Intramolecular Reorientational Motion in Trypsinogen Studied by Perturbed Angular Correlation of ^{199m}Hg Labels

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Time-differential perturbed angular correlation measurements of the nuclear quadrupole interaction of ^{199m}Hg labels introduced into the disulfide bridge of the activation domain of bovine trypsinogen reveal intramolecular reorientational motion over a wide angular range with a correlation time $\tau_c = 11^{+6}_{-3}$ nsec. No dynamics in this time range was observed in the trypsinlike complex formed with pancreatic trypsin inhibitor, where the activation domain is rigid.

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The digestive enzyme trypsin is produced in the pancreas in the form of the inactive trypsinogen (Tg). The activation domain of bovine Tg is not visible in x-ray diffraction, in contrast to trypsin or the Tg complex formed with pancreatic trypsin inhibitor plus the dipeptide Ile-Val (Tg-PTI-I-V).[1] This feature is considered to prevent substrate binding and might be due to either dynamic flexibility or a superposition of different conformations. Since the activation domain of Tg remained invisible even at temperatures as low as 103 K it was considered predominantly statically disordered.[2] Yet, the mere existence of conformational heterogeneity enables collective conformational transitions to occur.

In this Letter we report on the first observation of dynamic flexibility of the activation domain in Tg in the 10-nsec range by means of time-differential perturbed angular correlation (TDPAC) of γ rays emitted from radioactive ^{199m}Hg labels. In the case of Tg there is no possibility to attach fluorescence[3,4] or chemical[5] labels rigidly to the activation domain without inducing the undesired rigid trypsinlike structure. However, atomic mercury between the disulfide bridge CYS191 and CYS220 does not destroy the flexibility and leads to minor structural modifications only.[2] The use of the isomeric decay of ^{199m}Hg ($T_{1/2} = 43$ min) avoids ambiguities in the interpretation of the results due to aftereffects encountered with preceding β or electron capture (EC) decays and the associated label transmutation.[6]

The TDPAC measurements were performed on the (375-158)-keV γ-γ cascade of ^{199m}Hg, whose 158-keV state has a half-life $\tau_{1/2} = 2.35$ nsec. A conventional four-detector apparatus of the fast-slow coincidence type was used.

In analogy to time-differential fluorescence depolarization,[4] in TDPAC the anisotropy A_2 of the emission probability of the second γ quantum with respect to the first, which is a consequence of angular momentum conservation, is perturbed by extranuclear fields. For a nuclear spin $I = \frac{5}{2}$ and static quadrupole interaction, i.e., constant interaction strength averaged over fluctuations fast compared to nuclear precession frequencies ω, the perturbation function reads[7]

$$G_{22}^{\text{static}}(t) = a_0 + \sum_{i=1}^{3} a_i \exp(-\tfrac{1}{2}\delta^2\omega_i^2 t^2)\cos\omega_i t, \qquad (1)$$

$$\omega_3 = \omega_1 + \omega_2 .$$

For random powder and an axially symmetric electric field gradient (EFG) eq we have $a_0 = 0.2$, $a_1 = 0.371$, $a_2 = 0.286$, $a_3 = 0.143$, and $\omega_2/\omega_1 = 2$ with $\omega_1 = 3\pi e^2 qQ/10h$ (Ref. 7) (eQ denotes the quadrupole moment of the 158-keV state). The exponential damping factors in front of the cosine terms account for a (Gaussian) frequency dis-

tribution. If the EFG tensor changes its orientation during times shorter than or of the order of $2\pi/\omega$, the perturbation factor reads for the following limiting cases (spherical rotor)

$$G_{22}(t) = \exp(-\lambda t) \quad (2)$$

for fast relaxation[8] or

$$G_{22}(t) = \exp(-t/\tau_c) G_{22}{}^{\text{static}}(t) \quad (3)$$

for slow relaxation.[9] Here, λ is proportional to $\langle (eq)^2 \rangle \tau_c$, where τ_c is the reorientational correction time. In both cases $G_{22}(t)$ goes to zero for sufficiently long times, contrary to the static inhomogeneous case, where the "hard-core" a_0 survives. The finite resolving time of our apparatus (2.4 nsec full width at half maximum) was accounted for.

For all sample preparations 2-mg HgO (enriched in ^{198}Hg by $\geq 85\%$) were irradiated in a thermal neutron flux of 10^{13} n/cm^2 sec and subsequently dissolved in 100 μl of 50% acetic acid (henceforth called ^{199m}Hg acetate). Several control experiments were performed in order to facilitate the interpretation of the protein results:

(a) HgO, polycrystalline, at 300 K, was studied as irradiated (not annealed).

(b) As a model compound for the CYS-Hg-CYS bridge we studied Hg-dithiothreitol (Hg-DTT) at 300 K: ^{199m}Hg acetate was added to excess DTT in 2 ml of phosphate buffer (pH = 6.5). Hg-DDT precipitates immediately. The supernatant liquid was not removed.

(c) Since all protein solutions contained ethylenediaminetetraacetic acid (EDTA) in order to avoid nonspecific bonding of Hg, Hg-EDTA was studied at 258 K: ^{199m}Hg acetate was added to excess EDTA in 2-ml phosphate buffer and frozen to 258 K.

The reduction of the disulfide bridge in Tg was carried out in analogy to the procedure described in Ref. 2. The reduced Tg was frozen and stored in dry ice for up to 2 h until it was made to react with ^{199m}Hg acetate.[10]

It would be desirable to perform the protein measurements under physiological conditions. However, the tumbling motion of Tg in a liquid might overshadow the effect due to intramolecular flexibility. The reorientational correlation time for trypsin, e.g., is $\tau_c = 12.9$ nsec (at 25 °C, 1 cP),[4] sufficiently short to obscure any additional motion on the same time scale. Keeping in mind that all the following procedures had to be completed within 30 min after irradiation because of the short lifetime of ^{199m}Hg, we therefore tried the following to slow down the tumbling motion:

After reacting Tg in phosphate buffer with ^{199m}Hg acetate for 3–5 min we added 0.5 g Sephadex or 0.5 g polyethyleneglycol (PEG4000). These methods proved unsatisfactory because it was impossible to adjust the viscosity reproducibly.

(d) Next, we froze the solution to 258 K. All these three methods have the disadvantage that the Hg-EDTA spectrum appears superimposed on the HgTg spectrum.

(e) Therefore we precipitated HgTg after mercuration with 2 ml of methanol, centrifuged for 3 min at 3000 rpm, and removed the supernatant Hg-EDTA–containing liquid. Inspection of the radioactivity revealed that (40–60)% of the Hg was bound to the protein, in agreement with chemical determinations.

(f) HgTg-PTI-I-V was formed by addition of 60 mg of PTI and 10 mg of I-V to HgTg prior to precipitation (1–2 min reaction time). The observed spectra, which are a sum over two to three separate samples, are shown in Fig. 1. The solid lines represent least-squares-fitted functions of the type of Eq. (1) [Figs. 1(a)–1(c), 1(d), and 1(f)] and Eq. (3) [Fig. 1(e)]. The relevant parameters, A_2, ω_1, asymmetry parameter η, 2δ, and τ_c, are compiled in Table I. The spectra obtained using Sephadex or PEG4000 (not shown here) were similar to the one shown in Fig. 1(e) with the exception that a fast relaxation-type decay [Eq. (2)] with $1/\lambda = 10$–20 nsec was superimposed. The average anisotropy $A_2 = +0.12(1)$ is in good agreement with Ref. 11.

From these results we can draw the following conclusions:

(i) The frozen EDTA chelate yields a frequency of 350 Mrad/sec, compatible with a nearly perfect tetrahedral coordination of Hg. The high frequency around 900 Mrad/sec observed in HgTg and HgTg-PTI-I-V results from CYS-Hg-CYS. Since it is not very much lower compared to the results for the twofold coordinated systems with linear bonds HgO and $HgCl_2$,[11] this would suggest a twofold liganded Hg in the protein as well (our model substance Hg-DTT exhibits an even higher coupling strength; unfortunately the coordination of Hg in Hg-DTT is unknown). This suggestion, however, is in conflict with a S-Hg-S bond angle of about 105°, indicative of tetrahedral coordination, which is obtained from the difference Fourier map of HgTg-PTI-I-V/Tg-PTI-I-V.[2] Unfortunately only the two sulfur ligands are visible crystallographically. Hence, no definite conclusion about the detailed ligation of Hg is possi-

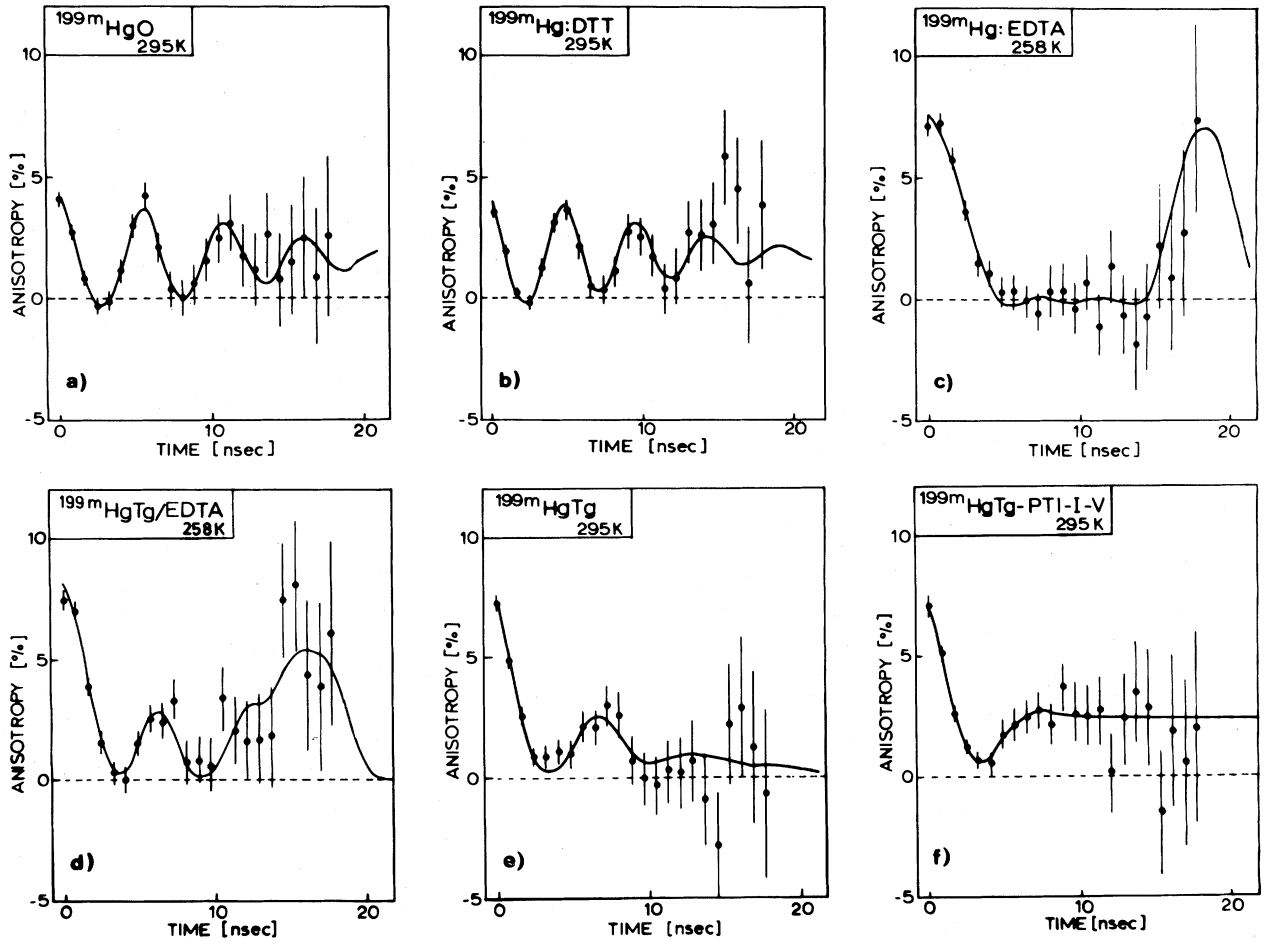

FIG. 1. TDPAC spectra showing nuclear quadrupole interaction of ^{199m}Hg for (a) HgO; (b) Hg-DTT; (c) Hg-EDTA; (d) HgTg/EDTA; (e) HgTg; and (f) HgTg-PTI-I-V. All spectra show a static, in some cases strongly inhomogenous, interaction with the exception of (e) which displays slow relaxation behavior.

ble at present.

(ii) The frozen HgTg [experiment (d)] revealed the Hg-EDTA component and an apparently sharp, static HgTg component, indicative of a lack of flexibility and static disorder. Both effects might be a consequence of solvent freezing and will not be discussed further. Yet, the observed ratio of Hg-EDTA to HgTg of about 1:1 is in good agreement with other previous determinations. The spectra obtained with Sephadex and PEG4000 suggest that Hg-EDTA still rotates rapidly. No conclusions concerning the flexibility in HgTg can be drawn because of insufficient accuracy of these spectra.

(iii) The static spectrum of HgTg-PTI-I-V demonstrates that there is no wide-angle intramolecular reorientational motion in the nanosecond range (translation is possible). The broad inhomogeneous distribution of EFG's results from a substantial heterogeneity of the Hg bond length and/or bond angles, possibly exceeding those of the three-site model proposed in Ref. 2.

(iv) The precipitated HgTg [experiment (e)] clearly displays the slow relaxation behavior [total loss of anisotropy, Eq. (3)]. Thus apart from fast atomic vibrations, conformational transformations occur with a reorientational correlation time $\tau_c = 11^{+6}_{-3}$ nsec, at least as far as the Hg environment is concerned. Although an initial conformational change might have been induced by the recoil energy of 0.38 eV due to the 375-keV γ emission, this recoil is not responsible for the relaxation behavior because it is absent in HgTg-PTI-I-V. Since a total loss of anisotropy would require isotropic reorientation (0° to 90° suffices because of EFG tensor symmetries), we conclude that reorientation of the EFG tensor over a substantial angular range oc-

TABLE I. Results obtained from least-squares fits: anisotropy A_2, precession frequency ω_1, asymmetry parameter η, frequency distribution 2δ, and reorientational correlation time τ_c. When no error limits are quoted the corresponding quantity was kept constant.

Compound	Temp. (K)	A_2 (%)	ω_1 (Mrad/sec)	η	2δ (%)	τ_c (nsec)
HgO	300	11.8(1)	1190(32)	0[a]	14(6)	•••
Hg-DTT	300	13.7(1.4)	1341(39)	0[a]	16(6)	•••
Hg-EDTA	258	8.4(5)	354(20)	0	0	•••
HgTg/EDTA	258	12.6(1.3)	406(40)[b]	0	0	•••
			1051(84)	0[c]	0	•••
HgTg	300	14.0(1.7)	975(105)	0[c]	32(18)	11^{+6}_{-3}
HgTg-PTI-I-V	300	12.3(1)	842(183)	0[c]	70(18)	•••

[a] ω_2 is not detectable because of time resolution limitations. η can be close to either 0 or 1. The choice $\eta=1$ would give better agreement with A_2 of Hg-EDTA.

[b] Two-component spectrum with relative weight 48(12)% low frequency, 52% high frequency.

[c] η unknown. Neither ω_1 nor τ_c depends sensitively on the choice for η.

curs. For a linear S-Hg-S bond, e.g., the largest component principal axis coincides with this bond axis and a rotation of this segment and possibly the neighboring peptide chain would be required.

The magnitude of the transformation enthalpy, which is the subject of further investigations, ultimately decides whether the invisibility of the activation domain in x-ray diffraction at 103 K is still a result of rapid conformational transformations or due to a freezing of the system into many different substates (glassy state).

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[10] We used the following reagents: 200 mg Tg, 20 mg benzamidine hydrochloride, 2 mg EDTA, 50 μl 4*M* K_3PO_4 buffer, 100 μl 4*M* NaH_2PO_4 buffer, 200 μl $NaBH_4$ solution, 1 ml H_2O, 70 μl acetone. Tg was found to be mercurated to 60% by protein and Hg analysis after separation from the reagents via a Sephadex column.

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