAWSKA, Adam SLIWOWSKI
Institute of Biostructure, School of Medicine, ul.Chałbińskiego 5, 02–004 Warsaw, Poland

Lech WOJTCZAK
Department of Cellular Biochemistry, Nencki Institute of Experimental Biology, Pasteura 3, 02-093 Warsaw, Poland

and

Jacek MICHALIK, Wacław STACHOWICZ
Department of Radiation Chemistry, Institute of Nuclear Research, ul.Dorodna 16, 03–195 Warsaw, Poland

Received 5 November 1975

1. Introduction

The problem of the origin and nature of intramitochondrial mineral deposits is widely discussed because of the possible role of mitochondria in the calcium metabolism of the cell [1,2]. These are suggestions that physiologically calcium-loaded mitochondria are involved in the early stages of skeletal tissue mineralization [1,3]. At the First International Conference on Matrix Vesicle Calcification (New York 1974) evidence was given for a relationship between Ca ion transfer from mitochondria to the plasma membrane and the matrix vesicles associated with endochondrial ossification [4,5]. Intramitochondrial accumulation of inorganic Ca salts is observed in a variety of physiological conditions in vivo and also as the result of in vitro incubation of fragments of tissues, cells or isolated mitochondria in media with a high concentration of Ca^{2+} [6]. Amorphous granules as well as crystalline needle-shaped mineral deposits were described in mitochondria from various tissues [6]. On the other hand, only amorphous calcium phosphate has been observed to accumulate in isolated mitochondria [1,7,8]. Electron microscopy of blue crab hepatopancreas mitochondria shows bundles of needle-like crystals [9]. The way of preparation and preservation of mitochondria can influence the physico-chemical state of these deposits [10].

Various methods as chemical analysis, electron microscopy, electron diffraction, X-ray diffraction as well as X-ray and electron probe analyses have been used to study intramitochondrial mineral deposits.

In this paper a new approach is suggested which might help in the analysis of different kinds of intracellular mineral deposits. It was shown [11] that with the use of electron spin resonance (ESR) spectrometry one can detect the crystalline fraction of the tissue mineral after irradiation of the sample with ionizing radiation. The irradiation produces paramagnetic defects in the crystalline lattice of hydroxyapatite [11–13]. The number of these paramagnetic centers detected and measured by the ESR technique related to the total ash content of the sample gives information on the crystallinity of the tissue mineral [14].

The advantage of ESR spectrometry in the detection of hydroxyapatite is connected with the fact that other forms of calcium salts present in the sample do not interfere with the detection of the signal derived from the paramagnetic centers induced in the hydroxyapatite crystals. The high sensitivity of this method allows the detection of 10^{-9} mole equivalent of spins in the sample.

 North-Holland Publishing Company – Amsterdam

The application of the ESR method to Ca^{2+}-loaded rat liver mitochondria, as described in this paper, shows no evidence for the crystallinity of calcium phosphate deposits, this being in good agreement with previous reports from the Lehninger's group [1,7,8] based on different methods.

2. Material and methods

Mitochondria from livers of albino rats, isolated according to Hogeboom [15], were used throughout in these investigations. Massive loading with calcium was performed by incubating mitochondria in a NaCl medium containing $CaCl_2$, phosphate, succinate and ATP, as described by Thomas and Greenawalt [8]. After 20 min at 30°C the mitochondria were sedimented by centrifugation. The pellet was resuspended in 250 mM sucrose–6 mM succinate and sedimented again. It was then extracted with chloroform-methanol (2:1, v/v), followed by chloroform and ethyl ether and dried in air at room temperature. Control samples were treated in the same way except that $CaCl_2$ and ATP were omitted in the incubation medium. Extraction of mitochondria with organic solvents was necessary to remove some stable free radicals, e.g. those derived from ubiquinone, which may interfere in ESR analysis. This procedure substantially lowers the 'native' ESR signal of the samples.

Samples weighing about 0.010 g were placed in tubes 3 mm in diameter made of special quality ceric glass which does not show any gamma ray-induced ESR signal after prolonged irradiation at room temperature. The samples were irradiated in a 'Gammacell 220' AECL ^{60}Co source with a dose of 10 Mrads (dose rate 205 rads/min). The absorbed dose was measured with a ferrous–ferric Fricke dosimeter. In order to suppress the ESR signals arising from organic constituents the samples were stored after irradiation for 3 weeks at room temperature with access of air. The first derivative ESR spectra were recorded and accumulated 16 times with an X-band 100 kc magnetic field modulation computerized JES-ME-3X JEOL spectrometer.

The dry weight of the samples was recorded before and after ashing with the use of Electrobalance Cahn G-2 with accuracy in the range of 10^{-6} g and systematic error of 0.1 per cent. Ashing was performed on a platinum foil in a Perkin-Elmer 240 Elemental Analyzer at 980°C for 8 min. The amount of released C, N, and H was automatically registered by a Perkin-Elmer MC-1 Microanalytical Computer.

Since no crystalline hydroxyapatite was found by the ESR method additional experiments were performed to determine the threshold detectability of hydroxyapatite in mitochondrial samples by addition of synthetic hydroxyapatite.

With synthetic amorphous calcium phosphate and calcium-loaded mitochondrial samples parallel experiments on recrystallization according to the procedure described by Boskey and Posner [16] were done. The effects of this procedure were controlled by electron microscopy.

3. Results

Ash content as well as percentage of N, C and H in samples of Ca-loaded rat liver mitochondria from two different experiments and in control ones are shown in table 1.

Incineration of Ca-loaded mitochondria left 10–15% of ash as compared to 1% in delipidated control

Table 1
Content of ash, nitrogen (N), carbon (C) and hydrogen (H) in calcium-loaded rat liver mitochondria

Mitochondria	Number of determinations	Ash	N	C	H
Control	6	1.2 ± 0.2	11.4 ± 0.04	46.1 ± 0.12	6.9 ± 0.02
Ca-loaded (Expt. No. 1)	6	15.4 ± 0.3	9.9 ± 0.06	37.5 ± 0.10	6.0 ± 0.04
Ca-loaded (Expt. No. 2)	8	10.3 ± 0.3	12.8 ± 0.03	40.8 ± 0.12	6.5 ± 0.05

The values are expressed in percentage of the delipidated dry weight ± standard error.

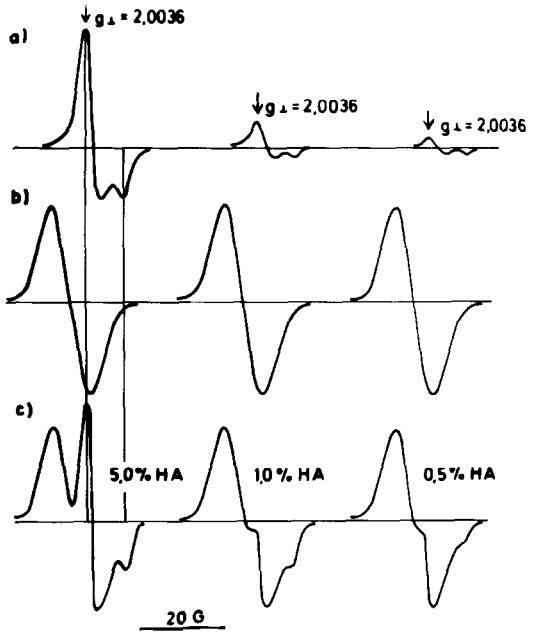

Fig.1. The first derivative ESR spectra of Ca-loaded mitochondria mixed with different amounts of synthetic hydroxyapatite (average crystal size along c-axis about 250 Å) after irradiation with 10 Mrads (^{60}Co source). (a) Asymmetric singlet $g_\perp = 2.0036$, $g_{\parallel} = 1.9978$ derived from stable paramagnetic centers induced by radiation in hydroxyapatite crystals of the samples weighing 0.50 mg, 0.10 mg and 0.05 mg respectively. (b) Symmetric singlet associated with native paramagnetic species in the three samples of mitochondria of about 10 mg each. (c) Superimposed (a) and (b) signals in samples of mitochondria containing a different percentage of hydroxyapatite (HA).

mitochondria. Assuming that the ash in mainly calcium phosphate and that the rest is protein one can calculate that about 1100–1600 nmol Ca was accumulated per 1 mg of protein. This is within the range observed for massive loading of liver mitochondria [7].

When the material was subjected to gamma irradiation and examined for ESR three weeks later, no appearance of the asymmetric singlet characteristic for crystalline hydroxyapatite [11–13] could be observed (fig.1).

For the evaluation of the threshold detectability of this singlet against the background of the residual native ESR signal of the mitochondrial samples, standard amounts of irradiated synthetic hydroxyapatite of average crystal size about 250 Å, as established by X-ray diffraction, were added. It was found that the lowest quantity of hydroxyapatite which still gave an ESR signal when measured together with mitochondria was 0.035 mg (fig.1). Since the weight of mitochondrial samples was about 10 mg each, the content of crystalline hydroxyapatite in the samples may not exceed 0.35 per cent. Since the Ca-loaded mitochondria contain 10–15% of ash, the amount of the crystalline fraction of the inorganic constituents of these samples can not exceed 3.5–2.33% of the deposited mineral. This conclusion is valid only under the assumption that the average size of mitochondrial hydroxyapatite crystals would be the same or similar as in our standard. The yield of radiation-induced paramagnetic centers in apatite crystals depends on their average size [11].

An attempt was made to convert calcium phosphates present in Ca-loaded mitochondria into hydroxyapatite by incubating the samples in 0.15 M Tris buffer, pH 8.5, at 45°C for 10 days [16]. After subsequent irradiation ESR spectra were recorded. It was found that the asymmetric singlet characteristic for stable radiation-induced paramagnetic centers in hydroxyapatite never appeared. This was confirmed by electron microscopic pictures. The shape of granular mineral deposits was not changed.

The control samples of $CaHPO_4 \cdot 2\ H_2O$ hydrolyzed in identical conditions were completely converted into hydroxyapatite in 7 days. The initial ESR measurements showed complex spectra derived from the products of radiolysis of $CaHPO_4$. After 7 days the conversion is completed and only the asymmetric ESR singlet is seen.

4. Discussion

The amorphous character of granular intramitochondrial aggregates was described by several authors [7,8]. In some cases needle-like crystals have also been observed [6,9].

It was suggested that the granular amorphous mineral deposit which is usually observed in the Ca-loaded rat liver mitochondria can be completely converted into crystalline apatite under the influence of different additives to the medium [10]. The situation is complicated by the observation of Bonucci et al. [6] that in different mitochondria, even in the same cell,

different types of calcium salt aggregates, i.e. granular or needle-shaped, could be found. The influence of the organic constituents of cells on the shape of the aggregates is suggested by these authors. Thermal stability and crystallizability of the ash obtained from the Ca-loaded rat liver mitochondria was demonstrated in the heating experiments of Thomas and Greenawalt [8]. From these experiments, based on electron diffraction analysis, the authors conclude that the amorphous original intramitochondrial deposit is the submicrocrystalline precursor of calcium-deficient hydroxyapatite. The crystallized ash was tentatively shown to be tricalcium phosphate (whitelockite). Chen et al. [9] working on hepatopancreas mitochondria of blue crab and liver mitochondria of rat, in vitro loaded with calcium, point to differences in the physico-chemical state of intramitochondrial mineral deposits depending on the species. When comparing electron micrographs of crab and rat mitochondria needle-shaped crystals and amorphous deposits of calcium phosphates were found, respectively, 'under similar conditions'.

Betts et al. [17] studied the nature and stability of extramitochondrial intracellular calcium phosphate deposits in the hepatopancreas of blue crab. They found that ATP and Mg^{2+} present in physiological concentrations stabilize amorphous calcium phosphates and interfere with their conversion into hydroxyapatite in vivo and in vitro. This was confirmed in model experiments with synthetic calcium phosphates. A similar statement could be found in an earlier paper by Termine et al. [18] who found that conversion of calcium phosphate into hydroxyapatite might be inhibited in mitochondria by Mg^{2+} and CO_3^{2-} or amino-acid residues of proteins such as lysyl or phosphoseryl which stabilize calcium phosphates.

We tried to show in this paper that the ESR technique is useful for analysis of the physico-chemical state of intracellular mineral deposits. It allows to describe in a quantitative way the degree of crystallinity of mineral aggregates.

References

[1] Lehninger, A. L. (1970) Biochem. J. 119, 129–138.
[2] Barnard, T. and Afzelius, B. A. (1972) Sub-Cell. Biochem. 1, 375–389.
[3] Martin, J. H. and Matthewes, J. L. (1969) Calc. Tiss. Res. 3, 184–193.
[4] Brighton, C. T. (1975) 1st Intern. Conference on Matrix Vesicles Calcification, N. Y. 1974, Suppl. to Feration Proc. in the press.
[5] Ali, S. Y. (1975) 1st Intern. Conference on Matrix Vesicles Calcification, N. Y. 1974, Suppl. Federation Proc. in the press.
[6] Bonucci, E., Derenzini, M. and Marinozzi, V. (1973) J. Cell. Biol. 59, 185–211.
[7] Greenawalt, J. W., Rossi, C. S. and Lehninger, A. L. (1964) J. Cell Biol. 23, 21–38.
[8] Thomas, R. S. and Greenawalt, J. W. (1968) J. Cell Biol. 39, 55–76.
[9] Chen, C., Greenawalt, J. W. and Lehninger, A. L. (1974) J. Cell Biol. 61, 801–815.
[10] Matthews, J. L., Martin, J. H., Kennedy III J. W. and Collins, E. J. (1974) Hard Tissue Growth Repair and Remineralization, Ciba Found. Symp. 11, p. 187–211, in discussion.
[11] Ostrowski, K., Dziedzic-Goclawska, A., Stachowicz, W. and Michalik, J. (1974) Ann. N. Y. Ac. Sci. 238, 186–201.
[12] Stachowicz, W., Ostrowski, K., Dziedzic-Goclawska, A. and Komender, A. (1970) Sterilization and Preservation of Biological Tissues by Ionizing Radiation, Int. Atomic Energy Agency, PL 333/3, p. 15–27.
[13] Stachowicz, W., Michalik, J., Dziedzic-Goclawska, A. and Ostrowski, K. (1972) Nukleonika, 17, 425–431.
[14] Ostrowski, K., Dziedzic-Goclawska, A. Stachowicz, W. and Michalik, J. (1972) Histochemie, 32, 343–351.
[15] Hogeboom, G. H. (1955) Methods in Enzymology 1, 16–19.
[16] Boskey, A. L. and Posner, A. S. (1973) J. Phys. Chem. 77, 2313–2317.
[17] Betts, F., Blumenthal, N. C., Posner, A. S., Becker, G. and Lehninger, A. L. (1975) Proc. Natl. Ac. Sci. USA 72, 2088–2090.
[18] Termine, J. D., Peckauskas, R. A. and Posner, A. S. (1970) Arch. Bioch. Biophys. 140, 318–325.