New and Notable

Definite Answers from Random Events

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Dissection of the free energy changes that produce each level of protein structure is a central aim in equilibrium studies of protein folding. The noncovalent enthalpic forces and the many sources of entropy that govern secondary and tertiary structures have been extensively studied in many proteins, as have quaternary interactions in multisubunit proteins. The next, quinary, level of structure that is seen in proteins that polymerize, often important for function and pathology, has not been examined as intensively. Although many of the same kinds of forces are concerned, protein polymerization presents new problems if a full description of the stabilizing interactions is desired.

In this issue, Cao and Ferrone (1997) analyze the polymerization of sickle cell hemoglobin (HbS) and characterize the entropic and enthalpic interactions that produce the characteristic fiber. In particular they show that a large vibrational entropy results from polymerization and, hence, that this, along with other entropically dependent forces as well as bond enthalpies, must be taken into account if the final equilibrium is to be correctly characterized.

Deoxygenated HbS polymerizes to form long, rodlike fibers. Because the polymerization is responsible for pathogenesis in sickle cell disease, characterization of the equilibrium is important for clinical pathology as well as as an example of protein aggregation into large assemblies. The polymerization (see review, Eaton and Hofrichter, 1990) can be treated as a phase change characterized by a solubility, with a solution concentration, c_s, in equilibrium with the polymeric phase.

Cao and Ferrone measure the entropic and enthalpic contributions to the four chemical potentials that contribute to HbS solution-polymer equilibrium, $\mu_{TR} + RT \ln \gamma_s c_s = \mu_{PC} + \mu_{PV}$. The solution terms on the left represent, respectively, the translational and rotational chemical potential of monomers in solution (μ_{RT}), which is lost when the large polymer, treated as a crystal, is formed, and the usual solubility term. The terms on the right represent the polymer contact bonding free energy at the interface (μ_{PC}) and the additional polymer vibrational free energy (μ_{PV}) that arises when monomers associate. The importance of the last term, μ_{PV}, in protein aggregation was originally demonstrated by Steinberg and Scheraga (1963), who showed that the gain in internal degrees of vibrational freedom resulting from aggregation is significant and cannot be neglected in the energetic balance.

The central problem lies in the determination of μ_{PC}. Once determined, the extent to which new vibrational chemical potentials compensate for the loss of translational and rotational chemical potentials can be calculated: $\Delta\mu \equiv \mu_{TR} - \mu_{PV} = \mu_{PC} - RT \ln \gamma_s c_s$; when temperature-dependent data are obtained, each chemical potential can be broken down into enthalpic and entropic contributions. The strategy for determining μ_{PV} rests on characterization of two equilibria: 1) the solution-polymer equilibrium, and 2) the solution-nucleus equilibrium, which exists under the assumption that in this nucleation-controlled process, the most unstable aggregate, the nucleus, is in rapid equilibrium with monomers. Because the nucleus has essentially the same structure as a short piece of fiber but has on average fewer bonds per monomer than does the fiber because of end effects, the scaling of the number of bonds, which gives rise to the contact energy, μ_{PC}, to the number of monomers, which gives rise to vibrational modes, is different in nuclei and long polymers; i.e., the relative contributions of contact and vibrational energies to stability are different in nuclei from what they are in large polymeric fibers. This permits quantitative separation of the μ_{PC} from μ_{PV}. That the nucleus and polymer have the same structure is a key point in the argument and is supported by the success of the double nucleation model (Ferrone et al., 1985), which assumes this, in predicting extensive kinetic observations. The model, which postulates homogeneous nucleation in bulk solution, heterogeneous nucleation on the surface of preexisting fibers, and fiber elongation, is further supported by direct visual evidence of homogeneous and heterogeneous nucleating events and fiber growth by differential interference microscopy (Briehl, 1995).

Within this framework, Cao and Ferrone characterize the thermodynamics of (equilibrium) nucleation in two independent ways: 1) μ_{PC} was obtained by fitting light-scattering progress curves of polymerization to the independent parameters in the double nucleation model. 2) Homogeneous nucleation is a random event; hence, the polymerization that follows it as well as the domains that form exhibit random properties that arise from the original nucleating events. Cao and Ferrone measure the distribution of times needed to produce a fixed amount of polymer, a distribution that depends on the stochastic nature of the initiating nucleations. From the distribution they obtain a definite (average) homogeneous nucleation rate and from it, μ_{PC}. This measurement of μ_{PC} is key in the their work; as it were, stochastic events have been used to provide a definite value for a chemical potential and, in turn, as outlined below, have led to quantitative measurement of vibrational chemical potential.

Extraction of the (average) homogeneous nucleation rate from the distri-

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bution of times needed to reach a specific polymer concentration is a problem in first passage times, i.e., the distribution of times needed for a stochastic process to reach a fixed level for the first time (e.g., Feller, 1968; Weiss, 1967).

Using additional results from direct observations on the fiber growth rate kinetic constant (Samuel et al., 1990), k_+, which is one of the independent variables in the double nucleation model, Cao and Ferrone then find that the two measures of μ_{PC} give essentially the same result. Once μ_{PC} is obtained, $\Delta\mu$ is known. Hence, how much of the lost solution translational and rotational chemical potential is replaced by vibrational chemical potential in the polymer can be calculated, which is the primary aim.

The conclusion is that the vibrational chemical potential is large and must be taken into account in predictions of solubility. Cao and Ferrone then complete the puzzle by showing that the molar enthalpy in μ_{PV} is the same as that in μ_{TR}. Consequently, the difference in the two chemical potentials lies only in entropic contributions. Thus, it is vibrational entropy that must be taken into account in the thermodynamic bookkeeping for solubility. They also show from temperature-dependent data that h_{PC}, the molar enthalpy in μ_{PC}, calculated from fitting the double nucleation model, is the same as that for the polymer solubility term, h_s, obtained by solubility measurements. Because this should be the case, it provides further support for the validity of model fitting to obtain the critical parameters.

The significance of these results extends beyond an analysis of the interesting HbS system. It shows that the growing zoo of entropic forces in protein polymerization and interactions includes more than simple mixing entropy and generally recognized effects including: 1) entropic contributions to hydrophobic interactions and other interactions concerned with the entropy of water (Kauzmann, 1959) and/or other solution moieties, 2) configurational terms should changes in folding occur, 3) excluded volume solution nonideality ("crowding"; (Minton, 1981), and, 4) for rodlike particles, excluded volume-driven nematic alignment (Onsager, 1949; Flory, 1956). Along with translational and rotational chemical potentials, vibrational chemical potentials need to be considered, and means to measure them experimentally must be devised for each system individually.

Cao and Ferrone have not only shown the importance of vibrational terms and applied them to an important protein, but they have also made two useful experimental contributions: 1) they have devised an innovative way, based on equilibrium aspects of a nucleation process, to separate the contact potential from the overall solubility term for HbS, no mean accomplishment in a field in which previous analyses, lacking methods for obtaining direct experimental data, have focused largely on theoretical analyses of vibrational energies; and 2) they have devised an optical apparatus for collecting the large volume of data needed to obtain distributions of stochastic processes in a reasonable time by parallel measurements on a single sample.

Finally, this work is significant for efforts to develop a treatment for sickle cell disease and its myriad consequences, which arise from the intracellular polymerization of HbS. Attempts to inhibit polymerization have used many strategies, including covalent and noncovalent modifications of hemoglobin and attempts to alter hemoglobin synthesis to produce increased amounts of fetal hemoglobin, which inhibits polymerization. The work of Cao and Ferrone argues that such attempts should consider more than the binding contact energies such as those that arise from hydrogen bonding, hydrophobic interactions, and charge interactions. Binding geometries and bonding that are associated with large (negative) vibrational chemical potentials will stabilize polymers, and conversely, less negative vibrational chemical potentials can produce the desired inhibition of polymerization without alteration of contact binding energies. Because the vibrational chemical potentials are large compared with the final energies of a few kcal/mol that govern solubility, vibrational changes might produce sufficient alteration of solubility to protect against polymerization.

The interesting possibility of addressing inhibition of polymerization through changes in site geometry suggests that the use of site-specific recombinant hemoglobins may be a fruitful avenue, both for further analysis of the role of vibrational chemical potentials and for the ultimate therapeutic goal.

(References cited above emphasize seminal articles or references that treat general aspects of pertinent issues; also see references in Cao and Ferrone (1987), especially by Horton and Lewis, 1992; Hofrichter, 1986; Szabo, 1988; and Tidor and Karplus, 1994.)

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