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IDENTIFICATION OF CADMIUM BINDING SITES WITHIN LIVING HUMAN CELLS BY PERTURBED ANGULAR CORRELATION SPECTROSCOPY

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1. Introduction

For the first time the spectroscopic technique of perturbed angular correlation of gamma rays (PAC) has been applied to a study of binding modes of Cd(II) in living cells. This method only needs trace amounts of species to obtain structural information about metal binding sites. The cells studied were human epitheloid cells designated HE_{100} [1,2], with a high content of the protein metallothionein (MT) [3]. PAC studies of MT-bound Cd(II) have successfully been done using pure MT isolated from rabbit liver [4]. The purposes of this study were to test if it is possible with the PAC technique to get information about Cd(II) binding within living cells and, if so, to determine which cell components Cd(II) is bound to. The results show that:

(1) The PAC technique is capable of giving significant information about the number and types of Cd(II) binding sites available in living cultured cells;
(2) The cellular Cd is mainly bound to MT;
(3) The Cd-containing MT molecules are freely suspended in the cell cytoplasm.

Abbreviations: PAC, perturbed angular correlation of gamma rays; MT, metallothionein; NQI, nuclear quadrupole interaction; HE_{100}, epitheloid cells used [1,2]; Tris, tris (hydroxymethyl) aminomethane sulfate

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2. Experimental

2.1. *PAC method*

The general theoretical basis for perturbed angular correlations of gamma rays (PAC) has been reviewed in [5–7]. However, briefer introductions to the method of PAC are also available [8]. This method has in fact proved to be a valuable tool in the study of metal-site structures in macromolecules such as proteins both in the solid and solvated state [9–12]. The ^{111}Cd-isomer used in this work is particularly well suited for such studies. The coincident counting rate $W(\theta,t)$ was measured at fixed angles 0–180°, and 0–90° as a function of the delay time t between the emission of the 2 gamma rays. θ is the angle between the detectors of the 2 gamma rays. The measurements and data handling were made with equipment described in [9]. The nuclear quadrupole interaction NQI parameters ω ($\omega = 6\,e\,q\,Q/2I\,(I-1)\hbar$) and η were calculated according to eq. (1) [5,8]:

$$\frac{W(180°, t)}{W(90°, t)} = \frac{1 + A\,G(t, \omega, \eta)}{1 - \frac{1}{2}A\,G(t, \omega, \eta)} \qquad (1)$$

where A is the amplitude at $t = 0$ for the 150–247 keV cascade of ^{111}Cd and the perturbation factor $G(t, \omega, \eta)$ is a known function of t, ω and η [5,8]. For a highly viscous medium where $\omega\tau_R >> 1$ where τ_R is the rotational tumbling time we have assuming spherical molecules [13]:

$$G(t, \omega, \eta) = \exp(-t/\tau_R)\,G_{stat}(t, \omega, \eta) \qquad (2)$$

where $G_{stat}(t, \omega, \eta)$ is the perturbation factor for

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$\tau_R = \infty$. For $\omega\tau_R \approx 1$ the relaxation effects quickly produce disalignment of the nuclear spin and very little structure in the time spectrum is observed, i.e., a fast exponential decay of the observed anisotropy.

The 49 min ^{111}Cd-isomer at 397 keV was produced through the reaction ^{108}Pd (21 MeV α,n) ^{111}Cd with the Tandem-Accelerator at the Niels Bohr Institute. ^{111}Cd was chemically separated carrier free from Pd-metal as in [14]. The final ^{111}Cd(II)-solution was evaporated to 100 μl and pH-adjusted to 7.4 with 1 M $NaHCO_3$. 200 μl culture medium was then added to this solution, thus making a 300 μl sample of radioactive culture medium. The amount of Cd present is $<10^{-12}$ mol.

2.2. *Cell processing*

The cells used were a substrain of human epitheloid cells derived from normal skin [2]. The cells had been made resistant to 100 μmol Cd/l culture medium and had been designated HE_{100} [1]. The induced Cd-binding protein, which constitutes 2–3% of the total cell protein in these cells, has been disclosed as MT [2]. HE_{100} were routinely maintained in plastic flasks with Dulbecco's modified Eagle's medium as in [2]. The culture medium was replaced by a Cd-free medium 24 h before use to reduce the cellular content of Cd. For experiments, cells in almost confluent monolayers were rinsed once with 5 ml and then loosened from the plastic substratum by gentle shaking after incubation for 5 min at 37°C with 5 ml 0.25% trypsin in a buffered 0.02% EDTA solution. The cell suspensions were centrifuged at 800 × *g* for 5 min.

For the PAC-measurements the cell pellets thus produced were resuspended in the 300 μl sample of radioactive medium and then incubated at 37°C for 15–20 min prior to a new centrifugation. The medium was removed and the cells were rinsed once by resuspension in a Cd-free medium and subsequent centrifugation. About 10% of the total radioactivity was found in the supernatant, the remaining 90% being bound to the cell pellet. A second rinsing procedure did only remove additional 1–2% of this cell-bound activity. Thus one rinsing was found sufficient.

Two PAC-measurements were performed on living cells. For both experiments cells from 4 culture flasks (~4 × 10^7 cells) were used. To keep the cells alive during the measurement period (2–3 h) the cell-pellets were resuspended in 200 μl Cd-free medium and kept in sealed plastic tubes. During the first PAC-measurement the cell suspension was kept at 36°C. During the second measurement the cell suspension was kept at 4°C to increase the viscosity of the cellular components and to reduce the metabolic activity. After each measurement the cells were replated (1:1) in new plastic culture flasks and further incubated at optimum conditions [2]. The vitality of the cells was judged by phase contrast microscopy over 24 h. No other test of cell survival was done at the time of experiments, but a subsequent dye-exclusion test (using trypan blue) was performed under quite similar conditions.

A third measurement was done on a crude fraction of MT obtained by Sephadex G-75 gel filtration of cytosol from HE_{100} cells (1.6 × 90 cm column eluted at 4°C with 10 mM phosphate buffer (pH 8.0)). To a 100 μl sample of radioactive Cd-solution buffered to pH 7.4 by 1 M $NaHCO_3$ was added 200 μl MT-solution (0.5 mg metallothionein/ml). The MT bound fraction of Cd was then isolated through a second chromatography step using a 0.9 × 10 cm column with Sephadex G-25 eluted at room temperature with 50 mmol/l Tris–SO_4 buffer (pH 7.4) (this fraction is referred to below as pure MT). To a 1 ml eluate fraction containing ~40% of the radioactive Cd was added 1.85 g sucrose giving a 65% sucrose solution.

3. Results and discussion

The spectra obtained from the different PAC-measurements are shown in fig.1. The spectrum from living cells at 36°C shows a rapid attenuation in time and therefore the spectrum reveals very little structure. This is characteristic of a rapidly rotating molecule, i.e., the rotational time τ_R is in the region where $\omega\tau_R$ is close to 1. This fact gives the important conclusion that Cd within the cell is bound to a molecule dissolved in the cytoplasm and therefore not immobilized by binding to membrane or other heavy cellular components. The spectrum from living cells at 4°C shows more structure and is characteristic of a slowly rotating molecule i.e. $\omega\tau_R$ much larger than 1. The change in rotational speed between the 36°C and 4°C experiment can arise either from an increased cell viscosity, polymerisation, or attachment of smaller molecules into heavier molecules.

PAC analysis of the 2 last spectra are given in table 1. It is seen that both spectra have been decomposed into 2 different NQI, i.e., 2 different types of Cd(II) sites are detected. The first NQI (ω_1) for both

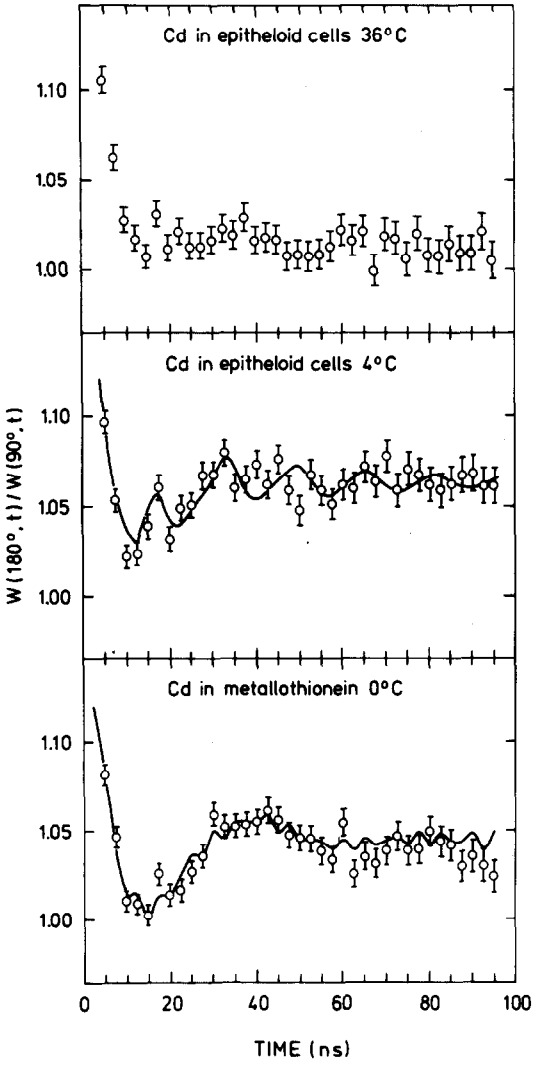

Fig.1. PAC time spectra. For the spectra of metallothionein and epitheloid cells at 4°C the full drawn curves represent least square fits to the spectra with NQIs as stated in table 1.

spectra shows a large frequency broadening (denoted δ in table 1), whereas the second NQI (ω_2) essentially shows no frequency broadening. The NQI parameters of pure MT are in good agreement with those found in [4]. We refer to this work for a detailed explanation of the origin of these two NQIs.

The spectral parameters for living cells at 4°C and those from pure MT exhibit the following equality: The first NQI (ω_1) detected for pure MT is present to 80% in the case of living cells at 4°C. Thus we infer that $\geqslant$66% of Cd(II) within the cell is bound to metallothionein.

The first NQI (ω_1) detected at 4°C for living cells exhibits a much stronger frequency broadening than that obtained for pure MT (table 1). As no definite cause for the frequency broadening has been extracted for pure MT [4] we defer any explanation of this difference in frequency broadening. The second NQI (ω_2) decreases from pure MT to living cells by a factor of 2. This decrease could originate either from a drastic change in the coordination geometry for this Cd(II) site in metallothionein or from the possibility that this second NQI does not arise from Cd bound to MT in the cells but rather to some other molecule in the cell.

The vitality of the cells during and after PAC-measurements was demonstrated by the subsequent replating of the used cells. The cells were attached to the plastic substratum immediately after the experiments, and after 8–15 h mitoses were seen. The dye exclusion test performed later showed 92% surviving cells after measurements at 36°C and 76% at 4°C. Thus, we have for the first time demonstrated that PAC-measurements are applicable to studies on living mammalian cells in culture. This points towards a possible future pharmacological and toxicological usefulness of this technique.

Table 1
NQI parameters for metallothionein

Condition	ω_1 (MHz)	η_1	ω_2 (MHz)	η_2	P_1 (%)	P_2 (%)	δ_1 (%)[a]	δ_2 (%)[a]
4°C living cells[b]	127	0.45	374 ± 20	0.17 ± 0.25	81 ± 8	19 ± 8	50	5
0°C metallothionein	127 ± 11	0.45 ± 0.10	802 ± 40	0.58 ± 0.11	76 ± 9	24 ± 9	25	0

[a] The frequencies ω are broadened by $e^{-\frac{1}{2}\delta^2\omega^2t^2}$ (see [5])
[b] ω_1 and η_1 are fixed at the values for metallothionein

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