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PERTURBED ANGULAR CORRELATION γ RAY (PAC) SPECTROSCOPY OF ^{111}Cd CARBOXYPEPTIDASE Aα‡

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SUMMARY

The active center of Cd(II) carboxypeptidase A has been examined by means of perturbed angular correlation of gamma rays as a function of pH. This method simultaneously detects changes in coordination geometry, in the charge of groups within 5 Å of the metal nucleus and delineates multiple states of the active center. Carboxypeptidase A crystals exhibit one predominant state while several are present in solution. Interpretation of the experimental data in terms of Angular Overlap Theory suggests the species characteristic of the crystals has a distorted tetrahedral coordination geometry and is in equilibrium with a pentacoordinated species in solution.

Perturbed Angular Correlation (PAC) of gamma rays detects the nuclear quadropole interaction (NQI) between a ^{111}Cd(II) nucleus and its environment. We have previously demonstrated that this technique uniquely elucidates specific structural features of the active site of metalloenzymes, not discernible by other general spectroscopic procedures (1,2). The theory underlying this method was discussed nearly two decades ago (3) but has been applied to the study of enzymes only quite recently (1,2). Its potential is somewhat analogous to that of Mössbauer spectroscopy for ^{57}Fe containing enzymes, though its scope is even greater and applicable to any system into which ^{111}Cd can be incorporated at a specific binding site. The method is sensitive to charge-dependent and conformational changes within 5Å of the

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^{111}Cd nucleus and allows inferences regarding the detailed coordination geometry of the metal at an active site, even when in different physical states. Changes in the environment of the metal result in multiple, different PAC spectra, and the application of Angular Overlap Theory (4) allows the extraction of detailed features bearing on microscopic structure. We have now performed PAC studies of ^{111}Cd carboxypeptidase both in solution and in crystals over the pH range 5.5 to 10.5. The NQI parameters which have been derived demonstrate significant differences between the two physical states. While the spectrum of crystals is very simple and suggests the existence of but one dominant conformation, in solution the behaviour is quite variable and complex.

EXPERIMENTAL

Bovine carboxypeptidase A was obtained from Sigma Chemical Co., and recrystallized before use. Apocarboxypeptidase crystals were prepared as described (5). The ^{111}Cd isotope was produced by bombardment of metallic Pd (enriched ^{108}Pd) with 21 Mev α-particles. The ^{111}Cd isotope prepared in this manner exists in its 497 kev excited state with a $T_{1/2}$ of 49 min. Each PAC experiment must be performed within a period compatible with this $T_{1/2}$. ^{111}Cd(II) carboxypeptidase was obtained by adding ^{111}Cd(II) to a suspension of 2 mg of apocarboxypeptidase crystals in 2 ml 0.005M Hepes pH 7.0. After 5 minutes, the crystals were washed three times with the same buffer and finally suspended in 1-2 ml of the buffer for the experiment. For solution experiments the ^{111}Cd(II) carboxypeptidase crystals were dissolved in a small volume of buffer containing 1M NaCl, passed over a Sephadex G-25 column (0.9 x 6cm) equilibrated in the buffer of the experiment and added to a solution of sucrose. The final volume was 5 ml, the sucrose concentration 50.8% W/W and the enzyme concentration about 10^{-6}M. The amount of radioactive ^{111}Cd is less than 10^{-12} moles. No carrier Cd was added. PAC spectra were recorded with a four-detector slow-fast coincidence spectrometer (1) at 22 ± 1°C. The theory and technique of perturbed angular correlation of gamma rays and its application to studies of metalloenzymes have been described, (1,2), and essentially the same procedures and treatment of the data have been employed in the present work. The coincident counting ratio $W(\theta, t)$ was measured at fixed angles 0-180°, and 0°-90° as a function of the delay time, t, between the emission of the two gamma rays. θ is the angle between the detectors of the two gamma rays. From the coincident counting rates which were determined experimentally, $W(180°,t)$ and $W(90°,t)$, the NQI parameters ω and η were calculated according to equation [1] (1).

$$\frac{W\ (180^\circ,\ t)}{W\ (90^\circ\ ,\ t)} = \frac{1 + A\ G(t,\ \omega,\ \eta)}{1 - 1/2\ A\ G(t,\ \omega,\ \eta)} \qquad [1]$$

where A is the amplitude at t = 0, equal to 0.18 for the 150-247 Kev cascade of ^{111}Cd (6). Thus, from equation [1] the perturbation function $G(t, \omega, \eta)$ can be obtained and ω and η can be determined in turn (1).

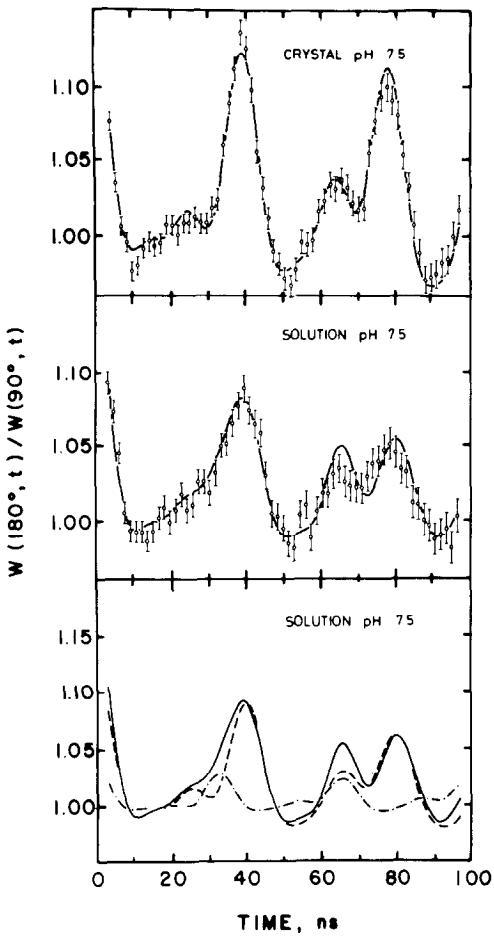

Figure 1. The two upper panels show PAC spectra of ^{111}Cd carboxypeptidase A in crystals (above) and solutions (center), pH 7.5. The line drawn through the experimental points represents the best calculated fit. The bars indicate ± one standard deviation. The lower panel represents the resolution of the spectrum in the center, the solid line, into its two components A(---) and B(-·-).

RESULTS AND DISCUSSION

Figure 1 (top) shows the PAC spectra of solutions and crystals of ^{111}Cd carboxypeptidase at pH 7.5. A single set of NQI parameters defines the crystal spectrum very well, but at this pH two such sets are required to match the solution spectrum (Figure 1, center and bottom; Table I). A species identical to the one present in the crystals accounts for about 75% of the solution spectrum but the remaining 25% differs, indicating that in solution at pH 7.5 the enzyme exists in at least two forms. Near pH 6 and in the vicinity of pH 9, the PAC spectra of ^{111}Cd carboxypeptidase crystals change

TABLE I

NQI Parameters for ^{111}Cd Carboxypeptidase at pH 7.5[a]

Physical State	ω_A(MHz)	η_A	ω_B(MHz)	η_B	%A	%B
Crystal	163±2	0.23±0.06	-	-	100	-
Solution	156±4	0.26±0.09	191±14	0.18±0.20	75±12	25±12

a) NQI parameters for A and B will be identified later as species I and IV (Table II).

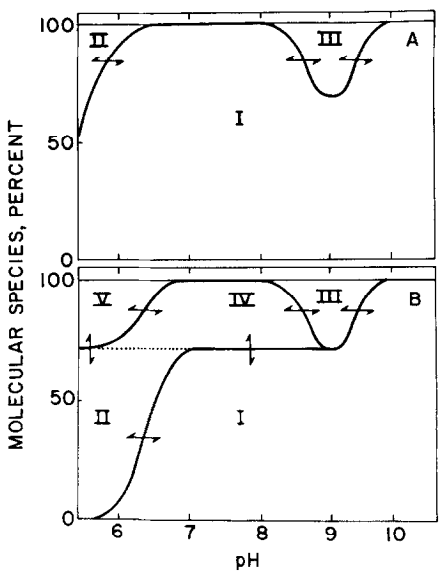

Figure 2. Schematic representation of the relative distribution of ^{111}Cd carboxypeptidase A among its various molecular species as a function of pH in crystals (A) and solution (B). The Roman numerals denote the various species detected (see text) and the solid lines represent the percent contribution of each to the total composition at any given pH. The dotted line in (B) is the boundary between species II and IV. The double headed arrows designate species in equilibrium. When parallel to the abscissa, the equilibrium is pH dependent; when perpendicular, the equilibrium is not pH dependent.

from that at pH 7.5, accounted for by the presence of three different species of the enzyme designated I, II and III in the neutral, acidic and alkaline regions, respectively (Table II). Figure 2A shows their relative abundance as a function of pH.

In solution, the ^{111}Cd carboxypeptidase PAC spectra cannot be fitted using only the three sets of NQI parameters detected for the crystal spectra

TABLE II

AVERAGE OF NQI PARAMETERS FOR ^{111}Cd CARBOXYPEPTIDASE

Crystals

ω_I	= 160±1 MHz	η_I	= 0.25±0.03
ω_{II}	= 122±10 MHz	η_{II}	= 0.48±0.16
ω_{III}	= 197±9 MHz	η_{III}	= 0.87±0.08

Solutions

ω_I	= 159±2 MHz	η_I	= 0.29±0.05
ω_{II}	= 111±4 MHz	η_{II}	= 0.65±0.07
ω_{III}	= 193±28 MHz	η_{III}	= 0.79±0.19
ω_{IV}	= 196±10 MHz	η_{IV}	= 0.29±0.15
ω_V	= 162±10 MHz	η_V	= 0.96±0.24

between pH 5.5 and 10.5, but require in addition two new sets of NQI parameters, i.e., two new species exist in solution. At pH 7.5, one of them, IV, represents about 25% of the molecular species, and at pH 5.5 and 6.5, the other, V, represents about the same percentage (Table II). Figure 2B shows the percentage of each of the species in solution as a function of pH.†

The data show that between pH 7.5 and 10.5 PAC does not detect any ionization of solvent either in the crystals or in solution. It follows, therefore, that mechanisms can be ruled out which call for such ionization(s) within this pH range either at or within 5 Å of the metal atom.

The PAC method in conjunction with the Angular Overlap Theory can identify coordination geometries, as has been shown previously for low molecular weight Cd complexes based on comparison of structural data obtained from X-ray diffraction analysis (7). The semiempirical Angular Overlap Model relates the NQI parameters ω and η to structural parameters

†The five sets of NQI parameters identified will be referred to interchangeably as molecular species of the enzyme.

proportional to the squared overlap integrals and polar angles of coordination for each of the ligands to the metal (7). The various quantum mechanical approximations and assumptions made in the Angular Overlap Theory have been detailed (4,7).

Using the Angular Overlap Model simultaneous analysis of the five sets of NQI parameters I to V have been performed for carboxypeptidase. We conclude that those three sets of NQI parameters, which exist both in solution and in the crystals i.e., I, II, and III, reflect structures that are independent of the physical state (Table II).

Calculations of coordination geometry from the PAC spectra and Angular Overlap Theory are based on the participation of four ligands: one each from nitrogens of two different histidines, one carboxyl oxygen and one solvent molecule. A distorted tetrahedral coordination with one of the nitrogens about 30^{o} off the tetrahedral position is the only geometry consistent with the data for molecular species I and II, entirely in accord with the conclusions from X-ray diffraction analysis (8). The NQI parameters of species I and II differ by the equivalent of a half a unit of charge on that solvent molecule which functions as a metal ligand. A proton bridge shared between this metal coordinated water molecule and an ionized carboxyl group could account for this fractional charge.

Such calculations further demonstrate that a weakly bound, fifth ligand opposite the carboxyl oxygen in molecular species IV and V constitutes their characteristic difference from I and II. Finally, the NQI parameters defining species III are consistent with an ionization of this fifth, weakly bound ligand. Thus, the NQI parameters for species III, IV and V are all consistent with pentacoordination.

PAC combined with Angular Overlap Theory has a number of characteristics which prove valuable in the discrimination of different molecular species, independent of the physical phase. The theory predicts the spectra; hence, discrete sets of the NQI parameters which define the spectrum can be extracted

to identify molecular species whose concentrations can then be determined to within 5-10%. Such discrete sets of NQI parameters may arise either from changes in charge of one or more groups within 5 Å of the cadmium atom or from the simultaneous existence of species with different local metal geometries. These two sources can be discriminated by study of the pH dependence of the NQI parameters. Application of the Angular Overlap Theory then defines the coordination geometry.

The present studies of carboxypeptidase emphasize the flexibility of protein molecules and demonstrate the existence of multiple and interconvertible molecular forms, both in solution and in crystals. Owing to its inherent characteristics, this method allows the inspection within a radius of five angstroms of the metal nucleus of microscopic details and thus changes at the active site of metalloenzymes, greatly potentiating the scope of mechanistic investigations.

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