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The Entropically Favored Osmotic "Compression" of Sickle Cell Hemoglobin Gels

Abstract: *Contrary to the accurate, hard-sphere depiction of monomeric hemoglobin in solution, sickle cell hemoglobin (HbS) polymerization/gelation requires attention to molecular interactions. From the temperature dependence of the osmotic compressibility of HbS gels, we were able to extract the entropy increase for concentrating HbS in this phase. Normalized per mole of water removed, the entropy increase from gel compression* ΔS^{gel} *is four times the previously measured* ΔS^{trans}*, for the transition from monomeric HbS solution to HbS gel. The positive entropy change cannot emerge from the assembly of hard spheres but can indicate remodeling of HbS fibers driven by release of ordered water. The fourfold difference in* ΔS^{gel} *and* ΔS^{trans} *suggests that the act of initial fiber/gel formation from monomeric solution differs from the process of further polymerization due to tighter packing within the gel phase.* © 2001 John Wiley & Sons, Inc.* Biopolymers 59: 120–124, 2001

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INTRODUCTION

From the formation of amyloid plaques in the brains of Alzheimer patients, to the aggregation of prion proteins in Creutzfeldt–Jakob disease, to the polymerization of mutant deoxygenated hemoglobin in sickle cell anemia, it is becoming increasingly recognized that disease can result from abnormal protein aggregation. Sickle cell hemoglobin (HbS) has a single-point mutation in each of its two β-chains [Glu(β6) → Val]. Changing these two negatively charged glutamates for hydrophobic valines (2 out of a total of 574 residues) creates pathological aggregation. In vitro, under physiological conditions, HbS polymerizes to form self-associating fibers similar to those observed in the anemia where these fibers stiffen red blood cells and transform them from a normal biconcave to a rigid "sickled" shape (see, e.g., Ref. 1, for review).

In vitro, at temperatures greater than 3°C, deoxygenated HbS confined within a dialysis bag can be

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induced, by controlled changes in osmotic pressure/stress, to undergo a transition from monomer solution to viscous gel[2] (Figure 1a). The higher the temperature, the lower the osmotic stress required to cause this sol–gel transition and the greater the difference between the saturated solution and the coexisting gel concentrations. After the gel is formed, it can be further concentrated/compressed osmotically with a bemusing linearity between total HbS concentration and applied stress. The slope is essentially independent of temperature.[2] From the temperature dependence of osmotic pressure required to maintain constant total HbS concentration, it is possible to "map" the entropy of the gel under osmotic compression.

THERMODYNAMIC ANALYSIS OF HbS GEL DATA

The chemical potential of the HbS confined within the dialysis bag μ_{HbS} changes as a function of T and Π, while salt, buffer, and pH are kept constant.

$$d\mu_{HbS} = -S(T, \Pi)dT + V_{aq}(T, \Pi)d\Pi \qquad (1)$$

V_{aq} is the volume of exchangeable aqueous solution per mole HbS.[3] The chemical potentials of water and other exchangeable components are set by the external reservoir. Applying a Legendre transform $\mu_{HbS} \rightarrow (\mu_{HbS} - V_{aq}\Pi)$ to Eq. (1) results in

$$d(\mu_{HbS} - V_{aq}\Pi) = -S(T, V_{aq})dT - \Pi(T, V_{aq})dV_{aq} \qquad (2)$$

which yields a Maxwell cross-relation:

$$\left(\frac{\partial S(T, V_{aq})}{\partial V_{aq}}\right)_T = \left(\frac{\partial \Pi(T, V_{aq})}{\partial T}\right)_{V_{aq}} \qquad (3)$$

The measured change in osmotic pressure necessary to maintain constant V_{aq} with varied temperature immediately gives the change in entropy versus volume.

Experimentally, c, the total concentration of HbS inside the bag, and not V_{aq} is measured as a function of Π. Because the amount of HbS inside the dialysis bag is fixed, its contribution, V_{HbS}, to the total volume $V_{tot} = V_{aq} + V_{HbS}$ remains constant. Therefore when c is in molar units,

$$dV_{aq} \equiv d\left(\frac{1}{c}\right) = -\frac{dc}{c^2} \qquad (4)$$

Integrating the Maxwell relation, Eq. (3), as a function of c, Eq. (4), gives

$$\Delta S = \int_{c(V_0,T)}^{c(V_1,T)} \partial S = -\int_{c_0}^{c_1} \frac{1}{c^2}\left(\frac{\partial \Pi}{\partial T}\right)_c dc \qquad (5)$$

Changes in c are reciprocal to changes in V_{aq} so that a process that maintains constant V_{aq} is equivalent to one at constant concentration.

Surprisingly and conveniently, the dependence of Π on c in the gel phase can be expressed in a linear form (Table I and Figures 1a and 1b):

$$\Pi = A(T) + B(T)c \qquad (6)$$

Substituting Eq. (6) into Eq. (5) gives

$$\Delta S = \frac{dA}{dT}\frac{1}{c}\Big|_{c_0}^{c_1} - \frac{dB}{dT}\ln c\Big|_{c_0}^{c_1} \qquad (7)$$

Note that for a dilute solution (van't Hoff limit), $\Pi = RTc$, $A(T) \equiv 0$, and $B(T) = RT$ in Eq. (6), then Eq. (7) reduces to the familiar ideal case where $\Delta S = -R\ln(c_1/c_0)$ at constant T.

From the experimental data, the temperature variation in $B(T)$ is statisticaly insignificant ($B(T) \equiv B = 8.0 \pm 0.3$ kPa mM^{-1}) (Figure 1b). Constant $B(T)$ reduces Eq. (7) to;

$$\Delta S^{gel} = \frac{dA}{dT}\left(\frac{1}{c_1} - \frac{1}{c_0}\right) \equiv \frac{dA}{dT}(V_1 - V_0) \qquad (8)$$

Taking $T = 30$°C as a representative point, the average slope of A(30°C),

$$\frac{dA(30°C)}{dT} = \left(\frac{\partial S^{gel}}{\partial V_{aq}}\right)_{T=30°C} = -1.1 \text{ kPa K}^{-1} \text{ HbS}^{-1}$$

gives (Table I, footnote c):

$$\Delta S^{gel}(30°C) = -1.1\left(\frac{1}{c}\right)\Big|_{c_0}^{c_1} \equiv -1.1(V_1 - V_0)$$

$$= -1.1\Delta V \qquad (9)$$

(ΔS is in J K^{-1} HbS^{-1}, c in M and V in L HbS^{-1}).

The entropy of the bag and reservoir *increases* when water is removed from the gel phase ($V_1 < V_0$). Operationally we express ΔS^{gel} (30°C) as the entropy change when 18 mL of aqueous solution (per mole HbS) are squeezed out of the gel. This is essentially

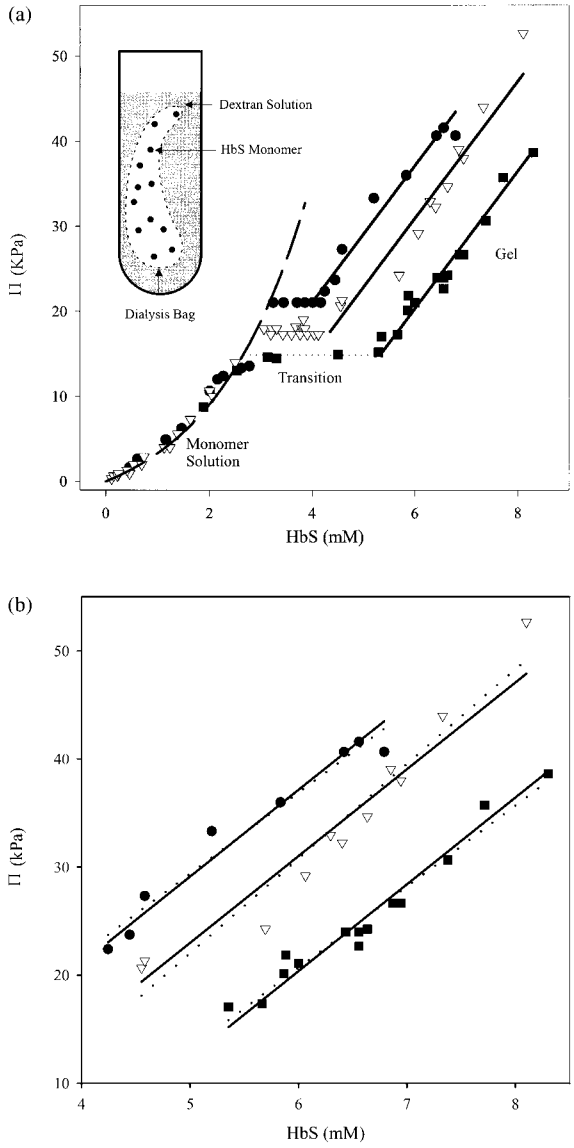

FIGURE 1 (a) The osmostic pressure vs concentration of HbS at T = 20°C (●), 30°C (▽), and 37°C (■). Heavy dashed line is from Ross and Minton[5] hard-sphere model for monomeric hemoglobins. Heavy solid lines indicate the linear fit to HbS gel data (see text and Figure 1b). Inset: a schematic of the experimental setup. After equilibration against T500 dextran (MW 500,000 in 0.15M phosphate buffer at pH 7.4), the deoxygenated HbS gel inside the dialysis bag was carefully extracted and depolymerized either by cooling of oxygenating to measure concentration (see Prouty et al.[2] for experimental details). For reference, 1 atm is 101.3 kPa; the intercellular concentration of hemoglobin in red blood cells is approximately 34%, which can be achieved by applying approximately 45 kPa[2]. (b) The gel region of HbS [T = 20°C (●), 30°C (▽), and 37°C (■)], determined by Prouty et al.,[2] fitted either with a constant B or a temperature variable $B(T)$ (date fitted using SigmaPlot 5.0 by SPSS). $A(T)$ was the result of fitting the gel region data to a linear model [Eq. (3) in Table I,

the same as saying "the entropy increase when one mole of water is removed per mole HbS in the gel." Table I reports this entropy as ΔS^{gel}_{18mL} (30°C).

How does this ΔS^{gel}_{18mL} (30°C), based on gel compression, compare to the analogous ΔS^{trans}_{18mL} (30°C) determined by Prouty et al.[2] for the sol–gel transition? At 30°C, ΔS^{gel}_{18mL} is almost 4 times ΔS^{trans}_{18mL} (Table I). This difference stems directly from the measured difference in $(\partial\Pi/\partial TV_{aq})$ in the gel vs $d\Pi_{trans}/dT_{trans} = \Delta S_{trans}/\Delta V_{aq}$ for the transition (Table I). Normalizing ΔS^{trans} per ΔV_{aq} = 18 mL removed is for comparison only. This normalization is natural for the continuous process of osmotic gel compression. For the discontinuous sol–gel transition, a specific ΔV_{aq} of water is removed[2] all at once.

DISCUSSION

The factor-of-four inequality between $d\Pi_{trans}/dT_{trans}$ of the sol–gel transition and $(\Delta\Pi/\Pi T)_{V_{aq}}$ of osmotic gel compression (Table I) strongly suggests that gel formation from monomer and gel compression are dominated by at least two different processes. Herzfeld et al.[4] have convincingly argued in many places that sol–gel transition creates a heterogeneous population of aggregates while gel compression involves a change in the mix of this population as well as interactions between aggregates themselves. The positive entropies of gel formation and compression a priori rule out the dominance of steric (hard-sphere, configurational) interactions occurring *in a continuous and featureless medium,* to the free energy of gel formation or compression. This situation contrasts strongly with models for the compression of the monomer phase where hard-sphere steric entropy neatly explains the entire set of osmotic pressure data[5].

The source of positive net entropy is usually taken to reflect the temperature dependence of the polymerization reaction or of the direct (i.e. non-steric) interaction of polymers. Prouty et al.[2] and Han and Herzfeld[4] have suggested that the entropy increase for both the sol–gel transition and the osmotic concentration of HbS gels might be due to the release of ordered water. In the extreme limit, where this ordered water is ice-like, the entropy of its release

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footnote c] where a common $B(T) \equiv B$ is assumed (solid lines). Allowing $B(T)$ (dotted lines) to vary with temperature improves the date fit; however, F-test analysis shows that the data do not support rejection of the simpler common slope model ($F_{2,27}$ = 1.52, F_{crit} = 5.49 at 1%, $F_{2,27} < Fcrit$).[8]

Table I Numerical Results

									Two-Phase Model Applied to Gel	
T (°C)	Π_{trans}[a] (kPa)	c_{sat} (mM)	$A(T)$[b] (kPa)	$(\partial\Pi/\partial T)_c$[c] (kPa K^{-1})	ΔS^{gel}_{18mL}[c] (J K^{-1} HbS^{-1})	$d\Pi_{trans}/dT_{trans}$[d] (kPa K^{-1})	ΔS^{trans}_{18mL}[d] (J K^{-1} HbS^{-1})	Moles H_2O Released[e]	Moles HbS Polymerized[f]	Moles H_2O Released/ Moles HbS Polymerized
20	21.1	3.16	-11.0 ± 2.1	—	—	—	—			
30	17.7	2.85	-17.2 ± 2.4	-1.1	0.020	-0.27	0.0049	9.1×10^{-4}	6.0×10^{-5}	15
37	16.3	2.58	-27.8 ± 2.5	—	—	—	—			

(where $\bar{V}_{HbS} = 0.75$ mL g^{-1} and GMW of HbS = 64,800)

[a] Values derived from data by Prouty et al.[2] (Figure 1a). They differ slightly different from those reported in Table I of Prouty et al.[2]

[b] Results of fitting $\Pi = A(T) + B(T)c$; $B(T) = 8.0 \pm 0.3$ kPa mM^{-1} (see Figure 1b).

[c] $dA(30)/dT = \frac{1}{2}\{[A(30) - A(20)]/[30 - 20] + [A(37) - A(30)]/[37 - 30]\} = -1.1$. ΔS^{gel}_{18mL} (30°C) is calculated for $\Delta V = V_1 - V_0 = 18$ mL.

[d] For ΔS^{trans}_{18mL}, we can use the average slope of Π_{trans} at 30°C as calculated in footnote c. $\Delta S^{trans} = -0.27(1/c)|_{c_0}^{c_1} \equiv -0.27(V_1 - V_0)$.

[e] $\Delta S^{gel}_{18mL}/\Delta S_{fusion}(\Delta S_{fusion} = 22$ J K^{-1} $H_2O^{-1})$.

[f] $C_{sat} = n_{HbS}/(V_{aq} + V_{HbS}) = n_{HbS}/(V_{aq} + \bar{V}_{HbS}n_{HbS})$, where n_{HbS} is number of HbS monomers, V_{aq} is the aqueous solution volume, V_{HbS} is the volume of HbS monomers, and $\bar{V}_{HbS}$ is the molar volume of HbS monomers (0.75 mL g^{-1})[1].

resembles an entropy of fusion. For lack of a better measure, we divide ΔS^{gel}_{18mL} (Table I) by 22 J K^{-1} H_2O^{-1}, the ΔS_{fusion} of ice at 0°C, to estimate qualitatively an equivalent number of frozen waters released while compressing the gel by 18 mL. At 30°C this corresponds to a minuscule 9.1×10^{-4} moles "melted off" per mole water (18 mL) squeezed from the gel (Table I, footnote e). This estimate relies heavily on the arbitrary choice of ΔS_{fusion} for "melting."

Experiments done in the *absence* of an osmotic pressure constraint have suggested to others that the HbS gel phase consists of two regions that act as separate phases, a saturated monomer solution in local equilibrium with condensed polymer.[1] The condensed polymer was determined to have a concentration of approximately 69 g dL^{-1} (10.6 mM), similar to that of deoxy-HbS single crystals.[1] This "two-phase" approach has been used with great success to interpret equilibria and kinetics of HbS polymerization.[1] In this approach, the experimentally observed changes in HbS gel concentrations are thought of as changing the fraction of HbS in a saturated monomer solution phase versus polymer phase. Applying this model to osmotic gel compression, the estimated 9.1×10^{-4} moles of released "frozen" water calculated above, correspond to 15 molecules of water per HbS polymerized (Table I, footnote f). For comparison, the amount of such waters released per HbS polymerized at the sol–gel transition based on ΔS at 303 K determined by Prouty et al.[2] would only be 3.6.

In experiments done under osmotic pressure/chemical potential of water constraint, the polymer composition of the "gel" phase is not well established. It is not clear whether the "gel" is in fact a single phase or a mixture of phases that have not come to true phase equilibrium. According to the phase rule, a two-component (water and HbS) preparation fixed by three intensive variables (hydrostatic pressure, temperature, osmotic stress/chemical potential of water) can exist only in one phase away from the sol–gel transition/coexistence point in the c-Π plane. By this thermodynamic constraint, the 15 water molecules per HbS molecule calculated in the previous paragraph rests precariously on an assumed two-phase gel that cannot exist at true equilibrium over a range of osmotic stress above of the sol–gel transition.

What feature of the "gel" resists applied osmotic stress? Even in the sol–gel transition, HbS does not exhibit "ideal," conveniently clear two-state behavior. In contrast lysozyme, subjected to sufficient osmotic stress, precipitates/crystallizes to consume all monomers and the concentration of lysozyme in the crystals remain constant under higher applied stress.[2] HbS gels continue to increase in concentration under additive stress after the sol–gel transition. Phrased another way, what keeps the HbS gel concentration from immediately "jumping" to a limiting value after the transition? Is the gel a bag of polymer straw whose stiffness resists osmotic compression but whose disorder protects unstressed cavities of saturated monomer solution? This picture violates the very thermodynamics that inspired it.

More reasonably, Han and Herzfeld[4] argue that there is a dominant soft repulsion between polymer fibers while the relatively few HbS monomers contribute little to gel osmotic pressure. The low osmotic pressure sufficient to induce polymerization is unlikely to cause HbS to undergo significant volume change. Ross and Minton[5] have shown that at comparable osmotic pressures both monomeric oxygenated HbS and normal hemoglobin are well modeled

by hard spheres. According to Han and Herzfeld,[4] gel compression entails continuous remorphing of the polymer population through interactions between polymers themselves. It would be pleasing to see how the positive entropy of compressing such a mélange can vary linearly with its water content. The release of water bound to hemoglobin monomers or to ends of fibers, may be the source of this entropy increase. If so, then linearity in entropy with water volume implies proportionality between water volume and unjoined hemoglobins. The observed decrease in gel volume with increasing temperature would suggest a concomitant decrease in the total number of oligomers and monomers.

HbS is one of many soluble biological macromolecules that exhibit ordered aggregation with increased temperature. Collagen[6] and Mn^{2+}DNA,[7] for example, also assemble at elevated temperatures. There too osmotic stress applied at different temperatures reveals positive entropies of condensation, entropies that vary continuously with protein or DNA concentration. Similarities between these systems may point to a novel mechanism by which nature harnesses the normally disordering power of entropy to form complex higher order structures from interaction between intricately structured polar and nonpolar macromolecular surfaces.

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