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30. Immunoelectron microscopy studies indicate that snRNPs and SC-35 localize to interchromatin granule clusters (*7, 21*). These clusters likely correlate with transcript domains, but their precise spatial relation remains unresolved, particularly with respect to their relative boundaries. Interestingly, the interchromatin granule clusters (which may be poly(A) RNA–rich) do not label as heavily as some other areas of the nucleoplasm when [^{3}H]uridine is incorporated into total nuclear RNA (*5*). Only a small fraction of nuclear RNA is heterogeneous nuclear RNA, and 80% of this fraction is not polyadenylated [B. Lewin, *Cell* **4**, 1 (1975)]. Much of this non-poly(A) RNA likely resides outside transcript domains as indicated by general RNA staining with fluorescent dyes (K. C. Carter and J. B. Lawrence, unpublished data).
31. Fixation, in situ hybridization, and fluorescence detection were done on the basis of our previously developed methods for the preservation and detection of cellular constituents including intact RNA [C. V. Johnson, R. H. Singer, J. B. Lawrence, *Methods Cell Biol.* **35**, 73 (1991)]. Cells were briefly treated with 0.5% Triton X-100 (<30 s), fixed in 2% or 4% paraformaldehyde (10 min), and stored in 70% ethanol. Poly(A) RNA detection with biotinylated deoxy(T)$_{55}$ and double-labeling of RNA and specific proteins were performed as described (*9*). Transcript domains were detectable in living cells and in cells fixed by a variety of methods (*9*), but it remains to be determined whether the finer details of the images shown here are dependent on the type of fixation.
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TECHNICAL COMMENTS

The Role of Water in Hemoglobin Function and Stability

The report by M. F. Colombo *et al.* (*1*) and the Perspective by R. P. Rand (*2*) state that a large number of water molecules (about 60) take part in the allosteric regulation of human hemoglobin A (HbA). In our microscopic approach, published 1 year earlier (*3*), we derived not only the number of water molecules involved in the allosteric mechanism (about 75, the "correct" number being largely a matter of definition) but also their entropic contribution to the allosteric constant. To this purpose, we combined data about the viscosity and density of solutions of monohydric alcohols with earlier data from a study by L. Cordone *et al.* (*4*) concerning the effects of the same alcohols on the oxygen equilibrium of HbA. We used an extrapolation to zero alcohol concentration; Cordone *et al.* subtracted contributions resulting from changes in the dielectric constant in alcohol solutions. This procedure is at variance with Colombo *et al.*'s method of using considerable polysaccharide concentrations, which surely affect the dielectric constant. Also, we commented (*3*) on the significance of a (HbA + number of water molecules) unit. The latter (compared with the bare HbA) presents a greatly expanded set of microscopic states that concur in one and the same functional state (or set of functional states) (*3, 5*). This confers to the (protein + number of water molecules) unit the long-searched-for (*5*) thermodynamic stability of a semimacroscopic machine.

The enthalpic contribution of the approximately 75 water molecules to the allosteric mechanism has also been derived (*6*). In addition, recent simulations (*7*) and experiments on myoglobin (*8*) suggest that many more water molecules similarly contribute to forces responsible for the structure and dynamics of the whole protein. Such molecules would further extend the thermodynamic probability of the functional state of the (protein and water) system.

Solvent-induced forces (SIFs) are at least as important as other forces (for example, electrostatic) acting through the solvent (*9*). Perhaps, as a consequence of their subtle nature, SIFs are overlooked or related to some (ill-defined) "hydration water" that is erroneously supposed to have necessarily extra long residence or rotational times around solutes.

Average values of SIFs can be understood in terms of the solute-solvent potential energy, U_{SW}, and of its thermodynamic average, $\langle U_{SW} \rangle$, over all solvent configurations (*10*). In the averaging process the entropic contribution is brought in, so that $\langle U_{SW} \rangle$ measures the free energy of solute-solvent interactions. When the mutual presentation and distance (R) of two solutes is slowly varied from R_0 to R there is a change of free energy, $\Delta \langle U_{SW} \rangle$, resulting from work performed by SIFs in the change of mutual position. It follows that $\bar{F}_{q_i}$, the average SIF component acting between the given solutes along the q_i coordinate, is given by the derivative of $\langle U_{SW} \rangle$ along q_i. SIFs on a protein would change on the space scale of individual atoms or residues and on the time scale of the solvent rearrangement times among configurations available to the solvent. What counts in determining a given $\bar{F}_{q_i}$ are the structural and dynamic configurations available to the solvent (compatible with constraints imposed by the given and nearby solutes) and the *relative statistical weight* of these configurations, rather than the structural rearrangement time scale (*5, 11*). In itself, a change of the latter reflects changes of inherent structures (*12*) caused by the presence of solute or solutes (*11*).

SIFs also affect the overall thermodynamic stability of biomolecular solutions (*11, 13*). The instability regions (as encompassed by their respective spinodal lines) of solutions of HbA and hemoglobin S (HbS) in standard conditions, together with the similar region for HbA in high phosphate (*13*), are shown in Fig. 1. The location of these regions in the temperature–concentration plane indicates the tendency of solutions toward demixing, as distinct from gelation (*13, 14*). Instability regions are obtained by extrapolation from experiments in the accessible temperature interval (*13*). A mean-field approximation holds for the derivation of these spinodal lines (*13*), which allows quantitative values for Flory-Huggins enthalpies and entropies to be determined (*13*). Modulation of the solvent (by phosphate concentration) or the solute (by the Glu-Val substitution corresponding to the HbA-HbS mutation) affects both the instability region (a mean-field effect) and the molecular local recognitive interactions needed for the orderly self-assembly of the

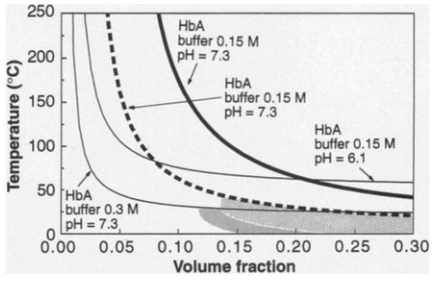

Fig. 1. Spinodal lines of solutions of human hemoglobins showing the lower temperature boundaries of the respective instability regions. Data from (*13, 16*). Gelation regions shown as gray and hatched areas, as given in (*15*).

gel, which is a microscopic effect (Fig. 1).

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Response: We thank Bulone *et al.* for pointing out the remarkable agreement between two different ways of estimating changes in hemoglobin hydration upon switching functional state. Our number for the hemoglobin hydration change, 60 ± 2 water molecules per tetramer, is built on an empirical model-independent linkage between water and oxygen activities (*1*); it represents a change in the number of waters that exclude osmotically stressing solute (extrapolated to zero concentration) as hemoglobin goes from its fully deoxygenated "T" form to its fully oxygenated "R" form. The solution dielectric constant does not seem to be a key variable. Identical results were obtained with the osmotic pressure of four different osmotic stress agents—triethylene glycol, octaethylene glycol, disaccharide sucrose, and tetrasaccharide stachyose (*1*)—that create different solution dielectric constants at the same osmotic stress.

The number of water molecules found by Bulone *et al.* (*75*) is based on the entropy modulation by alcohols that can create long-lived, high-connectivity hydrogen bonds combined with the concomitant entropic effect of these alcohols on the functional states of hemoglobin. There could be a mutual disturbance of the hydrogen-bond cages created by alcohol and hemoglobin.

There is no reason a priori why these two measures of solvation should give such similar results. If the similarity in the number of water molecules is not fortuitous, then the good agreement might indicate properties of boundary water that are not yet fully appreciated.

Last, the idea of a "solvent-induced force" is familiar to us. We and our coworkers have been measuring hydration forces between lipids (*2*), nucleic acids (*3*), polysaccharides (*4*), and proteins (*5*) for more than 15 years. We are delighted to see these kinds of forces explicitly recognized in macromolecular function and stability.

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