NMR Order Parameters and Free Energy: An Analytical Approach and Its Application to Cooperative Ca^{2+} Binding by Calbindin D_{9k}

Mikael Akke,† Rafael Brüschweiler,‡ and Arthur G. Palmer, III*,§

Department of Physical Chemistry 2
University of Lund, S-221 00 Lund, Sweden
Department of Molecular Biology
The Scripps Research Institute
La Jolla, California 92037
Department of Biochemistry and
Molecular Biophysics, Columbia University
New York, New York 10032

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Magnitudes and time scales of intramolecular conformational fluctuations of several proteins have been characterized by using NMR spectroscopy to measure ^{15}N and ^{13}C nuclear spin relaxation rate constants (for a review, see ref 1). The spin relaxation data usually are interpreted using the "model-free" formalism, which parametrizes intramolecular dynamics in terms of an overall tumbling correlation time τ_c, generalized order parameters S^2, and effective internal correlation times τ_e.[2] As described in this communication, contributions to the Gibbs free energy from fast time scale intramolecular fluctuations ($\tau_e < \tau_c$) can be estimated from experimentally-determined order parameters. As an illustration, contributions to the free energy of cooperative calcium ion binding by calbindin D_{9k} are obtained from generalized order parameters of the backbone ^{15}N nuclei.[3,4] Although the available experimental data sample only a subset of the motional modes of the protein, the results support the hypothesis that changes in the distribution of intramolecular fluctuations can contribute significantly to the free energy of cooperativity.[5]

Dipolar spin relaxation of a protonated ^{13}C or ^{15}N nucleus is mediated by the orientational fluctuations of the internuclear vector between the heteronucleus and the proton. The relationship between atom coordinate fluctuations, which lead to reorientation of the bond vector, and the Helmholtz free energy is expressed by[6]

$$A = -kT \ln Q \tag{1}$$

in which Q is the canonical partition function. Assuming that the orientations of the bond vectors are statistically independent of other degrees of freedom of the molecule,

$$Q = Q_u Q_o = Q_u \prod_{j=1}^{N} q_j \tag{2}$$

in which Q_o is the contribution from the observed orientational fluctuations of the N bond vectors and Q_u is the contribution from unobserved sources. The q_j are partition functions for the individual bond vectors and are given by

$$q_j = \sum_i \exp(-E_j^{(i)}/kT) = \exp(-E_j^0/kT)\sum_i \exp(-\Delta E_j^{(i)}/kT) = \exp(-E_j^0/kT)\tilde{q}_j \tag{3}$$

in which $E_j^{(i)}$ is the energy of the jth bond vector in the ith orientation, $\Delta E_j^{(i)} = E_j^{(i)} - E_j^0$, and E_j^0 is the minimum energy of the potential energy surface for the jth bond vector. The assumption of independence will lead to an overestimation of the partition function Q; however, this treatment is a reasonable first-order approximation for the fast fluctuations governed by random, thermal motions.[7] Neglecting contributions from pressure–volume work, the change in Gibbs free energy between two states, denoted 1 and 2, of the system is $\Delta G = G_2 - G_1 \approx A_2 - A_1$ and is given by

$$\Delta G = -kT \ln(Q_2/Q_1) = \Delta G_u + \Delta G_o = \Delta G_u + \sum_{j=1}^{N} [\Delta E_j^0 - kT \ln(\tilde{q}_{j,2}/\tilde{q}_{j,1})] \tag{4}$$

in which ΔG_o contains experimental contributions from bond vector fluctuations, ΔG_u contains contributions from unobserved sources, and $\Delta E_j^0 = E_{j,2}^0 - E_{j,1}^0$. The generalized order parameter, S_j^2, is defined as[2]

$$S_j^2 = \frac{4\pi}{5}\sum_{m=-2}^{2}\left|\sum_i p_j^{(i)} Y_2^m(\theta_j^{(i)},\varphi_j^{(i)})\right|^2 \tag{5}$$

in which $Y_2^m(\theta_j^{(i)},\varphi_j^{(i)})$ are spherical harmonics,[8] $\theta_j^{(i)}$ and $\varphi_j^{(i)}$ define the ith orientation of the jth bond vector in a molecular reference frame, and the equilibrium probability distribution is

$$p_j^{(i)} = \exp(-E_j^{(i)}/kT)/q_j = \exp(-\Delta E_j^{(i)}/kT)/\tilde{q}_j \tag{6}$$

Hence, S_j^2 only depends on relative energies with respect to E_j^0. Herein, motions of the bond vectors are assumed to be axially symmetric; thus, only the term with $m = 0$ in eq 5 is non-zero, and $\Delta E_j^{(i)}$, $p_j^{(i)}$, $\tilde{q}_j$, and S_j^2 depend solely on the polar angle $\theta_j^{(j)}$.

Numerical calculations were performed using three simple models to describe the orientations of the bond vectors: (i) free diffusion within a cone with $\Delta E_j(\theta,\varphi) = 0$ if $\theta \le \theta_{jo}$ and $\Delta E_j(\theta,\varphi) \to \infty$ otherwise;[9,10] (ii) axially symmetric parabolic potential energy function with $\Delta E_j(\theta,\varphi)/kT = \alpha_j\theta^2$;[11] (iii) maximum entropy potential function with $\Delta E_j(\theta,\varphi)/kT = \alpha_j \sin^2\theta$.[12] Each model contains a single characteristic parameter, α_j or θ_{jo}. Models ii and iii represent the lowest order approximations to arbitrary potentials in θ and $\sin\theta$, respectively. To proceed, α_j or θ_{jo} is obtained from S_j^2 by numerical inversion of eq 5, $\tilde{q}_j$ is calculated according to eq 3, and ΔG_o is obtained from eq 4 together with the simplifying assumption that $\Delta E_j^0 = 0$. Graphs illustrating the dependence of $\tilde{q}_j$ on S_j^2 for the above models are presented in Figure 1; as can be seen, $\tilde{q}_j \propto (1 - S_j^2)$ for $S_j^2 > 0.5$. Thus, changes in the Gibbs free energy also can be calculated directly

† University of Lund.
‡ The Scripps Research Institute.
§ Columbia University.

(1) Palmer, A. G. *Curr. Opin. Biotechnol.* **1993**, *4*, 385–391.
(2) Lipari, G.; Szabo, A. *J. Am. Chem. Soc.* **1982**, *104*, 4546–4559.
(3) Kördel, J.; Skelton, N. J.; Akke, M.; Palmer, A. G.; Chazin, W. J. *Biochemistry* **1992**, *31*, 4856–4866.
(4) Akke, M.; Skelton, N. J.; Kördel, J.; Palmer, A. G.; Chazin, W. J. *Biochemistry* **1993**, *32*, 9832–9844.
(5) Cooper, A.; Dryden, D. T. F. *Eur. Biophys. J.* **1984**, *11*, 103–109.
(6) Hill, T. L. *An Introduction to Statistical Thermodynamics*; Dover: New York, 1986.
(7) Lipari, G.; Szabo, A.; Levy, R. M. *Nature* **1982**, *300*, 197–198.
(8) Brink, D. M.; Satchler, G. R. *Angular Momentum*; Clarendon Press: Oxford, 1968.
(9) Lipari, G.; Szabo, A. *Biophys. J.* **1980**, *30*, 489–506.
(10) Lipari, G.; Szabo, A. *J. Chem. Phys.* **1981**, *75*, 2971–2976.
(11) Pedersen, N. O.; Chan, S. I. *Biochemistry* **1977**, *16*, 2657–2667.
(12) The maximum entropy[13] model is obtained by determining the functional form for $p_j^{(i)}$ that maximizes the Shannon entropy subject to the constraint that the resulting probability distribution reproduces the experimental value of the order parameter. This potential is bimodal with minima at 0° and 180°, which reflects the insensitivity of S_j^2 to 180° flips of the internuclear vector.
(13) Jaynes, E. T. *Proc. IEEE* **1982**, *70*, 939–952.

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Table I. Generalized Order Parameters for Calbindin D_{9k}[a]

	apo		$(Cd^{2+})_1$		$(Ca^{2+})_2$	
residue	$S^2_{f,j}$	$S^2_{s,j}$	$S^2_{f,j}$	$S^2_{s,j}$	$S^2_{f,j}$	$S^2_{s,j}$
G57	0.77 ± 0.03	0.77 ± 0.03	0.84 ± 0.04	1.0	0.81 ± 0.04	1.0
D58	0.78 ± 0.03	0.81 ± 0.04	0.86 ± 0.04	1.0	0.85 ± 0.04	1.0
G59	0.83 ± 0.04	0.74 ± 0.03	0.90 ± 0.04	1.0	0.86 ± 0.04	1.0
E60	0.69 ± 0.03	1.0	0.88 ± 0.04	1.0	0.85 ± 0.04	1.0
S74	0.77 ± 0.04	1.0	0.67 ± 0.03	1.0	0.77 ± 0.03	0.78 ± 0.03
Q75	0.70	0.63 ± 0.03	0.70	0.47 ± 0.02	0.70	0.48 ± 0.02

[a] Order parameters are taken from refs 3 and 4. The generalized order parameter $S^2_j = S^2_{f,j}S^2_{s,j}$, in which motions on two separable time scales are characterized by order parameters $S^2_{f,j}$ and $S^2_{s,j}$, respectively.[16] If motions were observed on only one time scale, then $S^2_{s,j} = 1.0$. For Q75, $S^2_{f,j}$ was fixed at 0.70 for all analyses.

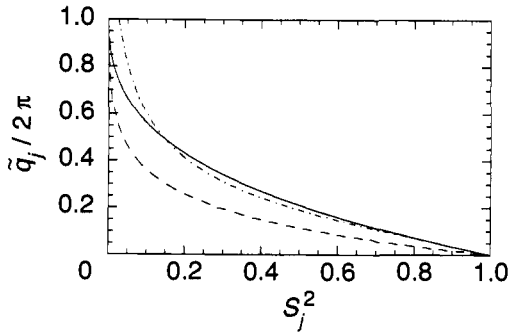

Figure 1. Calculates values of the partition function $\tilde{q}_j$ versus the generalized order parameter S^2_j for the (—) diffusion in a cone, (– – –) parabolic, and (– • –) maximum entropy potential energy functions. Graphs were constructed using the equations $\tilde{q}_j = \int_0^{2\pi}d\varphi\int_0^{\pi} \exp[-\Delta E_j(\theta,\varphi)/kT] \sin\theta d\theta$ and $S^2_j = \tilde{q}_j^{-1}\int_0^{2\pi}d\varphi\int_0^{\pi}P_2(\cos\theta) \exp[-\Delta E_j(\theta,\varphi)/kT] \sin\theta d\theta$, in which $P_2(\cos\theta) = (3\cos^2\theta - 1)/2$.

from order parameters if $S^2_{j,1}$, $S^2_{j,2} > 0.5$ by using

$$\Delta G_o = -kT\sum_{j=1}^{N} \ln[(1 - S^2_{j,2})/(1 - S^2_{j,1})] \qquad (7)$$

The linear relationship between $\tilde{q}_j$ and S^2_j does not hold for arbitrary model potentials (e.g., diffusion on the surface of a cone).

In the consecutive binding of calcium ions first to site II (the C-terminal site) and subsequently to site I (the N-terminal site) of calbindin D_{9k}, the cooperative phenomenon implies higher affinity for the second calcium ion than for the first calcium ion.[14,15] Hence, the total free energy of the cooperative phenomenon is $\Delta\Delta G = \Delta G_{I,II} - \Delta G_{II} < 0$, in which ΔG_{II} is the free energy of binding the first ion (to site II) and $\Delta G_{I,II}$ is the free energy of binding the second ion (to site I) after the first ion has bound. Estimates of the contributions to $\Delta\Delta G$ from conformational fluctuations of the backbone amide bond vectors ($\Delta\Delta G_o$) were calculated from the generalized order parameters for the apo, half-saturated [$(Cd^{2+})_1$], and fully saturated [$(Ca^{2+})_2$] states of calbindin D_{9k}. Only residues G57, D58, G59, and E60 in site II and S74 and Q75 at the C-terminus were included in the calculations, because order parameters for other individual residues do not vary significantly for the three states of the protein. Values of the order parameters are given in Table I. As shown in Table II, the values of $\Delta\Delta G_o$ estimated from the ^{15}N order parameters range from −13.2 ± 3.5 to −11.6 ± 3.2 kJ/mol. The dominant contribution to $\Delta\Delta G_o$ originates from the first binding event [apo → $(Cd^{2+})_1$], which essentially accounts for the free energy cost of stiffening the backbone of the protein. The free energy of cooperativity of calbindin D_{9k} is −7.7 kJ/mol at moderate salt concentration (0.05 M KCl), as determined by competitive Ca^{2+} titration in the presence of a fluorescent Ca^{2+} chelator.[17] The net free energy of cooperativity results from many contributions and tends to be small because of cancellation among large terms.[18] Nonetheless, the present calculations indicate that changes in free energy resulting from alterations in the intramolecular fluctuations of the protein are large enough to contribute significantly to overall cooperativity.

Table II. Contributions to $\Delta\Delta G_o$ from Fluctuations of the N–H Bond Vectors in Calbindin D_{9k}[a]

	$\Delta\Delta G_o$(kJ^{-1} mol)	
model	unimodal distribution	polymodal distribution
diffusion in a cone	−12.5 ± 3.3	−13.2 ± 3.4
parabolic potential	−12.8 ± 3.3	−13.2 ± 3.5
maximum entropy	−11.9 ± 3.3	−13.0 ± 3.4
linear approximation	−11.6 ± 3.2	−12.8 ± 3.0

[a] Residues G57, D58, G59, and Q75 exhibit intramolecular motions on two separable time scales. Analyses of the dynamics of these residues were treated in two fashions: in the unimodal treatment, $\tilde{q}_j$ was determined from S^2_j; in the polymodal treatment, the slower motion is modeled as a jump of the N–H bond vector between two distinct conformations, α and β, and the partition function is given by $\tilde{q}_j = (1 + \rho_\beta/\rho_\alpha)\tilde{q}^f_j$, in which ρ_α and ρ_β are the populations of the two conformers ($\rho_\alpha \geq \rho_\beta$) and $\tilde{q}^f_j$ is the partition function for the fluctuations described by $S^2_{f,j}$. For the calculations, $\rho_\alpha = \rho_\beta = 0.5$ was used.

At present, experimental relaxation data are available for only a limited set of bond vectors; however, fluctuations at other sites also contribute to Q and ΔG through Q_u and ΔG_u. In addition, slower time scale motions ($\tau_e \gg \tau_c$) of the bond vectors are not reflected in the order parameters[2] and are not incorporated within the present theory. Furthermore, factorization of Q into the product of q_j for individual bond vectors is not strictly justified if the motions of different internuclear vectors are correlated. Finally, possible contributions from ΔE^0_j have not been included in the estimates of ΔG_o. Experimental and theoretical investigations of these limitations are in progress.

In conclusion, this communication describes an analytical, approximate approach for extracting thermodynamic quantities from generalized order parameters and shows that changes in intramolecular fluctuations of calbindin D_{9k} contribute significantly to the cooperative phenomenon of calcium binding.

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(14) Akke, M.; Forsén, S.; Chazin, W. J. *J. Mol. Biol.* **1991**, *220*, 173–189.
(15) Akke, M. Molecular Basis for Cooperativity in Calcium Binding by Calbindin D_{9k} as Studied by NMR; Ph.D. Thesis, University of Lund, Sweden, 1993; pp 13–22.
(16) Clore, G. M.; Szabo, A.; Bax, A.; Kay, L. E.; Driscoll, P. C.; Gronenborn, A. M. *J. Am. Chem. Soc.* **1990**, *112*, 4989–4991.
(17) Linse, S.; Johansson, C.; Brodin, P.; Grundström, T.; Drakenberg, T.; Forsén, S. *Biochemistry* **1991**, *30*, 154–162.
(18) For instance, compensation of the electrostatic repulsion between the two calcium ions implies that the sum of the contributions associated with the protein reorganization upon ion binding is larger (more negative) than the net free energy of cooperativity.[14,15]