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Perturbed Angular Correlation Study of a Haptenic Molecule

(rotational label/anti-Dnp/radioactive/^{111}In)

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ABSTRACT **The angular correlation of the 173–247 keV gamma-ray cascade after the electron-capture decay of ^{111}In is strongly perturbed when the 1-*p*-nitrophenylethylenediaminetetraacetate chelate of $^{111}In^{3+}$ is added to a solution containing rabbit antibody to dinitrophenyl groups. The radioactive chelate can be displaced by the addition of dinitrophenyllysine or unlabeled chelate. The average association constant between the antibody and the labeled chelate has been estimated from perturbed angular correlation measurements; this value is compared to the results of equilibrium dialysis. These experiments provide good evidence that information concerning macromolecular behavior can be obtained from perturbed angular correlation experiments that use chemically specific labels.**

The potential of perturbed angular correlation (PAC) studies to yield motional and structural information about biological macromolecules has been a subject of considerable recent interest (1–10). The PAC to be expected from nuclei attached to molecules that reorient slowly (3), anisotropically (5), with internal rotation (5), by random jumps (6), or in one dimension (7) have been calculated by various authors. As the theoretical understanding of the effects of molecular motion on angular correlations has become more complete, PAC has gained considerable potential usefulness as a labeling method. Like rotational labeling techniques that use other physical methods, such as electron spin resonance (11), fluorescence depolarization (12), and nuclear magnetic resonance (13), PAC measurements can yield information concerning the localized behavior and structure of a molecule near the labeled site.

Several isotopes, such as ^{111}In (1, 4, 10), ^{111m}Cd (2, 9), ^{199m}Hg (14), ^{204m}Pb (15), or ^{62}Zn (16) can be used in PAC studies. Because of its 2.8-day half-life, ^{111}In (Fig. 1) is particularly convenient. The applicability of ^{111}In for studies of macromolecular behavior has been demonstrated (1, 4, 10). However, the lack of versatility in selective attachment of radioactive rotational labels to specific sites on macromolecules has been a severe limitation. This paper describes the use of a small bifunctional organic molecule, 1-*p*-nitrophenylethylenediaminetetraacetic acid (NO_2Ph-EDTA) (Fig. 2), that (*A*) chelates In^{3+} ions so that they are not released into aqueous buffer solutions at neutral pH and (*B*) binds to the combining site of rabbit antibody to dinitrophenyl groups (anti-Dnp). Presumably, the presence of the *p*-nitrophenyl group is primarily responsible for the crossreaction with anti-Dnp. The average association constant between anti-Dnp and the labeled hapten has been estimated from PAC data obtained with rabbit antiserum; these measurements are compared to the results of equilibrium dialysis. Further, it appears that the chemical selectivity available with other labeling techniques may be achieved for PAC, because NO_2Ph-EDTA is a potential intermediate to several other labels. Since PAC measurements can be performed *in vivo* (10), and with much of the sensitivity and instrumental simplicity of radioactive tracer experiments, the method is a useful complement to existing techniques.

THEORETICAL

The ability to use a gamma-ray cascade from a radioactive nucleus to monitor molecular motion arises from the fact that there can be a strong angular correlation between the directions of propagation of the gamma rays in the cascade. Since the direction of emission of a gamma ray depends on the orientation of the magnetic moment of the emitting nucleus, this angular correlation will be perturbed if the nucleus in the intermediate state is reoriented by interaction with its surroundings. In order that there be an angular correlation, the intermediate state of the cascade must have a nuclear spin greater than, or equal to, one. Such a nucleus can have a quadrupole moment, and the interaction of the quadrupole moment with external field gradients usually represents the major perturbing influence on the angular correlation (17, 18). This phenomenon is favorable for the study of molecular

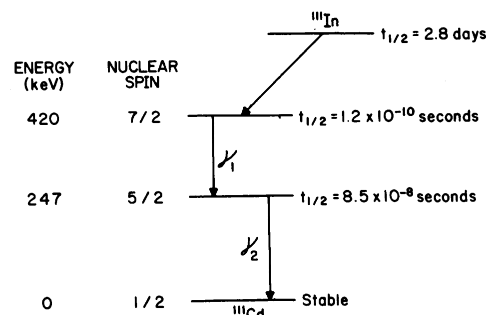

FIG. 1. The 173–247 keV gamma-ray cascade of ^{111}Cd after the electron-capture decay of ^{111}In (20).

Abbreviations: PAC, perturbed angular correlation; Anti-Dnp, rabbit antibody to dinitrophenylated bovine-serum albumin; PBS, 0.01 M phosphate–0.14 M NaCl (pH 7.0); NO_2Ph-EDTA, 1-*p*-nitrophenylethylenediaminetetraacetic acid; NO_2Ph-EDTA-(In), sodium 1-*p*-nitrophenylethylenediaminetetraacetatoindate-(III).

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rotational motion, because the nuclear reorientation rate due to a quadrupolar interaction is affected only by rotational motion, and not by relative translational motion or paramagnetic impurities (19).

Nuclear reorientation in liquids often can be characterized by a relaxation time, which may be obtained from a measurement of the perturbed angular correlation. This nuclear relaxation time can depend strongly on the rate of rotation of the molecule or group to which the nucleus is bound; therefore, it may be used to estimate the rotational correlation time, which is a measure of the rate of molecular reorientation.

For the 173–247 keV gamma-ray cascade in ^{111}Cd after the electron-capture decay of ^{111}In (Fig. 1), the coincidence counting rate, $W(\Theta,t)$, is given by (17)

$$W(\Theta,t) = \frac{e^{-t/\tau_N}}{\tau_N} [1 + A_{22}G_{22}(t)P_2(\cos \Theta)] \quad [1]$$

where $P_2(\cos \Theta)$ is the Legendre polynomial $^1/_2(3 \cos^2 \Theta - 1)$, τ_N (1.22×10^{-7} sec) (20) is the mean lifetime of the 247 keV state of ^{111}Cd, and A_{22} (-0.18) is a parameter that depends on the spins and multipolarities associated with the cascade. The differential perturbation factor, $G_{22}(t)$, completely describes the interaction of the nucleus in the intermediate state with external fields. For liquid samples in which the rotational correlation time, τ_C, of the molecule to which the emitting nucleus is bound is short compared to τ_N, the perturbation factor has been shown to be (18)

$$G_{22}(t) = e^{-t/T_1} \quad [2]$$

where

$$\frac{1}{T_1} = \frac{63}{1000} \frac{\langle (e^2qQ)^2 \rangle}{\hbar^2} \tau_C \quad [3]$$

for an axially symmetric electric-field gradient and a nuclear spin of 5/2. The brackets denote an ensemble average, and T_1 is the nuclear relaxation time. If $\tau_C \geq \tau_N$, G_{22} (t) is more complex (3, 5–7).

It is often experimentally advantageous to determine the time-integrated perturbation factor, $\overline{G_{22}(\infty)}$, where

$$\overline{G_{22}(\infty)} = \frac{1}{\tau_N} \int_0^\infty e^{-t/\tau_N} G_{22}(t) dt \quad [4]$$

since such a measurement may be performed with simpler apparatus and in less time than a measurement of $G_{22}(t)$. For large quadrupolar interactions, $\overline{G_{22}(\infty)}$ exhibits a strong dependence on the rotational correlation time when τ_C is between 0.01 and 10 nsec (3, 4). Therefore, it is straightforward to observe the interaction between a dissolved macromolecule and a small, tracer-containing molecule that binds noncovalently to sites on the macromolecule. If the exchange of the tracer molecules between these binding sites and the solvent is slow compared to the decay rate of the intermediate state of the gamma-ray cascade ($1/\tau_N$), then the measured perturbed angular correlation will be characteristic of two separate groups of nuclei that differ only in rotational correlation time. The contribution of each group to the measured value of the perturbation factor will then be proportional to the number of nuclei in that group. For the "slow exchange" situation, the differential perturbation

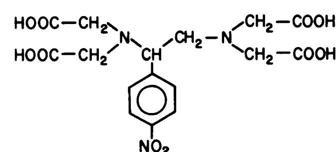

FIG. 2. 1-p-Nitrophenylethylenediaminetetraacetic acid (NO_2-Ph-EDTA).

factor will be given by:

$$G_{22}(t) = N_f G_f(t) + N_b G_b(t) \quad [5]$$

where $N_f + N_b = 1$, and where N_i is the population of group i and $G_i(t)$ is the corresponding differential perturbation factor. Similarly,

$$\overline{G_{22}(\infty)} = N_f \overline{G_f} + N_b \overline{G_b} \quad [6]$$

where $\overline{G_i}$ is the integral perturbation factor characteristic of group i. Thus, a knowledge of $\overline{G_f}$ and $\overline{G_b}$ permits the determination of N_b/N_f, the ratio between the number of tracer molecules bound to the macromolecules and the number free in solution. Therefore, under slow exchange conditions, one PAC measurement provides information similar to an equilibrium dialysis measurement, and it is possible to use PAC to determine antibody–hapten association constants (21, 22).

MATERIALS AND METHODS

The ^{111}In used in these experiments was prepared in the Oak Ridge Cyclotron by the ^{111}Cd$(p,n)^{111}$In reaction on enriched ^{111}CdO. It was obtained as a carrier-free solution in 0.05 N HCl. Angular correlation measurements were made with a four-detector fast/slow coincidence spectrometer that uses Amperex 56 DVP photomultiplier tubes and 5 cm by 5 cm NaI(Tl) crystals. Data for the differential angular correlation measurements were supplied to a Nuclear Data 2201 multichannel analyzer by an ORTEC time-to-pulse-height converter driven by EG&G fast electronics. Canberra NIM electronics were used for coincidence counting and energy analysis. Random coincidence rates were determined by the use of delayed pulse generators to compare 173 keV photons with 247 keV photons that had been delayed for 3 μsec. All measurements were corrected for the effect of the solid angle subtended by the detectors (23).

Rabbit antiserum to Dnp-bovine-serum albumin, 1.8 mg of antibody per ml of serum by precipitin test, was purchased from Miles–Yeda Ltd., Rehovot, Israel (lot no. 4-8P). It was dialyzed against 100 volumes of a solution 0.01 M in phosphate and 0.14 M in chloride at pH 7.0 (PBS). An Ouchterlony diffusion test (ref. 22, pp 200–209) revealed a single band of precipitate. Lyophilized normal control serum from Miles–Yeda (lot no. 13) was dissolved in PBS. Deferoxamine methylsulfonate was obtained from CIBA Pharmaceutical Co., Summit, N.J. The ε-Dnp-*l*-lysine (Dnp-lysine) was purchased from Schwarz/Mann, Orangeburg, N.Y. Chelex 100 and Dowex 50W × 2 ion-exchange resins were purchased from Bio-Rad Laboratories, Richmond, Calif. Microdialysis chambers (22) were a generous loan from Dr. Grace Rosenquist. Pierce "reacti-vials" were purchased from Pierce Chemical Co., Rockford, Ill. All binding studies were done at 24 ± 0.5°.

TABLE 1. *PAC titration*

NO_2Ph-EDTA(In) added (μl)	$\overline{G_{22}(\infty)}$	N_b/N_f	$\frac{r}{2-r}$	c (μM)
1	0.455	0.397	0.013	0.356
2	0.467	0.356	0.024	0.730
4	0.510	0.233	0.035	1.59
6	0.514	0.222	0.051	2.38
9	0.520	0.205	0.073	3.58
12	0.539	0.160	0.080	4.88
17	0.550	0.134	0.099	6.91
22	0.550	0.134	0.131	8.74

Initial antibody concentration, 1.12 nmol (based on a molecular weight of 160,000) in 0.20 ml of PBS; hapten concentration, 0.1 mM. Bound/free ratio, N_b/N_f; mol hapten bound per mol antibody, r; free hapten concentration, c. Calculations made with $\overline{G_f} = 0.618 \pm 0.003$ and $\overline{G_b} = 0.044$. In each case, the standard deviation in $\overline{G_{22}(\infty)}$ is 0.007.

Preparation of 1-p-Nitrophenylethylenediaminetetraacetic Acid. (Fig. 2). 1-Phenylglycinonitrile was reduced under 3 atm of hydrogen in the presence of Raney nickel and acetic anhydride (24). The resulting *N,N'*-diacetyl-1-phenylethylenediamine was then nitrated and hydrolyzed to the free amine. Carboxymethylation of the resulting 1-*p*-nitrophenylethylenediamine with iodoacetate or bromoacetate (25) gave 1-*p*-nitrophenylethylenediaminetetraacetic acid.

Anal. calcd. for $C_{16}H_{19}N_3O_{10}\cdot H_2O$: C, 44.54; H, 4.90; N, 9.74%. *Found:* C, 44.59; H, 4.83; N, 9.67%.

A complete description of the synthesis will be published elsewhere (M. W. Sundberg and L. Werthemann, manuscript in preparation).

Preparation of Sodium 1-p-Nitrophenylethylenediaminetetraacetatoindate(III) [NO_2Ph-EDTA(In)]. In a 300-μl reactivial, 5 μl of a 5 mM solution of deferoxamine methylsulfonate was added to 100 μl of the $^{111}InCl_3$ solution obtained from Oak Ridge. The chelate formed between ferric ion and deferoxamine is very stable (26); this step presumably removes free iron from the $^{111}InCl_3$ solution. After 10 min, 2 μl of a 0.1 mM solution of NO_2Ph-EDTA was added, and the reaction mixture was allowed to stand overnight. In order to remove free NO_2Ph-EDTA and the cationic deferoxamine chelates, the solution was passed through a column 0.5 cm in diameter containing 2 cm of copper–Chelex 100 prepared according to Siegel and Degens (27) and 4 cm of Dowex 50W × 2 in the Na^+ form that had been equilibrated with acetate at pH 5 and then thoroughly washed with water. The column was eluted with less than 1 ml of water, yielding a solution of NO_2Ph-EDTA(^{111}In).

Addition of NO_2Ph-EDTA(^{111}In) to PBS yielded $\overline{G_{22}(\infty)} = 0.71$, as did measurements of NO_2Ph-EDTA(^{111}In) in phosphate-free solutions at pH 4. Even after 4 days, the value of $\overline{G_{22}(\infty)}$ for NO_2Ph-EDTA(^{111}In) in PBS was within experimental error of the initial value. When $^{111}InCl_3$ was added to PBS, on the other hand, the resulting $\overline{G_{22}(\infty)}$ was 0.30, and much of the radioactivity adhered to the walls of the container (presumably due to the formation of insoluble phosphates). This result, which agrees with data reported by Saito and Tsuchimoto (28) for the ethylenediaminetetraacetate chelate of In^{3+}, indicates that the dissociation of NO_2Ph-EDTA(In) is negligible on the time scale required for labeling experiments.

Samples for PAC measurements were prepared by addition of 100-μl aliquots of a PBS solution containing about 10 μCi of ^{111}In as NO_2Ph-EDTA(^{111}In) to 100 μl of the antiserum. The initial concentration of NO_2Ph-EDTA chelate in the sample was less than 0.2 μM. This solution was then titrated

TABLE 2. *Association constants evaluated from the data in Fig. 4*

	a	K(M^{-1})	Linear-correlation coefficient
PAC	0.7	0.6 × 10^4	0.996
Equilibrium dialysis	0.7	1.6 × 10^4	0.987

The standard deviation of the measurement of the heterogeneity index, *a*, is about 0.08. The standard deviation of the mean association constant, *K*, is about a factor of four.

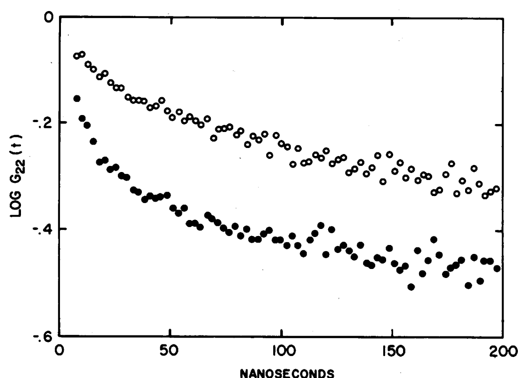

FIG. 3. The logarithm of the differential perturbation factor plotted against time for NO_2Ph-EDTA(^{111}In) in (●) rabbit antiserum to Dnp and (○) rabbit antiserum to Dnp containing excess Dnp-lysine.

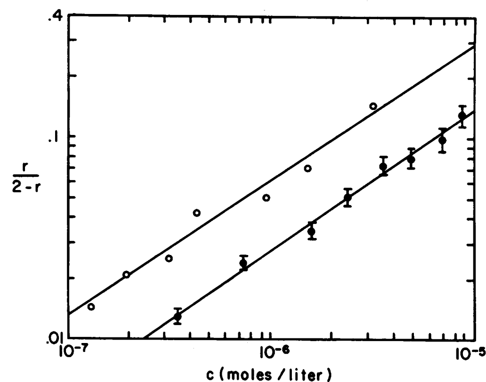

FIG. 4. Antibody–hapten binding data measured by (●) PAC and (○) equilibrium dialysis. Both sets of data are plotted according to the Sips equation, where *c* is the concentration of free hapten and *r* is the mol of hapten bound per mol of antibody. *Error bars* represent one standard deviation.

by the addition of microliter increments of a 0.1 mM solution of the NO_2Ph-EDTA chelate of nonradioactive In^{3+}. After each incremental addition, the sample was counted for about 35 min. The integral perturbation factors at each hapten concentration are given in Table 1.

The interaction between NO_2Ph-EDTA(In) and the antibody was also studied by equilibrium dialysis. Solutions of labeled NO_2Ph-EDTA(In) of various concentrations in PBS were dialyzed against rabbit serum or anti-serum to Dnp in microdialysis chambers. The concentration of free hapten at equilibrium was determined by measurement of the ^{111}In activity. It was not possible to measure the concentration of bound hapten because of the large volume changes due to the presence of serum proteins. To determine when equilibrium had been reached, a solution of hapten was dialyzed against PBS. A correction was made for nonspecific binding of the NO_2Ph-EDTA (In) to serum proteins by dialysis of NO_2Ph-EDTA(In) against normal serum, and the amount of hapten bound in that case was subtracted from the amount available in the antiserum dialysis. For the range of concentrations studied, the amount of nonspecifically bound hapten was about 10% of the amount added.

RESULTS

Fig. 3 shows a semilogarithmic plot of $G_{22}(t)$ for a solution containing NO_2Ph-EDTA(^{111}In) and rabbit antiserum to Dnp under conditions at which about one-third of the label is bound to the antibody. The data from a similar solution to which enough Dnp-lysine had been added to saturate the antibody-binding sites are also plotted.

From the data in Fig. 3, it is evident that the angular correlation is strongly perturbed when NO_2Ph-EDTA(^{111}In) is added to anti-Dnp. However, at times longer than 50 nsec the shapes of the two curves are quite similar, the only difference being a displacement along the y-axis by a distance approximately equal to log N_f. Therefore, it appears that the behavior of NO_2Ph-EDTA(^{111}In) in the presence of anti-Dnp can be described by Eq. [**5**]. This result substantiates the assumption that the exchange of hapten between the two environments is slow compared to $1/\tau_N$. For the bound NO_2Ph-EDTA(^{111}In), $G_b(t)$ is probably not a simple exponential function (3, 17, 18), but its true shape cannot be ascertained from the data in Fig. 3.

Since antibody populations are heterogeneous and, therefore, exhibit a distribution of binding constants, a Sips plot is often used to determine the average association constant and the width of the distribution. This method, which has been amply described elsewhere (21, 22), depends on the relation:

$$\log\frac{r}{n-r} = a \log c + a \log K \qquad [7]$$

where r is the number of mol of hapten bound per mol of antibody, n is the number of binding sites per antibody molecule ($n = 2$ for immunoglobin G), c is the concentration of free hapten, a is the heterogeneity index ($0 \leq a \leq 1$), and K is the average association constant or affinity. A plot of $\log[r/(n - r)]$ versus log c yields both a and K.

The data from a PAC titration of antiserum to Dnp are shown in Table 1 and are plotted along with the equilibrium dialysis results in Fig. 4. At free hapten concentrations larger than those shown, accurate measurements were difficult to obtain by either technique. This problem may be due to the low affinity and to the presence of serum proteins. The lack of overlap between the two sets of data suggests a small, systematic difference between the two methods of analysis. However, as shown in Table 2, both methods yield the same value for the heterogeneity index, $a = 0.7$. Also, considering the numerous experimental difficulties involved in measurements on crude antiserum, the value $K = 0.6 \times 10^4$ M^{-1} obtained by PAC is in reasonable agreement with the equilibrium dialysis res ılt, $K = 1.6 \times 10^4$ M^{-1}.

The value of $\overline{G_{22}(\infty)}$ for a solution of free (nonspecifically bound) hapten in the presence of serum proteins, $\overline{G_f} = 0.618$, was measured both by determination of $\overline{G_{22}(\infty)}$ after displacement of the NO_2Ph-EDTA chelate from rabbit anti-Dnp with Dnp-lysine and by determination of $\overline{G_{22}(\infty)}$ for a solution of NO_2Ph-EDTA(^{111}In) in normal rabbit serum. Both values fell within the experimental error limit. The value of $\overline{G_b}$ for a solution of hapten totally bound by antibody, $\overline{G_b} = 0.044$, was calculated by use of the adiabatic approximation (3, 5), with the assumption that the effective $\tau_C = 35$ nsec (11, 12);

$$\overline{G_b} = (1/5)\frac{1}{1 + \tau_N/\tau_C} = 0.044.$$

The parameters a and K are insensitive to the magnitude of $\overline{G_b}$; variation of $\overline{G_b}$ by ±100% does not significantly affect their values.

DISCUSSION

It is evident from this work that the NO_2Ph-EDTA chelate of $^{111}In^{3+}$ may be used to observe changes in molecular motion despite the disruptive aftereffects of the electron-capture decay that precedes the gamma-ray cascade. The agreement between the PAC results and the equilibrium dialysis measurements is particularly encouraging, since the low value for the antibody–hapten affinity in this system makes accurate measurements difficult.

The current work also illustrates several characteristics of PAC that make it unique as a labeling method. About 10^{10} molecules containing ^{111}In are required for a PAC experiment, so it is possible to make measurements on very small quantities of the macromolecule of interest. Quantitative PAC measurements are best made on a point source of radiation (23), so quite small volumes of sample can be studied as well. In this work, each titration was performed on antiserum containing 0.18 mg of antibody, and it should be possible to use much less.

With the apparatus used here, a PAC titration can be completed in about 4 hr, but this time requirement can be greatly reduced by the use of more instrumentation. Thus, measurements can be made on relatively unstable systems. Further, PAC provides a direct measure of bound and free label concentrations when $\overline{G_f}$ and $\overline{G_b}$ are known.

As with other motional labeling techniques, the label used for a PAC experiment must exhibit a detectable change in its rotational correlation time upon binding to the macromolecule, so it is desirable to minimize the size of the tracer molecule in order to make the change as large as possible. The current lack of versatile labeling molecules is a serious constraint, but a potential intermediate to several labels has been prepared by reduction of the nitro group of NO_2Ph-

EDTA to an amine. Work on the synthesis of such compounds is continuing.

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