ABSOLUTE MEASUREMENT OF ENHANCED FLUCTUATIONS IN ASSEMBLIES OF BIOMOLECULES BY ULTRASONIC TECHNIQUES

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ABSTRACT By expressing the fluctuation-dissipation theorem explicitly, equations are obtained for the ultrasonic relaxation amplitudes that contain one single molecular parameter, i.e., the fluctuation, or the sum of fluctuations. The absolute measurement of this parameter is therefore possible. The equations apply to a two-state system, to a multistate system and to a linear Ising chain as well. In an aqueous medium, where molar volume changes are important, the ultrasonic relaxation amplitudes are proportional to the volume fluctuations. For assemblies of biomolecules that exhibit enhanced ultrasonic absorption on assembly it is possible to measure the increase on assembly of the sum of fluctuations. In view of application to tobacco mosaic virus protein aggregates, examples are given in which the fluctuations associated with two normal modes of relaxation are equally enhanced when the difference of conformational stability of the states is reduced. The corresponding observable changes of the ultrasonic spectra are described.

INTRODUCTION

Protein and nucleoprotein self-assemblies have recently been found to exhibit increased ultrasonic absorption in comparison with their dissociated subunits. This effect, first observed for small icosahedral plant viruses and their capsids (1, 2), was found also for other self-assembled systems, in particular for tobacco mosaic virus (3–5), frog virus 3 (6), microtubules (4), and hemocyanins (4). It was interpreted as revealing structural fluctuations specific to assemblies (1, 2); possible sources for specific fluctuations were considered in the case of icosahedral plant viruses (7).

Here we show that ultrasonic relaxation experiments provide the absolute measurements of the fluctuations, or of the sum of fluctuations, as defined by Eqs. 5 and 7. This property, which is a consequence of the fluctuation-dissipation theorem (see Appendix), is derived in Multiple Relaxation from a simplified version of the linear response theory (8), that is well adapted to the study of Markovian processes considered here, such as the switching of a molecule among conformational states. We then describe enhanced conformational fluctuations, a process by which the observable absorption may be uniformly increased in a range of values of the ultrasonic frequency.

As our systems are aqueous solutions, the solute's contribution to the ultrasonic absorption is sensitive mainly to molar volume changes (9). Thus, only volume fluctuations will be considered here. Generalization of the results to fluctuations that would include both volume and enthalpy changes is straightforward.

SINGLE RELAXATION

Consider the two-state model and let Δv be the difference of the volumes of the states. It is readily seen that

$$\langle \delta v^2 \rangle = \langle v^2 \rangle - \langle v \rangle^2 = (\Delta v)^2 K(1 + K)^{-2}, \quad (1)$$

where K is the equilibrium constant. On the other hand, in aqueous dilute solution, the solute's contribution $\Delta\alpha$ to the ultrasonic absorption is (9)

$$\frac{\Delta\alpha}{\omega^2} = \frac{\rho c c_s (\Delta v)^2}{2kT} \frac{K}{(1 + K)^2} \frac{\tau}{1 + \omega^2\tau^2}, \quad (2)$$

where ρ is the density of the solution; c, the sound velocity; c_s, the number of systems per unit volume; k, Boltzmann's constant; T, the absolute temperature; τ, the relaxation time; and ω, the circular frequency.

If there are μ independent relaxing systems per molecule (or per assembly of molecules), and c_M is the (known) number of molecules (or of assemblies) per unit volume

$$c_s = \mu c_M. \quad (3)$$

Hence, $\Delta\alpha/\omega^2$ may be written

$$\frac{\Delta\alpha}{\omega^2} = \frac{\rho c c_M S}{2kT} \frac{\tau}{1 + \omega^2\tau^2}, \quad (4)$$

The laboratory was formerly named Laboratoire d'Acoustique Moléculaire.

where we set

$$S = \mu \langle \delta v^2 \rangle \tag{5}$$

and call this the sum of (volume) fluctuations. This term is preferred to that of total fluctuation, because one wishes to characterize the relaxing system rather than the entire molecule, or the assembly. Once the relaxation amplitude (equal here to the value of the righthand side of Eq. 4 for $\omega = 0$) and the relaxation time τ have been measured, Eq. 4 provides the absolute measurement of the only unknown, i.e., the sum of fluctuations S.

In aqueous media the solute's contributions to both the isothermal and adiabatic compressibilities are equal, and the compressibility of the relaxing system is simply denoted as β. By a derivation similar to that used in Multiple Relaxation, we recover from Eq. 1 the classical result

$$\langle \delta v^2 \rangle = kT \langle v \rangle \beta, \tag{6}$$

where $\langle v \rangle$ is the (mean) volume of the system.

When the relaxing systems are not all identical, the definition, Eq. 5, is generalized as follows

$$S = \sum_r \langle \delta v_r^2 \rangle, \tag{7}$$

where $\langle \delta v_r^2 \rangle$ is the volume fluctuation of the relaxing system r, and the summation extends to all independent relaxing systems of a molecule (or of an assembly). However, in the next section on multiple relaxation all systems are considered identical, i.e., Eq. 5 is assumed valid, except in the derivation of Eq. 28.

MULTIPLE RELAXATION

We follow the treatment described previously (8) for the case when the states differ by their energy values only. The number of states is assumed to be finite; the (Markovian) relaxing system to be considered is defined by the row-vector $[\mathbf{v}]$ of the volumes of its states and by the time-independent transition-rate matrix $\mathbf{Q}$ at zero perturbation

$$\mathbf{Q} = \lim_{\theta \to 0} \frac{\mathbf{P}(\theta) - \mathbf{I}}{\theta}, \tag{8}$$

where the elements $P_{lm}(\theta)$ of $\mathbf{P}(\theta)$ are the conditional probabilities that the system at time t in state I is in state m at the later time $t + \theta$.

The row-vector $[\mathbf{p}_0^0]$ of occupancies of the states for zero perturbation (lower index zero) and for stationary conditions (upper index zero) obeys the stationarity condition

$$[\mathbf{p}_0^0]\,\mathbf{Q} = 0. \tag{9}$$

The volume of the system is conveniently normalized to zero (as was done in our previous paper [reference 8] for the energy) by subtracting the mean volume, here denoted as V. Thus

$$[\mathbf{v}]\,\{\mathbf{p}_0^0\} = 0, \tag{10}$$

where $\{\mathbf{p}_0^0\}$ is the transposed (column-vector) of $[\mathbf{p}_0^0]$.

The normal relaxation modes are introduced by a real orthogonal transformation ($\mathbf{G}^{-1} = \tilde{\mathbf{G}}$), where the tilde denotes transposition

$$\mathbf{G}^{-1}\mathbf{B}^{1/2}\mathbf{Q}\mathbf{B}^{-1/2}\mathbf{G} = \mathbf{\Lambda}, \tag{11}$$

in which $\mathbf{B}$ is the diagonal matrix of the occupancies at rest (i.e., its nonzero elements are those of $[\mathbf{p}_0^0]$), and the elements of the diagonal matrix $\mathbf{\Lambda}$ are: $\lambda_j = -\tau_j^{-1}$, where τ_j are the relaxation times.

The transformed state variables y_j are introduced as elements of

$$[\mathbf{y}] = [\mathbf{v}]\,\mathbf{B}^{1/2}\mathbf{G}. \tag{12}$$

The volume fluctuation of the system is

$$\langle \delta v^2 \rangle = [\mathbf{v}]\,\mathbf{B}\,\{\mathbf{v}\} \tag{13}$$

or, in normal coordinates

$$\langle \delta v^2 \rangle = [\mathbf{y}]\,\{\mathbf{y}\} = \sum_j y_j^2, \tag{14}$$

where the summation extends over the normal modes of relaxation.

If the pressure is increased by the constant amount δP_c (the index c stands for constant), the occupancies vary by

$$[\delta \mathbf{p}_c^0] = -(kT)^{-1}\,[\mathbf{v}]\,\mathbf{B}\,\delta P_c \tag{15}$$

and the corresponding volume change of the system is

$$\delta v_c = [\delta \mathbf{p}_c^0]\,\{\mathbf{v}\} = -(kT)^{-1}\,[\mathbf{v}]\,\mathbf{B}\,\{\mathbf{v}\}\,\delta P_c = -(kT)^{-1}\,[\mathbf{y}]\,\{\mathbf{y}\}\,\delta P_c. \tag{16}$$

The quantity

$$\beta_j = (kTV)^{-1} y_j^2 \tag{17}$$

may be called the normal mode compressibility, since it represents the normal mode contribution to the system compressibility

$$\beta = \sum_j \beta_j. \tag{18}$$

Using Eq. 14, Eq. 6 with V in place of $\langle v \rangle$ is recovered for $\langle \delta v^2 \rangle$.

If, on the other hand, the applied pressure δP_s is sinusoidal (the index s stands for sinusoidal), the increment of the row-vector of occupancies is

$$[\delta \mathbf{p}_s] = (kT)^{-1}\,[\mathbf{v}]\,\mathbf{B}\,\mathbf{Q}\,(i\omega\mathbf{I} - \mathbf{Q})^{-1}\,\delta P_s, \tag{19}$$

where $\mathbf{I}$ is the unit matrix and $i = \sqrt{-1}$. Inversion of the matrix $i\omega\mathbf{I} - \mathbf{Q}$ is legitimate for $\omega > 0$ (8). The compressi-

bility of the system under sinusoidal pressure is

$$\beta_s = -(kTV)^{-1}\,[\mathbf{v}]\,\mathbf{B}\,\mathbf{Q}\,(i\omega\mathbf{I} - \mathbf{Q})^{-1}\,\{\mathbf{v}\}. \tag{20}$$

Taking the imaginary part of the solution's compressibility, $\beta_{s,sol} = c_\sigma V\,\beta_s$, the following result for the solute's contribution $\Delta\alpha$ to the ultrasonic absorption is obtained

$$\frac{\Delta\alpha}{\omega^2} = \frac{\rho\, c\, c_\sigma}{2kT}\sum_j \frac{y_j^2\,\tau_j}{1+\omega^2\tau_j^2}. \tag{21}$$

We may define the normal mode contribution to the sum of fluctuations

$$S_j = \mu y_j^2 \tag{22}$$

so that (see Eqs. 5 and 14)

$$S = \sum_j S_j. \tag{23}$$

Each term in Eq. 21 is proportional to S_j, and using Eq. 3 we have

$$\frac{\Delta\alpha}{\omega^2} = \frac{\rho\, c\, c_M}{2kT}\sum_j \frac{S_j\tau_j}{1+\omega^2\tau_j^2}. \tag{24}$$

Also, taking the real part of $\beta_{s,sol}$ gives the increase Δc in sound velocity over its zero-frequency value

$$\Delta c = \frac{\rho\, c^3\, c_M}{2kT}\sum_j \frac{S_j\omega^2\tau_j^2}{1+\omega^2\tau_j^2}. \tag{25}$$

In agreement with the result for the two-state model, each normal mode contribution to the sum of fluctuations is directly obtained from either one of the experimentally determined ultrasonic spectra, Eq. 24 or Eq. 25, provided deconvolution of these spectra in single relaxations can be achieved.

The sum of fluctuations can also be obtained, in principle, when the molecules (or assemblies) contain relaxing systems of different kinds, identified by the index i. We then write

$$\langle \delta v_i^2 \rangle = \sum_j y_{ij}^2 \tag{26}$$

and have

$$S = \sum_{i,j} \mu_i y_{ij}^2 = \sum_j \mu_i \langle \delta v_i^2 \rangle, \tag{27}$$

where μ_i is the number of relaxing systems i per molecule (or per assembly). Note that the set of relaxation times τ_j may depend on the relaxing system i considered.

The resulting equations will, however, be exploitable in practice only if either all systems i exhibit single relaxation, or all have the same relaxation times. In the latter case, we have, in place of Eq. 21

$$\frac{\Delta\alpha}{\omega^2} = \frac{\rho c}{2kT}\sum_{i,j} \frac{c_i y_{ij}^2\tau_j}{1+\omega^2\tau_j^2}, \tag{28}$$

where

$$c_i = \mu_i c_M, \tag{29}$$

with c_M again representing the number of molecules (or of assemblies) per unit volume.

Furthermore, when all systems have the same set of relaxation times the transformed state variable y_{ij} depends on i only through the index i itself, and the summation over i in Eq. 27 can be performed first

$$S = \sum_j \sum_i \mu_i y_{ij}^2 = \sum_j S_j'. \tag{30}$$

Summing then over i in Eq. 28 and using Eqs. 29 and 30, again produces Eq. 24, with S_j' in place of S_j.

THE LINEAR ISING-CHAIN

The starting point of kinetic theories of the helix-coil conformational change of peptide-chains is provided by Zimm and Bragg's (10) statistical treatment of the cooperative transition of those chains. In Schwarz's theory (11), the $4N$-5 kinetic equations are reduced to only four for an infinitely long chain of N residues. Of the corresponding four relaxation processes, only the slowest one, whose relaxation time at mid-transition is

$$\tau = 4(\sigma k_D)^{-1} \tag{31}$$

exhibits high cooperativity and need to be considered here. In Eq. 31, k_D is the rate constant associated with the growth of a disordered (coiled) sequence of residues, and σ is Zimm and Bragg's nucleation parameter. The reciprocal σ^{-1} of the nucleation parameter is a measure of the cooperativity of the chain. Thus, for a very long chain, the uninterrupted helical sequences are composed on the average of $\sigma^{-1/2}$ residues (cooperativity length). Taking into account (*a*) the volume change Δv_1 associated with the coil-to-helix transition of one residue, (*b*) the volume change Δv_2 associated with the loss of one disordered sequence per molecule, and neglecting the $(\Delta v_2)^2$ term, the excess ultrasonic absorption is

$$\frac{\Delta\alpha}{\omega^2} = \frac{\rho\, c\, c_R}{2kT}\frac{\partial}{\partial \ln s}\left[\theta_{AV}(\Delta v_1)^2 - 2N^{-1}\nu_{AV}\Delta v_1\cdot\Delta v_2\right] \cdot \frac{\tau}{1+\omega^2\tau^2}. \tag{32}$$

The two terms in Eq. 32, the first of which was derived by Schwarz (11), the second by the present author (12), stem from fluctuations of helicity and from fluctuations of the number of uninterrupted helix and coiled sequences per molecule, respectively; s is the relative statistical weight of a residue in the ordered (helix) conformation; ν_{AV}, the average of the number ν of ordered sequences per molecules; θ_{AV}, the average helix fraction (i.e., the helicity); and c_R, the number of residues per unit volume.

Let us show that these two contributions to the ultrasonic absorption can be cast in one single term that involves the overall volume fluctuation, whereupon Eq. 32 reduces to the form of Eq. 4. The volume of one (very long) chain, indeed, is

$$v = \text{constant} + n\,\Delta v_1 - \nu\Delta v_2, \tag{33}$$

where n is the number of residues per chain in the ordered conformation. Thus, neglecting the $(\Delta v_2)^2$ term

$$\langle \delta v^2 \rangle = \langle \delta n^2 \rangle (\Delta v_1)^2 - 2 \langle \delta n \cdot \delta \nu \rangle \Delta v_1 \cdot \Delta v_2. \tag{34}$$

The partition function is

$$Z = \text{constant} \cdot \Sigma s^n \sigma^\nu, \tag{35}$$

where the summation extends over all 2^N chain conformations, whence

$$\langle \delta n^2 \rangle = \frac{\partial^2 \ln Z}{\partial (\ln s)^2} \tag{36}$$

$$\langle \delta n \cdot \delta \nu \rangle = \frac{\partial^2 \ln Z}{\partial (\ln s) \cdot \partial (\ln \sigma)}. \tag{37}$$

Since

$$N \theta_{AV} = \frac{\partial \ln Z}{\partial \ln s} \tag{38}$$

$$\nu_{AV} = \frac{\partial \ln Z}{\partial \ln \sigma} \tag{39}$$

it follows (still neglecting the $[\Delta v_2]^2$ term) that

$$\langle \delta v^2 \rangle = (\partial / \partial \ln s)\,[N \theta_{AV} (\Delta v_1)^2 - 2\nu_{AV} \Delta v_1 \cdot \Delta v_2]. \tag{40}$$

Note that $\langle \delta v^2 \rangle$, as defined in Eq. 34, is the volume fluctuation of the whole chain and represents here the sum of fluctuations (i.e., $S = \langle \delta v^2 \rangle$). On the other hand, the number of molecules per unit volume is

$$c_M = N^{-1} c_R. \tag{41}$$

Inserting Eqs. 40 and 41 into Eq. 32 shows that the latter equation is identical to Eq. 4.

This example illustrates the general form taken by the equations when the dissipative properties are expressed in terms of the sum of fluctuations S. Eqs. 2 and 4 both are derived from the two-state model. Although Eq. 2 does not apply to the Ising-chain, for which the equilibrium constant K is not defined, Eq. 4 is valid for the slowest (and most cooperative) kinetic process exhibited by this chain.

ENHANCED CONFORMATIONAL FLUCTUATIONS

In studies of biomolecules in different states of aggregation the concentration c_M of the monomer was kept constant (1–6). The results of the preceding sections then show (see Eqs. 4, 24, and 25) that the increase of the relaxation amplitude that was observed on assembly expresses the increase of the sum of fluctuations S. In accordance with Eq. 5, this increase may, in turn, in limiting cases, be due to an increase on assembly of either the number μ of relaxing units, or of $\langle \delta v^2 \rangle$ for each relaxing unit. The latter process we call enhanced fluctuations in assembly. Separation of these two contributions to S may not be easy for a single relaxation process or for several independent single relaxation processes. The following examples, however, suggest that for multiple relaxation, under specified conditions, it will be possible to identify a process of enhanced fluctuations.

We consider the three-state model used earlier by us in connection with "slow temperature jump" measurements of Pohl (13) on pancreatic proteins. Consideration of a conformational state intermediate between the native and denaturated ones, according to Scheme I

$$\text{I} \rightleftharpoons \text{II} \rightleftharpoons \text{III}$$

was shown (14, 15) to provide a possible explanation for the apparently anomalous temperature dependence of the refolding rate constant reported by Pohl (who had assumed all-or-none conformational kinetics.[1] Of importance here are intermediate equations that we did not publish at the time (Eqs. 44 and 45 below).

For the rates of transitions between conformational states, and the corresponding transition-rate matrix $\mathbf{Q}$, we write explicitly

$$\mathbf{Q} = \begin{vmatrix} -q & q & 0 \\ qK & -(qK + q') & q' \\ 0 & q'K' & -q'K' \end{vmatrix} \tag{42}$$

with

$$K = \frac{p_I}{p_{II}};\ K' = \frac{p_{II}}{p_{III}}, \tag{43}$$

where the occupancies at rest are now written p_I, p_{II}, and p_{III}. The elements of $\mathbf{Q}$ follow from Eq. 9. Expressions of the two relaxation times $\tau_j = -\lambda_j^{-1}$ $(j = 1, 2)$ are derived along familiar lines.

The relaxation amplitudes, on the other hand, are

$$y_j = p_I^{1/2} \lambda_j g_j (qK)^{-1} \cdot [v_{II} + v_{III} (K')^{-1} (q'K' - q)(\lambda_j + q'K')^{-1}], \tag{44}$$

where y_j are the two nonzero elements of $[\mathbf{y}]$; v_{II} and v_{III} are the volumes of states II and III when v is normalized to zero (see Multiple Relaxation), and

$$g_j^2 = q\lambda_j^{-1} (\lambda_j - \lambda_k)^{-1} [\lambda_k + q(1 + k)]. \tag{45}$$

Here $k = 2$ when $j = 1$, and $k = 1$ when $j = 2$.

[1] Pohl later also made use of a three-state model to interpret his data (16).

Eqs. 44 and 45 are exact. We now consider the case when $K \gg 1$ (i.e., the reverse transition rate $P_{II,I} = qK$ is much higher than $P_{I,II} = q$), and K' is of the order of unity, as illustrated by the potential depicted in Fig. 1, where the wells correspond to states I to III.

Model *a*

Let all transition rates be fixed, except q, which is being increased by the factor $\gamma > 1$, keeping $K \gg 1$, as in the process shown by the arrow in Fig. 1. The quantities qK, $q'K' - q, \lambda_j, \lambda_j - \lambda_k, \lambda_j + q'K', \lambda_j + q(1 + K), p_I, v_{II}$, and v_{III}, then, all either have fixed values or are to the first approximation independent of q. Thus, from Eq. 45 the g_j^2 values and therefore the y_j^2 values are proportional to q.

When q is now increased by the factor γ, each normal mode contribution y_j^2 to the volume fluctuation $\langle \delta v^2 \rangle$ (Eq. 14), or each separate S_j (Eq. 22) as well as the sum of fluctuations S (Eq. 23), are multiplied by γ, i.e., the fluctuations are enhanced. On the other hand, the relaxation times $\tau_j = -\lambda_j^{-1}$, where the values of λ_j are the roots of the quadratic equation

$$\lambda^2 + [q(1 + K) + q'(1 + K')]\lambda + qq'[1 + K'(1 + K)] = 0 \quad (46)$$

are unchanged. According to Eqs. 24 and 25 the observables $\Delta\alpha/\omega^2$ and Δc are then multiplied by γ over the entire range of values of the frequency.

Model *b*

In the preceding example, fluctuations associated with two normal modes of relaxation were uniformly enhanced when state I was destabilized (see Fig. 1). Of course, only the relative stability of the states is of relevance. A more general model is shown in Figs. 2 *a* and 2 *b*. In Fig. 2 *a* state I is destabilized as before (i.e., the transition probability $P_{I,II} = q$ is multiplied by $\gamma > 1$), but states II and III are further stabilized by decreasing the transition probabilities $P_{II,I}$, $P_{II,III}$, and $P_{III,II}$, all of which we multiply by $\delta < 1$. The equilibrium constant K' is thus kept unchanged while K is multiplied by $\gamma^{-1}\delta$.

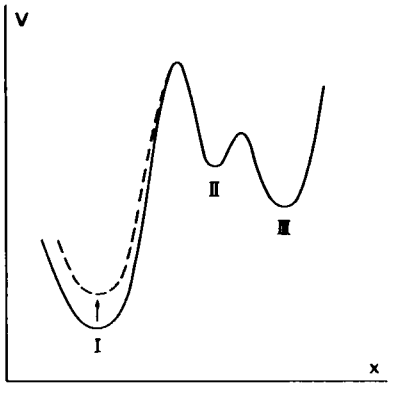

FIGURE 1 Potential function for model *a* considered in the text; I, II, and III are conformational states each defined by the corresponding value of x. State I is destabilized in the process represented by the arrow.

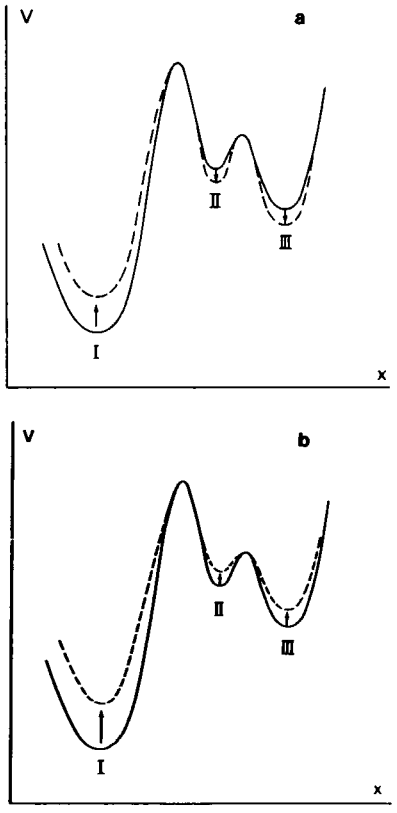

FIGURE 2 (*a*) Potential function for model *b*. While state I is destabilized in the process shown by the arrows, the two others are stabilized ($\delta < 1$). (*b*) Potential function for the alternative model *b*. The conditions are the same as in *a*, but with $\delta > 1$ instead of $\delta < 1$. The process shown by the arrows both lowers the potential barriers and destabilizes state I with respect to states II and III.

The difference in stability of the conformational states is clearly being reduced in both the processes shown by the arrows in Figs. 1 and 2 *a*. The reasoning used for model *a* (for which $\delta = 1$) shows, still assuming $K \gg 1$ and keeping $K \gg 1$, that for the process of Fig. 2 *a* each normal mode contribution y_j^2 to the volume fluctuation $\langle \delta v^2 \rangle$, or each separate S_j, is enhanced by the factor $\gamma^{\delta^{-1}} > 1$, as is readily verified using Eqs. 44–46. In the present case, however, the two relaxation times are multiplied by δ^{-1}, as follows from Eq. 46, or by noting that the times are proportional to $P_{II,I}^{-1}$. Uniform enhancement of $\Delta\alpha/\omega^2$ and Δc again occurs in semilogarithmic plots against ω, after a shift equal to $\log_{10}\delta$ has been effected in the frequency scale. The enhancement factors of $\Delta\alpha/\omega^2$ and Δc are equal to $\gamma\delta^{-2}$ and $\gamma\delta^{-1}$, respectively (see Eqs. 24 and 25). The preceding results are valid for both $\delta < 1$ and $\delta > 1$, and enhanced fluctuations still occur for $\delta > 1$, provided $\gamma\delta^{-1} > 1$. However, the relaxation times are lengthened for $\delta < 1$ and shortened for $\delta > 1$. A process for which $\delta > 1$ is represented by the arrows in Fig. 2 *b*; this process both lowers the potential barriers and decreases the stability of state I with respect to states II and III.

It will be shown in a forthcoming publication (Cerf, R., and Y. Dormoy, manuscript submitted for publication) that the increase of ultrasonic absorption observed (4, 5) in the disk → helix transition of tobacco mosaic virus (TMV) protein behaves exactly as has been described here for enhanced conformational fluctuations. Both models described in the present section were applied to the ultrasonic absorption data for the TMV-protein aggregates. This resulted in one single reduced ultrasonic spectrum for all aggregates. The enhancement factor of fluctuations in the disk → helix transition was found to be model independent and was close to four. This, then, raises the question of whether a quaternary structure that exhibits significant conformational fluctuations has to be present for the binding of the nucleic acid.

CONCLUSION

Application of the equations derived here is not restricted to biomolecular assemblies. Ultrasonic absorption and velocity measurements in a $K_2H\ PO_4(3H_2O)$ solution of α-chymotrypsin have revealed a relaxation at physiological pH in addition to the known relaxations due to proton-transfer reactions at acidic and basic pH. The ultrasonic absorption of the enzyme was four to five times larger than for the zymogen and about two times larger than for α-chymotrypsin treated with the inhibitor diisopropyl fluorophosphate. A publication with D. Rogez will present the data and an interpretation thereof based on the preceding theoretical results.

APPENDIX

Fluctuation-Dissipation Theorem

The derivation of Eq. 21 from Eq. 20 merely requires multiplication by the complex conjugate to $(i\omega\mathbf{I} - \mathbf{Q})^{-1}$. The relation of Eq. 21 to the fluctuation-dissipation theorem becomes apparent on noting that

$$[\mathbf{v}]\,\mathbf{B}\,\mathbf{Q}\,(i\omega\mathbf{I} - \mathbf{Q})^{-1}\,\{\mathbf{v}\} = \int_0^\infty [\mathbf{v}]\,\mathbf{B}\,\mathbf{Q}\exp(\mathbf{Q}\theta)\,\{\mathbf{v}\}\exp(-i\omega\theta)\,d\theta \quad \text{(A1)}$$

and that the matrix $\mathbf{P}(\theta)$ of Eq. 8 is (8)

$$\mathbf{P}(\theta) = \exp(\mathbf{Q}\theta). \quad \text{(A2)}$$

Furthermore, $[\mathbf{v}]\,\mathbf{B}\,\mathbf{P}(\theta)\,\{\mathbf{v}\}$ is the correlation function

$$\Psi(\theta) = \langle v(0)\,v(\theta)\rangle, \quad \text{(A3)}$$

where $v(t)$ is the volume of the state occupied at time t. Therefore

$$[\mathbf{v}]\,\mathbf{B}\,\mathbf{Q}\,(i\omega\mathbf{I} - \mathbf{Q})^{-1}\,\{\mathbf{v}\} = \int_0^\infty [d\Psi(\theta)/d\theta]\exp(-i\omega\theta)\,d\theta. \quad \text{(A4)}$$

Integration by parts and substitution of the imaginary part of $[\mathbf{v}]\,\mathbf{B}\,\mathbf{Q}\,(i\omega\mathbf{I} - \mathbf{Q})^{-1}\,\{\mathbf{v}\}$ into Eq. 20 then gives $\Delta\alpha$ in the form of a Kubo relation.

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