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Energy coupling between DNA binding and subunit association is responsible for the specificity of DNA–Arc interaction

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Abstract

The effects of several DNA molecules on the free energy of subunit association of Arc repressor were measured. The association studies under equilibrium conditions were performed by the dissociating perturbation of hydrostatic pressure. The magnitude of stabilization of the subunit interaction was determined by the specificity of the protein–DNA interaction. Operator DNA stabilized the free energy of association by about 2.2 kcal/mol of monomeric unit, whereas poly(dG–dC) stabilized the subunit interaction by only 0.26 kcal. Measurements of the stabilizing free energy at different DNA concentrations revealed a stoichiometry of two dimers per 21 bp for the operator DNA sequence and for the nonspecific DNA poly(dA–dT). However, the maximum stabilization was much larger for operator sequence (Δp = 1,750 bar) as compared for poly(dA–dT) (Δp = 750 bar). The importance of the free-energy linkage for the recognition process was corroborated by its absence in a mutant Arc protein (PL8) that binds to operator and nonspecific DNA sequences with equal, low affinity. We conclude that the coupling accounts for the high specificity of the Arc–operator DNA interaction. We hypothesize a mutual coupling between the protein subunits and the two DNA strands, in which the much higher persistency of the associated form when Arc is bound to operator would stabilize the interactions between the two DNA strands.

Keywords: DNA-binding protein; DNA recognition; free-energy linkage; mutual coupling; pressure dissociation

DNA-binding proteins are responsible for many cellular functions, including transcription, replication, and restriction. The DNA–protein recognition process is the primary event in the cellular regulation of transcription at the level of initiation. The mechanisms by which regulatory proteins recognize specific DNA sequences are not fully understood. Because of the very large potential for nonspecific binding, a large difference in affinity between binding of the protein to the cognate and all other DNA parts is required (Pabo & Sauer, 1984; Schleif, 1988; Brennan & Matthews, 1989; von Hippel & Berg, 1989). Repressors are generally dimeric or tetrameric proteins, and their oligomeric structure is believed to be important for their function. However, no quantitative appraisal of the coupling between DNA binding and protein–protein interactions has yet been performed.

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Arc repressor is a small, dimeric DNA-binding protein (M_r = 13,000) that represses transcription from the P_{ant} promoter of *Salmonella* bacteriophage P22 (Susskind, 1980; Sauer et al., 1983; Vershon et al., 1985). It belongs to a family of proteins that have an antiparallel β-sheet as the interfacial DNA-binding motif (Knight et al., 1989; Breg et al., 1990; Phillips, 1991). A tertiary structure model for Arc has been proposed (Breg et al., 1990) based upon homology between Arc and the *Escherichia coli* Met repressor and on two-dimensional NMR data (Breg et al., 1989; Zagorski et al., 1989). The proposed structure consists of an intertwined dimer, in which residues 8–14 of different monomers form an antiparallel β-sheet (see kinemages). It has been shown that the 21-bp operator can accommodate two dimers (Brown et al., 1990). Arc repressor does not tetramerize in solution even at high concentration, and the dimer reversibly dissociates into subunits (Silva et al., 1992). The dissociation can be obtained either by dilution at constant pressure or by an increase in pressure at a fixed protein concentration (Silva et al.,

1992). Dissociated Arc is compact with its nonpolar core partially exposed and has the conformational properties of a molten globule.

The main goal of this study is to evaluate the linkage between the specificity of operator DNA recognition and the monomer-monomer stability to pressure in Arc repressor under equilibrium conditions. Subunit dissociation studies are performed in the presence of different DNA sequences. Hydrostatic pressure (Heremans, 1982; Weber & Drickamer, 1983; Weber, 1987; Silva & Weber, 1993) was employed as the dissociating perturbation in order to permit equilibrium studies at concentrations of protein and DNA at which association is virtually complete at atmospheric pressure. The use of pressure permitted a unique characterization of the large coupling between intersubunit affinity and DNA binding as well as the stoichiometry in Arc-operator interaction.

Results

Each Arc repressor monomer contains a single tryptophan that is located at position 14 in the interface between subunits (Breg et al., 1990; Silva et al., 1992). The low polarity of the environment of Trp 14 when Arc repressor is in the dimeric state results in a blue-shifted fluorescence emission. In this condition, the wavelength of maximum emission is 328 nm, and the average energy of the emission (center of spectral mass) is 29,525 cm^{-1}. When the protein is dissociated into monomers, Trp 14 becomes exposed to the solvent and the center of spectral mass decreases to 28,053 cm^{-1} (Fig. 1A). The correlation between the emission at each pressure and the degree of dissociation has been confirmed by measurements of the hydrodynamic size by fluorescence polarization (Silva et al., 1992). We evaluated the effects of different DNA sequences on the dissociation of Arc repressor into monomers by measuring the changes in the average energy of emission of Trp 14. Sufficiently high concentrations of DNA and of the Arc protein were utilized to guarantee complete binding of protein to DNA at atmospheric pressure. We confirmed that Arc protein was bound to DNA at atmospheric pressure by measuring the increase in polarization on binding DNA (not shown).

Arc repressor binds with very high affinity to a 21-base operator sequence (Vershon et al., 1986). This sequence greatly stabilized the Arc subunit interaction (Fig. 1B), practically preventing its dissociation. Figure 2 shows the dissociation curves in the absence and in the presence of different DNAs. In the absence of DNA, 90% dissociation was achieved at 1,600 bar, whereas at this same pressure no significant dissociation occurred in the presence of operator (Fig. 2).

Nonspecific DNAs were much less effective than the operator in stabilizing the Arc subunit interaction (Fig. 2; Table 1). Vershon et al. (1986) showed that Arc repressor could bind to the plasmid pBR322 with an affinity 100-fold lower than its affinity for operator DNA. We found that the plasmid pBR322 shifted the $p_{1/2}$ by 530 bar, which corresponds to a stabilization of −0.63 kcal/mol for the subunit interaction (Fig. 2; Table 1). The synthetic DNA poly(dG-dC) stabilized the Arc subunit interaction

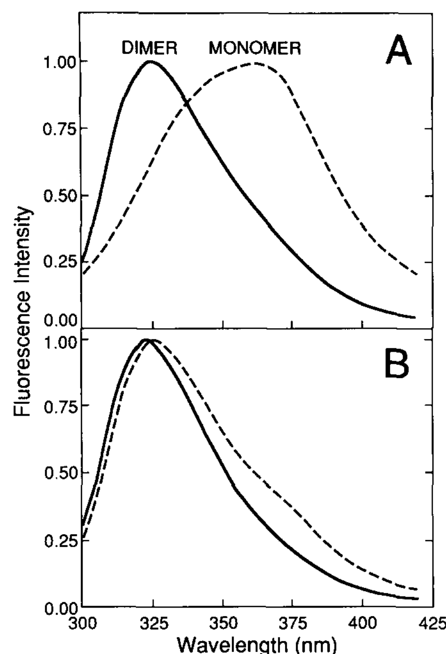

Fig. 1. Effect of operator DNA on the pressure-induced changes in fluorescence emission spectra of Arc repressor. The emission spectrum of the single tryptophan of Arc protein shifts to longer wavelengths when the dimer dissociates. **A:** Fluorescence emission spectra (λ_{exc} = 280 nm) of 1 μM Arc repressor at atmospheric pressure (1 bar) (——) and at 2,400 bar (----) in the absence of DNA. **B:** Fluorescence emission spectra of Arc repressor in the presence of the operator DNA sequence:

ATGATAGAAGCACTCTACTAT
TACTATCTTCGTGAGATGATA

at atmospheric pressure (——) and at 2,400 bar (----).

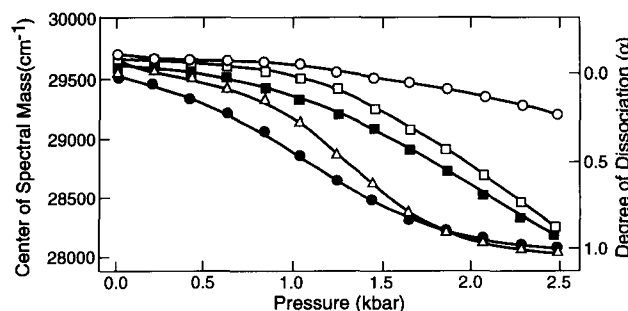

Fig. 2. Effects of different DNAs on the dissociation of Arc repressor. The pressure-induced dissociation of 1 μM Arc repressor was measured in the absence of DNA (●) or in the presence of 13.8 μg/mL operator DNA (○), poly(dG-dC) (△), poly(dA-dT) (□), or plasmid pBR322 (■). The value of center of mass for each pressure is the average of five measurements, and the standard deviation was between 5 and 15 cm^{-1}.

Table 1. *Effects of operator and nonspecific DNAs on the dissociation of Arc repressor and PL8 mutant protein*[a]

DNA	Protein	$\Delta p_{1/2}$ (kbar)	δG_s (kcal/mol)
Operator DNA	Arc	1.75 ± 0.1	−2.2
	PL8	0	0
Poly(dA-dT)	Arc	0.75 ± 0.05	−0.88
Poly(dG-dC)	Arc	0.23 ± 0.05	−0.26
	PL8	0	0
Poly(dG-m^{5}dC)	Arc	0.01	−0.01
Plasmid pBR322	Arc	0.53 ± 0.05	−0.63
	PL8	0	0

[a] $\Delta p_{1/2}$ is the difference between midpoint of the pressure-dissociation curves of the DNA-liganded and unliganded proteins and δG_s is the stabilizing free energy of association per monomer, calculated from Equation 9. The association volume change determined from the pressure dissociation data (Silva et al., 1992) is 50 mL/mol. The Arc protein concentration was 1 μM and the DNA concentration was 13.8 μg/mL.

even less, whereas poly(dA-dT) promoted a higher stabilization (Fig. 2; Table 1).

The magnitude of stabilization of the subunit interaction can be determined from the expression:

$$\delta G_s = \Delta\Delta G/n = \Delta G_D/n_D - \Delta G_0/n_0, \quad (1)$$

where δG_s is the stabilizing free energy per subunit, ΔG_D is the free energy of Arc subunit association in the presence of DNA, and ΔG_0 is its free energy without DNA. Both ΔG_D and ΔG_0 are divided by the respective number of subunits, n_D and n_0. In the absence of DNA, Arc is a dimer, but it forms a tetramer when bound to operator (Brown et al., 1990). In a pressure-dissociation experiment, δG_s can be calculated from

$$\delta G_s = -[(p_{1/2})_D \cdot \Delta V_D/n_D - (p_{1/2})_0 \cdot \Delta V_0/n_0], \quad (2)$$

where $(p_{1/2})_D$ and $(p_{1/2})_0$ are, respectively, the midpoints of pressure dissociation of the DNA-liganded and unliganded proteins, and $\Delta V_D/n_D$ and $\Delta V_0/n_0$ are the volume changes of association per subunit for DNA-liganded and unliganded Arc, respectively. ΔV was obtained from the slope of the plot of $\ln[\alpha_p^2/(1-\alpha_p)]$ vs. pressure for the unliganded protein (Silva et al., 1992) and from $\ln[\alpha_p^4/(1-\alpha_p)]$ vs. pressure for DNA-liganded protein. The fourth power is justified for the DNA-liganded protein in view of the literature evidence that two dimers bind to the 21-base operator (Brown et al., 1990) and by further stoichiometry data described below (Fig. 3). The values of $\Delta V_D/n_D$ and $\Delta V_0/n_0$ were very similar for different DNAs (between 45 and 50 mL/mol). Therefore Equation 2 can be simplified to the form:

$$\delta G_s = -[(p_{1/2})_D - (p_{1/2})_0] \cdot \Delta V = -\Delta p_{1/2} \cdot \Delta V, \quad (3)$$

where $\Delta V = \Delta V_D/n_D = \Delta V_0/n_0$.

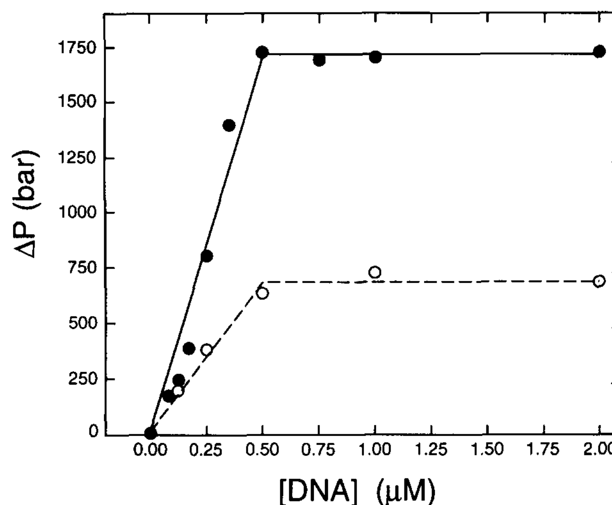

Fig. 3. Stoichiometry of the DNA-conferred subunit stabilization Plot of the changes in mid-pressure dissociation as a function of DNA concentration. Each point corresponds to a dissociation curve in the presence of operator (●) or poly(dA-dT) (○). For poly(dA-dT), the molar concentration was expressed in terms of 21 bp. The $p_{1/2}$ was determined and subtracted from the data in the absence of DNA. The concentration of Arc dimer was kept constant at 1.0 μM. Other conditions were as for Figure 1.

Operator DNA shifts the $p_{1/2}$ by more than 1,750 bar (Fig. 2; Table 1), which according to Equation 3 corresponds to an increase of 2.2 kcal/mol in the free energy of association per subunit. If the operator accommodates two dimers of Arc repressor (Brown et al., 1990), the total free energy of stabilization is 8.8 kcal/mol.

The values of free energy of stabilization (δG_s) derived from $\Delta p_{1/2}$ for the nonspecific DNAs are much smaller than that obtained for operator DNA (Table 1). Among the nonspecific DNAs, poly(dA-dT) promoted the largest stabilization (−0.88 kcal/mol) (Table 1). This result is consistent with the predominance of the bases A and T in the 21-base operator sequence (Vershon et al., 1989). Poly(dG-m^{5}dC) did not produce stabilization of subunit interaction in Arc (Table 1).

The effect of DNA binding on dissociation of a mutant Arc repressor was also investigated. The single amino acid replacement Pro 8 → Leu promotes a loss of DNA specificity (Vershon et al., 1986), such that PL8 binds both operator and nonspecific sequences with low affinity. In the absence of DNA, PL8 is also a dimer. The binding of either the operator sequence, the synthetic DNAs, or plasmid pBR322 did not affect the pressure dissociation of PL8 dimer (Table 1), indicating the absence of free-energy coupling.

The change in $p_{1/2}$ by increasing the concentration of DNA was measured for operator and poly(dA-dT) (Fig. 3). The concentration of Arc was kept fixed at 1 μM, and the DNA concentration was varied in the range 0–2 μM. In the case of the operator, the stabilization increases linearly and levels off at 0.5 μM DNA with a max-

imum Δp of 1,750 bar. These data clearly show that the stoichiometry of the binding is two dimers per DNA, confirming previous evidence obtained by gel retardation assays (Brown et al., 1990). The data for poly(dA–dT) show that two dimers of Arc repressor also bind to each set of 21 bp of AT. However the total amount of stabilization was much smaller ($\Delta p(\max) = 750$ bar).

Discussion

Our results demonstrate that the magnitude of stabilization of the subunit interaction is determined by the sequence of the DNA bound by the repressor. The more specific the DNA–Arc interaction, the greater the stabilizing energy (Table 1). The stabilization results from the linkage between the free energies of subunit association and DNA binding. The greatest free-energy coupling occurs for operator DNA. The coupling between subunit association and ligand binding is a feature of many oligomeric systems (Weber, 1975, 1984, 1992).

Figure 4 shows a species chemical potential diagram for the coupled reactions of Arc subunit association and binding to DNA. A DNA with the potential binding of two Arc dimers (D_4) was considered, given that for both operator and poly(dA–dT) a stoichiometry of two dimers per 21 bp DNA was obtained (Fig. 3). The species that are unlikely to be populated were represented by dashed lines. Particularly, the monomer bound to DNA has a chemical potential very close to the free species. It should be pointed

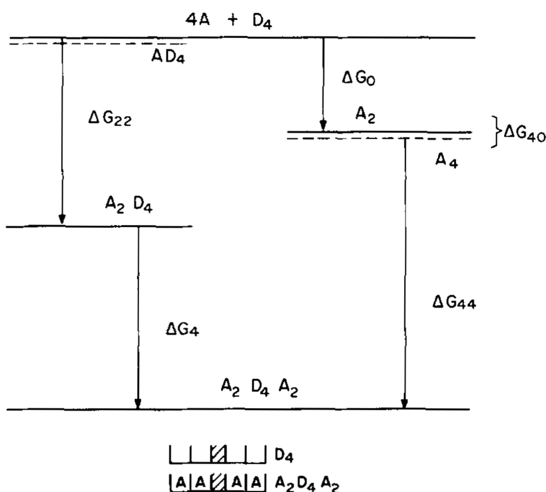

Fig. 4. Free energy levels of the relation between DNA binding and subunit association. A model for the DNA D_4 when it is empty and when it is fully occupied by two dimers is also depicted. A, free Arc monomer; A_2, free dimer; A_4, "free tetramer"; AD_4, monomeric Arc bound to DNA; A_2D_4, one dimer bound to DNA; $A_2D_4A_2$, two dimers bound to DNA; ΔG_{22}, the free energy change for dimerization in the presence of DNA; ΔG_4, free energy for formation of a second dimer bound to DNA; ΔG_0, free energy of dimer formation in the absence of DNA; ΔG_{40}, free energy of dimer-dimer association in the absence of DNA; ΔG_{44}, free energy stability of the whole complex.

out that we directly measured the fraction of monomers of Arc repressor, which permitted us to promptly relate the effect of different sequences of DNA to the stabilization of A–A interactions.

Utilizing free-energy conservation assumptions, we can deduce from the diagram of Figure 4:

$$\Delta G_{44} = (\Delta G_{22} + \Delta G_4) - (\Delta G_0 + \Delta G_{40}). \tag{4}$$

The free energy of association of dimer to tetramer in the absence of DNA (ΔG_{40}) is much smaller than ΔG_0, since no tetramerization is observed even at mmolar concentrations, and thus it can be disregarded. The sum ($\Delta G_{22} + \Delta G_4$) is the total free energy of subunit association in the presence of DNA (ΔG_D). Therefore, it follows from Equation 4:

$$\Delta G_{44} = (\Delta G_D - \Delta G_0) = -\Delta p_{1/2} \cdot \Delta V, \tag{5}$$

and from Equation 3, we infer that the stabilizing free-energy ($\delta G_s = -\Delta p_{1/2} \cdot \Delta V$) is equal to the free energy of stability (ΔG_{44}) of the protein–DNA complex ($A_2D_4A_2$).

We conclude that the difference in stability of the protein–protein interactions could drive the final stability of the DNA–protein interaction. This free-energy coupling is probably entropic and may reflect the correct positioning of the second subunit that is promoted by binding to the first subunit. Recently, it has been shown that coupling entropy plays an important role in allosteric enzymes (Reinhart et al., 1989). If the specific DNA-binding sites exist in tandem arrays, oligomerization with order higher than four may indeed occur. This seems to be the case for Met repressor of *E. coli* (Phillips et al., 1989; Rafferty et al., 1989), a protein with structural homology to Arc repressor (Breg et al., 1990).

Half-maximal binding of Arc to operator DNA occurs at a protein concentration of 10^{-10} M (Vershon et al., 1986), a concentration considerably lower than the dimer-monomer dissociation constant (10^{-8} M) (Bowie & Sauer, 1989; Silva et al., 1992). Thus, most of the stabilization conferred by operator DNA should be coupled to the strengthening of the interaction between subunits in the dimer (A–A). Mutational studies indicate that the amino acid residues in the intersubunit β-sheet (A–A interface) are important for the specificity of DNA binding (Knight & Sauer, 1992). A component of the stabilization could also be related to the interaction between dimers (A_2–A_2) in the complex $A_2D_4A_2$. This latter interaction would be similar to that found between lysozyme molecules on the surface of thyroglobulin (Rawitch & Weber, 1972).

The equilibrium constant for the breakage of two subunit contacts is determined by the rate constants of dissociation (k_-) and association (k_+). The dissociation rate constant for two subunit contacts can be expressed as:

$$k_- = k_+ \exp(\Delta G/RT). \tag{6}$$

As a general rule, the differences in equilibrium constant in protein interactions arise from modifications of the rate constant of dissociation (Weber, 1975). In the presence of DNA, the rate constant of association is difficult to estimate, but it is unlikely that it changes significantly as a function of the DNA sequence. Therefore, the ratio of dissociation rates as a function of the difference in stability when bound to different DNA sequences is

$$k_{-N}/k_{-S} = \exp[2(\Delta G_N - \Delta G_S)/RT], \quad (7)$$

where k_{-N} and k_{-S} are the subunit dissociation rate constants for Arc bound to a nonspecific and a specific DNA, respectively. ΔG_N and ΔG_S are the free energies of subunit association in the presence of a nonspecific and a specific DNA, respectively, and the difference is multiplied by two, because we are considering the interaction between two monomers. Looking at the data obtained for poly(dG-dC) and operator DNAs in Table 1, $2(\Delta G_N - \Delta G_S)$ is equal to 4.0 kcal/mol, and the derived ratio k_{-N}/k_{-S} is 1,000. This means that the half-time of the associated dimer will be at least 1,000 longer when it is complexed to the operator sequence in comparison to nonspecific sequences. Under the nonequilibrium conditions inside the cell, the *persistency* of the subunit interaction when bound to operator must be the determining factor of the recognition and of the resulting repression activity. If the association rate constant (k_+) is assumed to be 10^8 M^{-1} s^{-1}, the half-time of the dimer lengthens from 0.5 to 500 s when the protein switches from the nonspecific to the cognate sequence. A value of 500 s is close to the off rate constant for the dissociation of the Arc–operator complex measured by Brown et al. (1990).

The model of the structure of Arc repressor (Breg et al., 1990) and the structure of other related repressors (Rafferty et al., 1989; Phillips, 1991; Knight & Sauer, 1992) suggest the use of intersubunit β-strands for sequence recognition. The large free-energy linkage between DNA binding and subunit association elicits the attractive possibility of a mutual coupling between the intersubunit contacts and the two strands of DNA. In this way, not only the preferential binding to the operator, but the mechanism of repression can be rationalized. The Arc repressor promotes repression not by preventing the binding of RNA polymerase to the promoter but rather by blocking its isomerization from a closed to open complex (Vershon et al., 1987). This step requires melting the DNA strands. A mutual coupling implies that the increase in interaction between the two subunits of Arc repressor bound to DNA promotes an increase in the interaction between the two DNA strands. Therefore, the mutual coupling could prevent isomerization of bound RNA polymerase from the closed to open complex.

The magnitude of the Arc subunit stabilization promoted by the operator is large enough to account for the much higher affinity of the operator with respect to nonspecific DNA sequences. The presence of free-energy coupling in the wild-type Arc protein and its absence in the mutant PL8 corroborates this hypothesis. Our results further imply that a thermodynamic constraint ($\delta G_s = \Delta G_{(n)} - \Delta G_{(0)}$) may furnish the basis for the DNA recognition process. When a mutation compromises the operator DNA specificity, it affects δG_s, as in the case of the PL8 mutant. Further studies on other DNA-binding proteins will be necessary to determine whether a link between DNA specificity and repressor subunit association occurs as a general rule.

Materials and methods

Proteins and chemicals

An initial sample of Arc repressor and the mutant PL8 were kindly supplied by Professor Robert T. Sauer. Subsequent preparations of the protein were performed in our laboratory. The proteins were expressed in *E. coli* and purified as described previously (Vershon et al., 1985, 1986). Operator DNA was purchased from Genetic Design, Inc. (Houston, Texas). All other reagents were of analytical grade. Distilled water was filtered and deionized through a Millipore water purification system. Experiments were performed at 20 °C in the standard buffer: 0.05 M Tris chloride, 100 mM NaCl, pH 7.5.

Steady-state fluorescence spectra and anisotropy studies

The high-pressure bomb has been described by Paladini and Weber (1981). Fluorescence spectra were recorded on an ISS 200 spectrofluorometer (Champaign, Illinois). Fluorescence spectra at pressure p were quantified by specifying the center of spectral mass $\langle v_P \rangle$ in wave numbers (cm^{-1}).

$$\langle v_P \rangle = \Sigma v_i \cdot F_i / \Sigma F_i, \quad (8)$$

where F_i stands for the fluorescence emitted at wave number v_i, and the summation is carried out over the range of appreciable values of F. The degree of dissociation (α_p) is related to $\langle v_p \rangle$ by the expression:

$$\alpha_p = [1 + Q(\langle v_p \rangle - \langle v_F \rangle)/(\langle v_I \rangle - \langle v_P \rangle)]^{-1}, \quad (9)$$

where Q is the ratio of the quantum yields of dissociated and associated forms, $\langle v_p \rangle$ is the center of spectral mass at pressure p, and $\langle v_F \rangle$ and $\langle v_I \rangle$ are the corresponding quantities for dissociated and associated forms (Silva et al., 1986, 1989).

Fluorescence anisotropy measurements were made in an L-format polarization fluorescence instrument built in our laboratory (ICL 100). The corrections for the scram-

bling of the windows were performed as described by Paladini and Weber (1981).

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