Spinodal lines and Flory-Huggins free-energies for solutions of human hemoglobins HbS and HbA

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ABSTRACT Gelation of deoxygenated solutions of sickle-cell human Hemoglobin (HbS) is of high theoretical interest and it has serious pathological consequences. For this reason HbS is probably the most studied protein capable of self-organization. This notwithstanding, the location in the T, c plane of the region of thermodynamic instability of solutions of deoxy-HbS (as bounded by the spinodal line and as distinct from the gelation region) has remained unknown, along with related values of Flory-Huggins enthalpies and entropies. In the present work this information is derived from experiments for the two cases of (deoxy) HbS and of human adult hemoglobin (HbA). Experiments also show critical exponents having mean-field values, which validates a Flory-Huggins approach. Altogether, the present work offers a quantitative understanding of the thermodynamic effects of the genetic HbA → HbS mutation and it opens the way to similar quantitative evaluations of contributions of pH, salts, cosolutes, and single peptides (even for nongelling hemoglobins), and of potential therapeutic strategies.

INTRODUCTION

Self-organization of extended biomolecular structures from solutions covers vast theoretical, practical, and biological interest (1). Gelation of deoxygenated solutions of mutant sickle-cell human hemoglobin (HbS) has serious pathological consequences, making HbS probably the most studied protein capable of self-assembly (2). Intense efforts have succeeded in elucidating the microscopic, thermodynamic, and kinetic aspects of deoxy-HbS gelation (2). On the other hand, however, information on the shape and location in the T, c plane of the region of thermodynamic instability (bounded by the spinodal line) (3) of solutions as such, has remained unknown. Scarcity of this type of information, and of quantitative values of related Flory-Huggins (F-H) enthalpies and entropies appears to be surprisingly common for solutions of most proteins capable of self-assembly (4), and it has stimulated recent studies at our laboratory (5–7). The value of this type of thermodynamics consists in the fact that it measures at any given temperature the tendency of solutes to demix, that is to generate two sets of regions of higher and lower polymer concentrations, respectively. It should be borne in mind that demixing itself, when occurring, is a sol-sol transition whose thermodynamics is, theoretically as well as in fact, well distinct from that of self-assembly (1, 3, 8). For this very reason, F-H thermodynamic parameters relative to solutions of biomolecules capable of undergoing self-assembly illustrate an aspect of their behavior, which is different from that of self-assembly proper.

To make this clearer, let us briefly recall the expression of the coarse-grained (mesoscopic) density of the Gibbs free-energy of mixing of our solution according to the (mean-field) Flory-Huggins lattice approach (3). We call $\Phi_1(r)$ and $\Phi_0(r)$, respectively, the mesoscopic concentrations of the solute and solvent. We express them in volume fractions, defined as the volume occupied by each species, divided by the overall volume of the solution. We call n_1, n_0 the number of molecules of the two species; also, we introduce the mean-field, first-order interaction parameter χ (or Flory-Huggins parameter), expressing the free-energy associated with the appearance of pair-wise contacts of molecules of different species, i.e., with the process:

$$(S - S) + (W - W) = 2(W - S),$$

so that:

$$\chi = \chi_{sw} - \tfrac{1}{2}(\chi_{ss} + \chi_{ww}), \qquad (1)$$

where W and S stand for water and solute, respectively. The mesoscopic free-energy of mixing for a solution of pauci-disperse species of flexible polymers can be written as:

$$\frac{f[\Phi_1(r)]}{RT} = n_0 \ln \Phi_0(r) + n_1 \ln \Phi_1(r) + (n_0 + Pn_1)\chi\phi_0(r)\,\phi_1(r), \qquad (2)$$

where P is the number of monomers in a chain. In the present case of globular proteins it is $P = 1$ and χ represents the interaction of one globular protein with a neighbor "cell" (in the Flory-Huggins lattice) containing solvent molecules only. The volume of such cell is the

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same of that of one solute molecule. The region of thermodynamic instability is bounded by the spinodal line, which is obtained by equating $\partial^2 f/\partial\Phi_1^2$ to zero that is:

$$\frac{1}{1-\Phi_1} + \frac{1}{\Phi_1} - 2\chi = 0. \quad (3)$$

The shape of the spinodal line depends on the temperature-dependence of χ, that is, on the temperature dependence of generalized forces tending to associate solutes. In fact, χ contains an obvious enthalpic term and also an entropic term due to hydrophobic areas of the protein exposed to water (9–12). When the enthalpic term dominates, the instability region is reached by the homogeneous solution upon a temperature decrease and the opposite occurs in the so-called entropy-driven (or inverse temperature) case, that is, when the entropic term dominates (13). If the solution is brought in its region of thermodynamic instability, it demixes permanently, via a nonnucleated kinetic process due to the undamped character of fluctuations occurring in that region. The process is called spinodal demixing.

Experiments reported here have allowed determining the spinodal line and related F-H enthalpies and entropies for solutions of (deoxy) HbS and normal human adult Hemoglobin (HbA), notwithstanding the fact that for HbA actual demixing and gelation are both inaccessible to experiments. In this way the thermodynamic effects of the genetic HbA → HbS mutation (2) are brought to quantitative evaluation. The correctness of our approach is substantiated by the measured critical exponents, having mean-field values. The complete (T, c) phase diagram of HbS solutions, including instability and gelation regions and related values of F-H parameters becomes in this way available. The theoretical interest of these findings is related to the fact that the spinodal and gelation lines are inherently distinct (3, 8, 14). Crossing of the gelation line causes a topological phase transition marked by the onset of an infinite cluster of percolative cross-links (8, 14). Crossing of the spinodal line causes instead a sol-sol transition which generates regions of higher and lower polymer concentrations (3). For this reason, the thermodynamic parameter relative to gelation are conceptually and numerically different from those determining the position and shape of the spinodal line (3, 8, 14). Knowledge of the complete phase diagram provides a deeper insight in the process of self-assembly (3–8, 14). Further work in progress along the same line promises to provide a new type of quantitative thermodynamic evaluations of well known effects of pH, salts, co-solutes, mixtures with other Hemoglobins, etc. (2), as well as of potential therapeutic strategies.

HbS from homozygote donors (generously provided by Dr. W. A. Eaton) was treated as already reported (15), purified on DEAE (Pharmacia LKB Biotechnology, Upsala, Sweden) cellulose at 5°C and vacuum concentrated against 0.15 M phosphate buffer at pH = 7.35. The desired concentration of actual samples was obtained by dilution with the same buffer solution. Water was Millipore (Millipore Corporation, Bedford, MA) Super-Q throughout. Deoxygenation under gentle stirring in cold, water-saturated nitrogen atmosphere was followed by addition of sodium dithionite to a final concentration of 0.05 M. This could lower pH values to 7.2 at most (16). Samples were anaerobically transferred in thin glass capillaries, which were then fire sealed. Before and after each experiment, concentration, deoxygenation, and met-hemoglobin contents were spectrophotometrically checked. As a consequence of the presence of dithionite, Met-hemoglobin was initially absent. However, at the end of long experiments (24 to 36 h) its presence was often detected, but it never surpassed 3%. For Elastic and Quasi-Elastic Light Scattering (QELS) we used the apparatus already described (15), except for a new Transputer-controlled version of a Malvern K7023 (Malvern Instruments Ltd., Worchestershire, UK) 60-channel correlator (17).

Spinodal lines, and thus the regions of thermodynamic instability of solutions, were determined by the light-scattering method. The latter uses the proportionality predicted in a mean-field approximation between the reciprocal I^{-1} of the intensity of the light scattered by a homogeneous binary solution at a concentration C, and the second derivative of Gibbs' free energy of the same solution (18–20). In turn, this is expected to be proportional to $(T - T_{sp})/T_{sp}$, where T_{sp} is the spinodal temperature relative to that concentration. Samples were subjected to sufficiently slow upwards temperature scans (0.02°C/min) within the region of thermodynamic stability. A laser beam (~2 mW at 632.8) was shed on the sample and I values for light scattered at 90° were recorded. In these conditions, a check performed as in reference 15 showed that no effects attributed to laser heating were detectable. Straight-line fittings of the I^{-1} values vs. temperature proved satisfactory, (as illustrated in Fig. 1 for a typical case) and spinodal temperatures T_{sp} were obtained by extrapolation of linear best fittings. Several points of the spinodal line were obtained from experiments at different concentrations. Fitting these points in terms of Eq. 3, and of a (F-H) parameter written as $\chi = \Delta H/KT - \Delta S/K$ gave optimized values of the ΔH and ΔS F-H parameters. Under conditions of hydrodynamic regime (whose validity we preliminarily checked) the square of the mean size of concentration fluctuations is expected to be proportional to $(T - T_{sp})/T_{sp}$. This mean size was also measured, in the way already described (6) and its temperature dependence in a typical case is also shown in Fig. 1. The linear

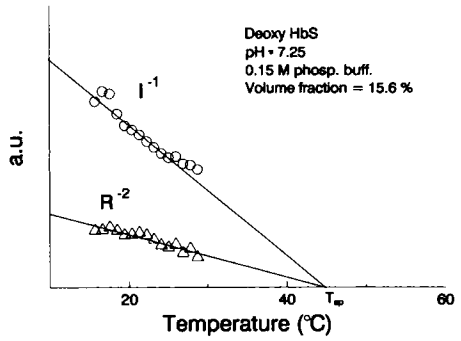

FIGURE 1 Typical linear best-fittings of the temperature dependence of I^{-1}, the reciprocal of light scattered at right angle, and R^{-2}, the reciprocal of the squared mean size of concentration fluctuations as measured by QELS. Note that the coincidence of intercepts of the two best-fitting straight lines is a systematic result of the fitting procedure, not an imposed condition.

temperature dependence of the type shown in Fig. 1, and the almost identical intercepts of the two best-fit straight lines with the temperature axis that we have consistently found, show that similarly to other cases recently studied (5–7), mean-field critical exponents appropriately described the divergence of concentration fluctuations on approaching the region of instability. QELS experiments as in reference 6 also proved that I-values contained no spurious contributions and that no detectable hemoglobin aggregation occurred in the course of the light-scattering experiments.

In Fig. 2 we show the relevant portions of the spinodal

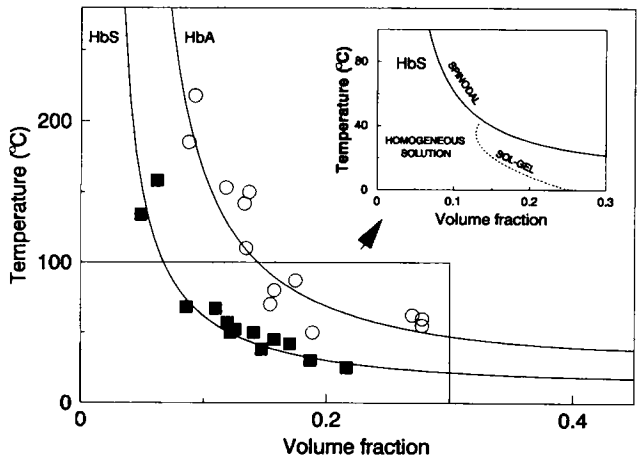

FIGURE 2 Relevant portions of spinodal lines for solutions of (deoxy) HbS and HbA at pH ≈ 7.25 (0.15 M phosph. buff.). Continuous lines are best-fits obtained by using the Flory-Huggins expression for the spinodal (see references 3 and 6). The inset refers to HbS only, and it also shows the well-known gelation line in the same pH and buffer conditions (*broken line* from data of references 17 and 2).

lines relative to solutions of (deoxy) HbS and HbA, as obtained from best fittings of experimental data in terms of Eq. 3. The latter could be confidently used because the measured mean-field exponents justified a mean-field treatment. In Table 1 values for F-H enthalpies and entropies were obtained from fittings.

Here $\Delta H/\Delta S$ ratios are given to show the consistency of present data with values close to 275° K usually found in processes involving hydrophobic interactions (21–22). Qualitatively, differences between HbA and HbS parameters shown above go in the expected direction (21–22). Quantitatively, they are well within the quoted uncertainties, for whose estimate we have conservatively included rather unsatisfactory fittings.

Values of ΔH and ΔS given above measure, respectively, the enthalpic and entropic contributions to the generalized forces determining the thermodynamic instability of solutions. Hence, they also measure the generalized forces responsible for the divergence of concentration fluctuations shown in Fig. 1. It should be noted that for reasons made clear above, they are not comprehensive of contributions such as of entropy of mixing, and of enthalpy (and entropy) of physical cross-linking/gelation, so that they should not be confused with analogous values relative to the sol-gel transition. Knowledge of the present numerical values allows us to understand quantitatively the thermodynamic effects of the Glu → Val substitution at the β6 position which is responsible for the HbA → HbS genetic mutation (2). This comparative information could never have been derived from the thermodynamics of gelation, simply because HbA does not undergo self-assembly and gelation.

The "solubility curve" of the gel obtained under the same conditions of ionic strength and pH as in the present work is shown in the inset of Fig. 2 as a broken line, redrawn from data in references 16 and 2. (The error bar in this curve is not much larger than the thickness of the line.) This curve is actually close to the gelation line and its operational definition is much simpler (2, 8, 14, 23, 24). The spinodal lines are obtained for both HbA and HbS notwithstanding the fact that they lie either beyond the gelation region (HbS) or beyond the denaturation temperature (HbA). The extrapolation method used here is thermodynamically sound (3, 5–7, 18–20), because the mean-field divergence of sizes and lifetimes of concentration fluctuations

TABLE 1

	ΔH	ΔS	$\Delta H/\Delta S$
	Kcal/mol	*cal/°K mol*	*°K*
HbS	15.5 ± 3	56 ± 8	276
HbA	7.5 ± 1	27.5 ± 4	272

is controlled by the distance of the representative point of the system from the spinodal line. Grossly, this divergence may be viewed as a transient demixing of longer and longer lifetime, capable of triggering the nucleation of fibers, the more easily as the lifetime becomes longer, that is, as the system is brought closer to its instability region. This possibility appears here appealing, because the nucleation of fibers depends upon an exceptionally high power of concentration (2).

From Fig. 2 we see that the spinodal line (and a fortiori the instability region) cannot be reached for HbS without crossing the gelation region. This rules out the possibility of interpreting Miller's proposal (25) by considering the gelation of HbS as a result of a *direct* spinodal mechanism starting from the homogeneous solution of isolated HbS molecules. However, as the spinodal line is approached, the diverging sizes and lifetimes of concentration fluctuations promote HbS polymerization, and the self-assembly of fibers occurs, probably via a double nucleation mechanism (26). There appears to be no reason for rejecting or confirming that under appropriate conditions the "new" solutes, that is the rod-like fibers obtained as a result of polymerization of individual HbS molecules, may find themselves in their instability region. The latter would in any case be conceptually different from the instability region encopassed by the spinodal line in Fig. 2. Indeed, it would concern a new phase diagram, that is, the one pertaining to a solution of rod-like fibers rather than a solution of single globular proteins.

In conclusion, the present approach offers *for both gelling and nongelling Hemoglobins:* (*a*) their instability regions, as bounded by the spinodal line and as distinct from the *gelation* region, and (*b*) the quantitative enthalpic and entropic contributions to the generalized forces responsible for driving their solutions toward instability. Such enthalpy and entropy are distinct from the homologous parameters relative to the topological phase transition of gelation (8, 14). In particular, the thermodynamic effects of the HbA → HbS genetic mutation are quantitatively understood. The present findings, apart from general theoretical interests (3–8, 14, 21–24), are thus seen to extend our understanding of HbS gelation and to open the way to further work. This includes comparisons of different (gelling as well as nongelling) Hemoglobins; of their hybrid or partially oxygen-saturated molecules (although with some caution, because in this case the system would no longer be binary); and of the effects of pH, salts, co-solutes and the like, for theoretical as well as practical purposes, such as evaluating whole classes of perspective therapeutic strategies.

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