lume is meant to provide an

nas Zeuthen, Copenhagen
ed D. Stein, Jerusalem

Protein–Water Interactions

V. Adrian Parsegian
Laboratory of Physical and Structural Biology, National Institute of Child Health and Human Development, NIH, Bethesda, Maryland 20892

By recognizing the forces that drive water transport across cell membranes or across tissues, we can see how water is driven to and from proteins. We learn from examples. When a dissolved protein (bovine serum albumin) accumulates water relative to small solutes, it effectively withdraws a number of water molecules from the bath; the number of water molecules changes with the identity but not with the concentration of small solutes. When a large ionic channel (VDAC or alamethicin) opens, it withdraws water from its bathing solution; excluded solute stabilizes the closed state in proportion to activity of water, the osmotic stress created by the solute, rather than in proportion to the activity of the solute itself. Hemoglobin too acts like an osmometer whose loading of oxygen shifts with the chemical potential of water. Assemblies of many macromolecules (proteins, nucleic acids, polysaccharides, lipids), subjected to the osmotic stress of completely excluded solutes, fight dehydration with powerful, exponentially varying intermolecular forces. Should we speak of these sensitivities and responses as solute effects or water effects? Intuitive but rigorous thermodynamics, developed in a set of appendixes, provides a surprisingly practical guide to alternatives in language.

KEY WORDS: Gibbs adsorption isotherm, Hydration forces, Intermolecular interactions, Ionic channels, Molecular assembly, Osmotic stress, Surface excess/deficit, Water effect.

I. Introduction

A channel on the outer membrane of a mitochondrion opens to pass ATP, while opening it pulls in ~1000 waters. Hemoglobin relaxes upon the binding of oxygen; it draws ~65 waters away from the small solutes in its bathing solution. An enzyme

skitters along a DNA double helix, pushes down onto its specific binding site, and squeezes out ~100 molecules of water.

We measure these numbers by watching the consequences of making it more or less difficult for the proteins to draw water from the bathing solution. Just as in bulk water transport, where water moves under the osmosis created by small thirsty solutes, so over the dimensions of macromolecules, water moves under stress. This can be on the millimeter scale of tissues, the micrometer scale of cells, or the nanometer scale of proteins (Fig. 1). Change stress; change flow. Proteins are osmometers. The same thermodynamic potentials that drive water transport also determine a single protein's swelling or a pair of proteins' expulsion of water in mating.

So linked are "water" and "protein" that a mere list with sketches of the techniques and ideologies connecting them would exceed reasonable limits. Surprisingly forgotten in such lists, at least until recent years, is the realization that proteins and other large molecules react directly to the activity of water much as they do to pH and to the activities of ligands. If there are two observable forms of a protein with different amounts of associated water, we can know the volume or number of waters transferred between the solution and the protein in its transit between states. When the protein has a more difficult time taking up water from the bath, the equilibrium ratio between states will shift to favor the drier state.

Why address protein–water interactions from the perspective of water effects? Water is a far more abundant ligand than any other species bathing a protein. Hydration forces have emerged as a major feature of the interactions between macromolecules nearing contact. Within the past few years, many systems have revealed responses that vary directly with osmotic stress.

Superficial interest in hydration deals with the differences in the amounts of water touched in various conformations and associations. More profound inquiry demands answers to questions such as:

- How much energy does it take for the protein to wrest water from the many other solutes in its milieu?

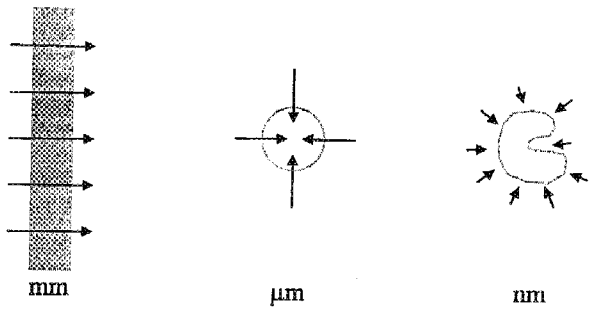


FIG. 1 Flow under osmotic stress, on the millimeter scale of tissues, the micrometer scale of cells, and the nanometer scale of proteins.

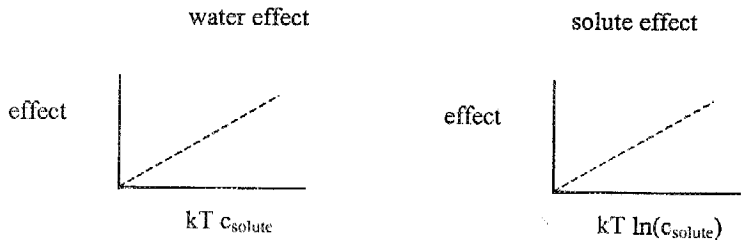


FIG. 2 The quantitative measure of hydration is the proportionality of effect to c_{solute}. The quantitative measure of solute effect is proportionality of effect to $kT \ln(c_{solute})$. The same effect cannot be linear in both c_s and $\ln(c_s)$. (It need not be linear in either.)

- How does one distinguish between the protein's work of wresting water and the protein's direct interactions with the solutes themselves?
- If we ignore the stress of water, do we miss essential features of the conditions under which proteins work?

We decide to speak explicitly of a "water effect" on a protein when the effect is proportional to the chemical potential (or osmotic pressure or stress)[1] of water rather than proportional to the chemical potential of small solutes (Fig. 2). Think of a solute s added to the solution bathing a protein. At low concentration c_s, its chemical potential μ_s varies as $kT \ln(c_s)$. At the same time, the chemical potential of water μ_w varies as $kT\, c_s$, the van't Hoff law for dilute solutions.

II. Baptism by Total Protein Immersion

By measuring solution vapor pressure vs added protein, Courtney *et al.* (2000) show how a protein takes water from small solutes that share its solution. Addition of each protein molecule increases solution osmolality by an increment that is virtually constant over a wide range of protein concentrations. Specifically, in a regime where the number density of proteins is negligible compared with the number density of the small solutes, each added protein surrounds itself

[1]Chemical potential denotes the work needed to transfer a number of molecules. In the particular case of water, equivalent to the chemical potential of water, osmotic stress (or pressure) refers to the energy available to move or to remove a volume of water (as opposed to a number of water molecules). The distinction is operational as well as linguistic. To know the work needed to transfer a volume V_w of water, multiply it by a quantity with units of pressure (Force/Area = Energy/Volume). If that pressure $\Pi_{osmotic}$ is changed by a small amount, $d\Pi_{osmotic}$, there is a change $V_w d\Pi_{osmotic}$ in the amount of work needed to transfer the volume V_w. If that volume V_w corresponds to N_w molecules of water, multiply by chemical potential μ_{water} for the work of transfer and think of the change in chemical potential $d\mu_{water}$ for the change $N_w\, d\mu_{water}$ in work. In either case, the operational measure of an osmotic effect is change in proportion to the chemical potential μ_{water}.

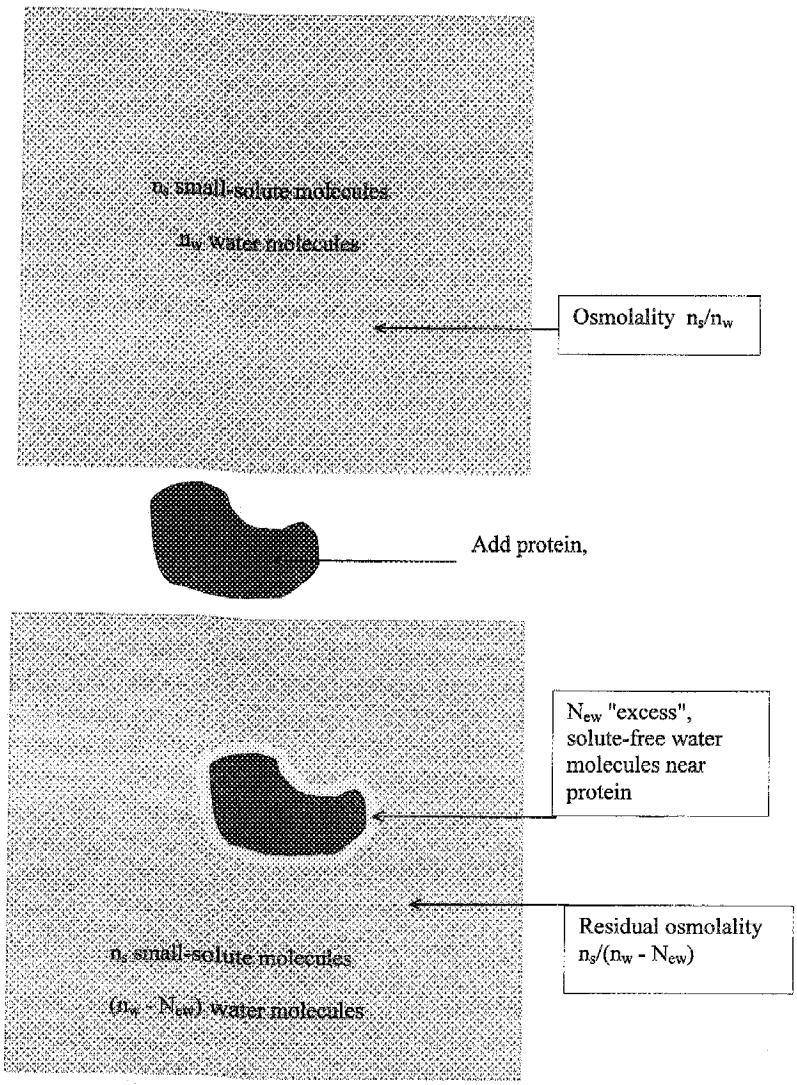


FIG. 3 Exclusion by the protein creates a thermodynamically defined excess number N_{ew} of solute-free waters near the protein (Appendix 1). It is possible to speak of the positive, excess N_{ew} in water molecules as a deficit (or negative excess) of solute, $N_{es} = -N_{ew}(n_s/n_w)$ [Appendix 2, Eq. (A2.12)].

with a region of solution depleted of solute. The consequence of the preferential attraction for water is to leave a bathing solution of higher solute concentration (Fig. 3).

More intriguing, the data (Fig. 4) show a factor of 7 difference in this preference for water, from glycerol—the least excluded from near the protein—to

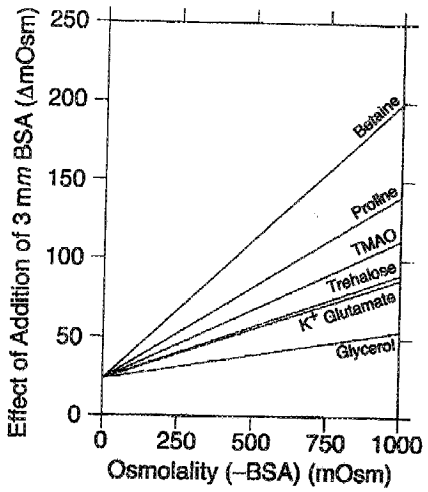


FIG. 4 Change in solution osmolality upon addition of 3 millimolal BSA vs osmolality of the two-component solution without BSA. The measured solution osmolality reflects the interplay of three species. (From Fig. 2 in Courtenay *et al.*, 2000.)

most-excluded betaine. That is, among these solutes, the amounts of water taken by the protein from the small-solute solutions differ by a factor of almost 7 (Table I).

There are many equivalent ways to express the three-way protein–solute–water competition. In the regime where the volume fraction of proteins is tiny, each protein molecule interacts independently with the surrounding solution.[2] In its fight for water (or solutes), the protein disturbs the small-molecule solution in its vicinity. This push–pull, excess–deficit ambiguity touches the limits on how we perceive. We can say "The protein repels solute more strongly than it repels water," or "The protein attracts water more strongly than it attracts solutes." Needless effort is spent dancing on such mental pinheads.

In the present case, there is less solute in this water around the protein than in a similar amount of water in the solution. Think "solute depletion" or "water excess"; it amounts to the same thing. The linearity between solution osmolality and protein molality shows that each added protein competes as effectively as its predecessors in fighting for water against the small solutes in the bath.

[2]The molecular mass of BSA is 66 kg/mol; protein density is ~4/3g/ml; 3 mmol BSA has a volume of $3 \times 10^{-3}\text{mol} \times \frac{66\text{kg}}{\text{mol}} \times \frac{3\text{L}}{4\text{kg}} = 0.15L$, compared to 1 L for 1 kg of water. There are 3×10^{-3} mol $\times$ 0.602×10^{24} molecules/mol $= 1.8 \times 10^{21}$ molecules per 1.15 L $= 1.15 \times 10^{3}\,\text{cm}^3 = 1.15 \times 10^{27}$ Å^3 of solution. Each protein is allowed an average volume $\sim 0.64 \times 10^{6}$ Å^3 or $\sim$ (86 Å)^3, a volume larger than the ~(44 Å)^3 volume of the protein. In fact (E. Courtenay, personal communication), in the actual experiments, there is some interaction between proteins; the relation between total osmolality is not strictly linear. "However, the preferential interaction coefficient at constant water chemical potential is not a function of protein concentration" (E.C.).

TABLE I

The Number of "Excess" Waters N_{ew} That Exclude Solute from the Vicinity of BSA Protein

Solute	No. excluding waters, N_{ew}	$\Gamma_{\mu 1,\mu 2}/m_3'$
Glycerol	555	−10
K^+ Glu^-	1110	−20
Trehalose	1170	−21
TMAO	1499	−27
Proline	1943	−35
Betaine	2775	−50

These N_{ew} are computed from column 2 Table 5 of Courtenay *et al.* (2000). Those authors list $\Gamma_{\mu 1,\mu 2}/m_3'$ as the number of solutes that *would be* excluded by the protein in a 1-molal solution, a partitioning of the listed solute between bulk water and a "local domain" one-water-layer thick around the protein. There are 55.5 waters per solute in a 1-*m* solution. The equivalent number of solute-excluding waters is therefore $55.5 \times \Gamma_{\mu 1,\mu 2}/m_3'$. Note that for those data, because $\Gamma_{\mu 1,\mu 2}/m_3'$ is said to be constant, N_{ew} is constant at all solute concentrations. Solute deficit $N_{es} = \Gamma_{\mu 1,\mu 2}$ is linear in solute concentration. See Appendix 3 for discussion of the different languages and Appendix 2 for the conversion between N_{ew} and N_{es}. The accessible surface area of BSA is given as 29,000 Å^2 (Courtenay *et al.*, 2000). Were the occupation area of a water molecule 9 Å^2, a monolayer of solute-excluding waters would contain $29{,}000/9 \approx 3225$ molecules.

The immersion of the protein raises the chemical potential of the water in the solution (Fig. 4). The magnitude of this consequence is linear in the chemical potential of the water in the pre-protein solution. This change with c_{solute} tells us that there is a change in the amount of work needed to insert the protein. Just as the Gibbs adsorption isotherm tells us that the energy to create an interface changes in proportion to the relative number of each component in its vicinity, so we can say that the change in proportion of solute to water near the protein is the key to connecting the work of immersion to the change in the ratio of small solute s and water w (Appendix 2, Eqs. (A2.7) and (A2.8), and Parsegian *et al.*, 2000):

$$d\mu_{\text{protein immersion}} = -N_{ew}\, d\mu_w = -N_{es} d\mu_s. \tag{1}$$

This formalism already asserts that if the numbers of s molecules and w molecules near the protein are in the same ratio as they are in the original bath, then there is no change on the immersion energy of the protein from adding s or w to the bath. We can ask, What makes this part of the solution around the protein different from the remainder of the solution? It is ratio of the total number of waters to the total number of solutes compared to the ratio (n_s/n_w) that matters.

N_{ew} and N_{es}, schematically portrayed in Fig. 3 and formally elaborated in the appendixes, have physical meaning. In the Gibbs spirit, N_{ew} is the number of waters around the protein that are in excess of the number of waters there would be if the protein did not redistribute w's and s's. We can say that the protein

m the Vicinity of BSA Protein

$\Gamma_{\mu 1,\mu 2}/m_3'$
−10
−20
−21
−27
−35
−50

rtenay *et al.* (2000). Those *be* excluded by the protein een bulk water and a "local e 55.5 waters per solute in waters is therefore 55.5 × is said to be constant, N_{ew} = $\Gamma_{\mu 1,\mu 2}$ is linear in solute languages and Appendix 2 ace area of BSA is given as a of a water molecule 9 Å^2, 00/9 ≈ 3225 molecules.

otential of the water in the e is linear in the chemical change with c_{solute} tells us nsert the protein. Just as the create an interface changes nt in its vicinity, so we can ar the protein is the key to ratio of small solute s and segian *et al.*, 2000):

$$N_{es} d\mu_s. \qquad (1)$$

nolecules and w molecules original bath, then there is adding s or w to the bath. l the protein different from nber of waters to the total atters.

formally elaborated in the rit, N_{ew} is the number of ber of waters there would can say that the protein

creates an excess number N_{ew} of waters free of solute. The protein might attract solutes relative to waters (or repel water relative to solutes), in which case N_{ew} is negative and N_{es} positive. The sensitivity of protein immersion to solute and solvent chemical potentials is a general idea, as general as the Gibbs isotherm for surface energy. It is the sensitivity of protein free energy to the activities of water and solutes that lets us measure these excesses and, sometimes, to gauge the shape of proteins.

If an experiment shows a linear response to μ_w, then we know that N_{ew} is a constant, and we can speak of a "water effect." In such a case, N_{es} cannot be a constant (Appendix 2). In their linear responses to solution molality, the Courtenay *et al.* (2000) data show precisely the number of water molecules that the bovine serum albumin (BSA) draws to itself from the various small-solute solutions.

The linearity of the data in Fig. 4 tells us that we have a water effect, a change in free energy proportional to changes in water activity. We could think of a solvent-accessible layer clear of solute, but it would be a layer of different thickness for different solutes. Its value depends on all the factors that drive solutes from or toward a surface. But there are many features of proteins that are not "surfaces." Rather than speak of data in terms of particular models, it is better to keep to the numbers N_{ew} that fall out neatly from basic definitions.

III. Transitions between States: Tipping Balances

Rather than look at proteins in only one state, it is more instructive to look at changes in protein–water interactions through transitions between functional states. In the simplest reaction scheme

$$a \leftrightarrow b \qquad (2)$$

between two states a and b, the probabilities of their occurrence follow the same kind of dependence on free energies that determine the extent of a chemical reaction (Parsegian *et al.*, 1995). These probabilities depend on the difference $(G_b - G_a)$ in the free energy needed to go from a to b. Think of this difference as the work it would take if we had the hands to bring the molecule from one form to the other without relying on the kick of thermal energy to effect this transition. To connect this energy difference with Boltzmann thermal distribution for the probability of being in one state vs. the other, measure $(G_b - G_a)$ in units of the thermal energy kT per molecule (or RT per mole). In reaction language, the ratio of concentrations or probabilities goes as

$$K_{eq} \equiv \frac{[b]}{[a]} = \frac{Pr(b)}{Pr(a)} = e^{-(G_b - G_a)/kT} \qquad (3a)$$

or

$$\ln(K_{eq}) = -(G_b - G_a)/kT. \tag{3b}$$

If these two states have different volumes V_a and V_b of solute-excluding water, an added osmotic pressure $\Pi_{osmotic}$ of the excluded nonbinding solute creates an additional work $\Pi_{osmotic}(V_b - V_a)$ to go between the two forms. Then K_{eq} changes with $\Pi_{osmotic}$ as

$$\ln(K_{eq}) = -(G_b - G_a)/kT - \Pi_{osmotic}(V_b - V_a)/kT. \tag{4}$$

If measured $kT\ln(K_{eq})$ is linear in $\Pi_{osmotic}$, the slope is the difference $(V_b - V_a)$ in aqueous volumes inaccessible to added solute.

A. Inversion, the Outside-in Surfaces of Ionic Channels

As an immediate corrective to the purely surface thinking of immersion, we turn now to cavities. The hydration event is the opening of an ionic channel bathed by excluded solutes. Then the act of opening requires extra work to separate water from excluded solute, work that necessarily varies with excluded-solute concentration.

Specifically, imagine a cavity too small for entry of a large solute (Fig. 5). It is as though a semipermeable membrane bounded the small space. Creating such a space requires work to purify the water that must be drawn away from the bathing solution to fill that space. Increasing concentrations of excluded solute mean increasing works of purification to draw a volume of water $V_{ew}^{open} - V_{ew}^{closed}$. With addition of osmotic stress, the free energy of the open form will increase by $V_{ew}^{open} \times \Pi_{osmotic}$ while that of the closed form will go up as $V_{ew}^{closed} \times \Pi_{osmotic}$. The ratio of open vs. closed states will change with $\Pi_{osmotic}$:

$$\left.\frac{[\text{Open}]}{[\text{Closed}]}\right|_{\Pi_{osmotic}} = \left.\frac{[\text{Open}]}{[\text{Closed}]}\right|_{\Pi_{osmotic}=0} e^{-\Pi(v_{ew}^{open} - v_{ew}^{closed})/kT}. \tag{5}$$

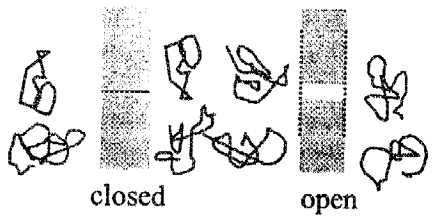


FIG. 5 Open vs closed channel under the osmotic stress of excluded solute.

(3b)

of solute-excluding water,
onbinding solute creates an
o forms. Then K_{eq} changes

$V_b - V_a)/kT.$ (4)

the difference $(V_b - V_a)$ in

Channels

ing of immersion, we turn
of an ionic channel bathed
es extra work to separate
aries with excluded-solute

of a large solute (Fig. 5).
the small space. Creating
st be drawn away from the
rations of excluded solute
e of water $V_{ew}^{open} - V_{ew}^{closed}$.
open form will increase by
as $V_{ew}^{closed} \times \Pi_{osmotic}$. The

$v_{ew}^{open} - v_{ew}^{closed})/kT.$ (5)

of excluded solute.

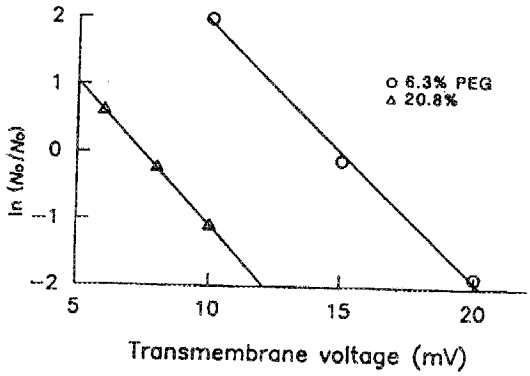


FIG. 6 Shift in [open]/[closed] ratio of the VDAC channel with addition of polyethylene glycol (PEG) polymer osmotic stress. Added PEG decreases the ratio of N_{open}/N_{closed} for the number of times the channel is periodically observed open vs closed. (Data from Vodyanoy *et al.*, 1993; Bezrukov and Vodyanoy, 1993.)

Plotting ln([Open]/[Closed]) vs $\Pi_{osmotic}$ will give a slope $-\left(V_{ew}^{open} - V_{ew}^{closed}\right)/KT$. That is, the open/closed ratio vs osmotic strength gives the difference in solute-inaccessible volumes of water associated with the two forms of the channel. Several mesoscopic channels, particularly VDAC (Zimmerberg and Parsegian, 1986) (Fig. 6) and alamethicin (Vodyanoy *et al.*, 1993; Bezrukov and Vodyanoy, 1993) (Figs. 7, 8), have been investigated.

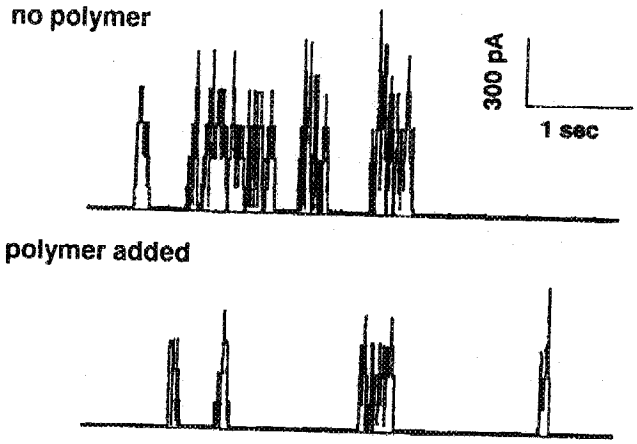


FIG. 7 Exposure to large-enough polymers of PEG suppresses alamethicin channel formation. Each successive opening requires about 100 additional water molecules to be drawn away from the large solutes in the bathing medium.

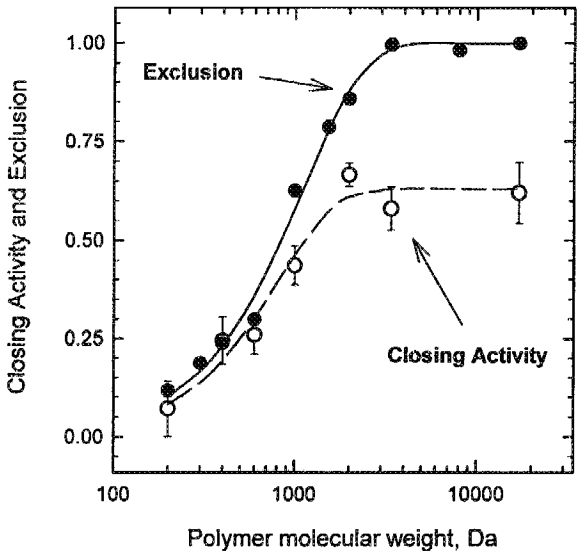
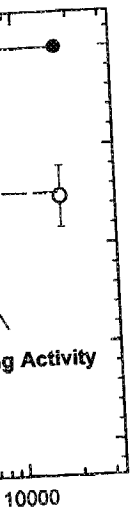


FIG. 8 Different-size PEGs reveal different volumes of solute-inaccessible water. Large PEGs are completely excluded from the open channel and exert maximum effect on closure. Smaller PEGs, able to enter the open channel, exert little power to close the channel. (Figure courtesy of S. Bezrukov, drawing on data from Bezrukov and Vodyanoy, 1993, and Vodyanoy *et al.*, 1993.) Closing activity (dashed line, open circles) is measured as $-\ln[([\text{open}]/[\text{closed}])_{\text{with PEG}}/([\text{open}]/[\text{closed}])_{\text{no PEG}}]$. Exclusion (solid circles, solid line) is derived from relative conductance of channels, those bathed in PEG solution vs channels in PEG-free solution.

With ionic channels, it is possible to watch the occurrence of open vs closed states by the amount of current flow permitted through them. Consider an electrical current burst of alamethicin channels with successive changes in conductance. Suppression of channel opening by addition of the polymer polyethylene glycol (PEG) to the bathing solutions on both sides of the membrane that houses the channel is seen in Fig. 7.

It is easy to visualize solute exclusion that operationally defines "large" solutes (Fig. 5). It is almost as easy to see that the degree of exclusion can vary with solute size. Large, completely excluded solutes are not equivalent to small solutes that enter the cavity. There will usually be a smaller apparent change in the difference $(V_{\text{open}} - V_{\text{closed}})$ in volume that is accessible to the smaller solutes. Figure 8 shows the variable power of PEGs to close alamethicin channels osmotically à la Eq. (5). Large PEGs exert a maximum osmotic stress and consequent closing activity (open circles, dashed lines). The power to close is almost lost with smaller PEGs. Measured open-channel conductance reveals that large polymers stay out of the channel, but smaller PEGs enter and obstruct ionic current (Vodyanoy *et al.*, 1993;



nt, Da

ccessible water. Large PEGs are
t on closure. Smaller PEGs, able to
e courtesy of S. Bezrukov, drawing
993.) Closing activity (dashed line,
'[closed])$_{no\ PEG}$]. Exclusion (solid
, those bathed in PEG solution vs

ccurrence of open vs closed
them. Consider an electrical
ve changes in conductance.
polymer polyethylene glycol
e membrane that houses the

onally defines "large" solutes
xclusion can vary with solute
uivalent to small solutes that
arent change in the difference
naller solutes. Figure 8 shows
nnels osmotically à la Eq. (5).
d consequent closing activity
lmost lost with smaller PEGs.
arge polymers stay out of the
current (Vodyanoy *et al.*, 1993;

Bezrukov and Vodyanoy, 1993). Figure 8 shows that the power to exert osmotic stress and the degree of polymer exclusion are strongly correlated.

B. Protein Surfaces and Cavities at the Same Time: Hemoglobin

Crudely but instructively, we can distinguish two kinds of protein association with water (cf. Peter Rand's chapter, "The Lipid–Water Interface: Revelations by Osmotic Stress.") First the protein "surface" perturbs water in its vicinity; this perturbation can exclude solutes, as indicated in the BSA-immersion measurements. Second, aqueous cavities such as some ionic channels will exclude large solutes though the cavity is large enough that water properties are not strongly perturbed. Hemoglobin shows both kinds of association.

Water is an effector of hemoglobin (Colombo *et al.*, 1992). Much as with the change in open/closed ratios of channels with the addition of excluded thirsty solutes, loading of oxygen is shifted with the addition of small solutes as various as NaCl, glucose, and PEG.

This classic allosteric protein was subjected to different pressures, pO_2, of oxygen at constant temperature, (atmospheric) pressure, pH, ionic conditions, but the oxygen titration was conducted in solutions with different concentrations of a neutral solute "s." The idea was to see how the presence of these solutes changes the oxygen loading curve and, consequently, to learn about the competition between these neutral solutes and water interacting with the hemoglobin molecule.

The chemical potential of oxygen will go as $d\mu_{O2} = kT\, d\ln(pO_2)$ because the pressure of oxygen is low enough for it to be treated as an ideal gas. The work done on a hemoglobin molecule by oxygen pressure and water/solute activity (at constant temperature and pressure) can be written in a general but impractical form as

$$d\mu_{\mathrm{Hb}} = -N_{\mathrm{O}_2}\, \mathrm{d}\mu_{\mathrm{O}_2} - N_w\, d\mu_w - N_s\, d\mu_s. \tag{6}$$

Because the chemical potential of added solute is connected with the chemical potential of water by the Gibbs–Duhem constraint, $n_s\, d\mu_s + n_w\, d\mu_w = 0$ (Eq. (A2.3)),

$$-d\mu_{\mathrm{Hb}} = N_{\mathrm{O}_2}\, d\mu_{\mathrm{O}_2} + N_{\mathrm{ew}}\, d\mu_w = N_{\mathrm{O}_2}\, d\mu_{\mathrm{O}_2} + N_{\mathrm{es}} \mathrm{d}\mu_s. \tag{7}$$

Consider, for example, the observation that hemoglobin takes up less oxygen at a given pO_2 when neutral solutes are added to the solution (Fig. 9). What can we learn from this observed decrease in bound oxygen N_{O_2} with added solute? Using the cross derivative, $\partial N_{O_2}/\partial \mu_s|_{\mu_{O_2}}$ can be interpreted as

(*a*) The decrease in the relative amount N_{es} of solute as oxygen is loaded:

$$\partial N_{\mathrm{O}_2}/\partial \mu_s\,|_{\mu_{\mathrm{O}_2}} = \partial N_{\mathrm{es}}/\partial \mu_{\mathrm{O}_2}|_{\mu_s}\,. \tag{8}$$

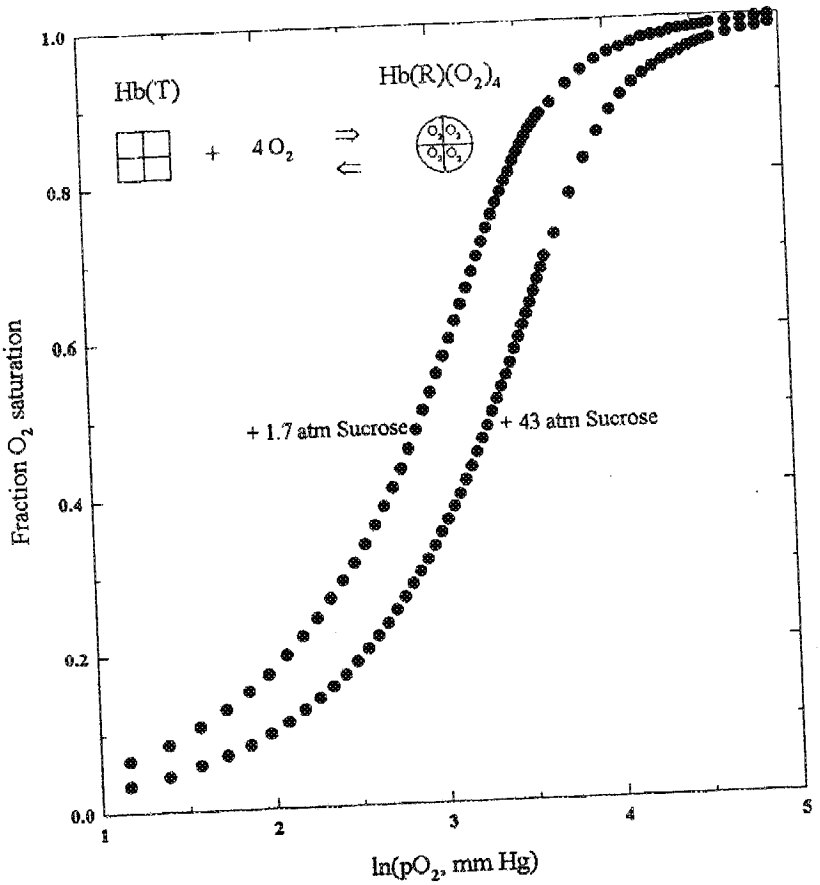
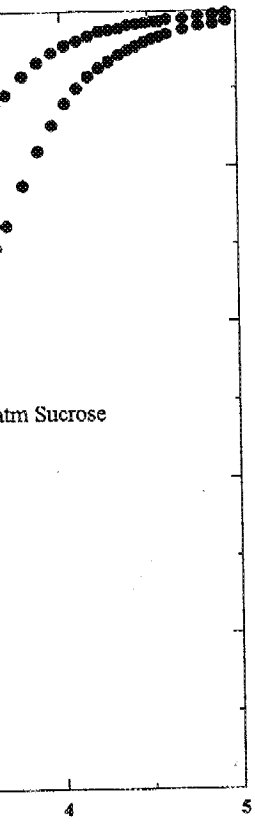


FIG. 9 Osmotic suppression of oxygen loading by tetrameric hemoglobin A.

(*b*) The increase in the relative amount of water $N_w\,[1 - (N_s/N_w)(n_w/n_s)]$ associated with hemoglobin as it loads oxygen:

$$\partial N_{O_2}/\partial \mu_w\,|_{\mu_{O_2}} = \partial N_{ew}/\partial \mu_{O_2}|_{\mu_w}\,. \tag{9}$$

We can assign these changes to the population of two forms of hemoglobin, or we can treat them as averages over all states, as written here. That is all that pure thermodynamics tells us (but we are allowed to use our brains to decide which kind of picture makes more sense). In either case, we see the combined acts of adding oxygen and adding solute as ways to work on hemoglobin. From the comparative action of each, it is possible to infer an excess or deficit of water or solute "bound" to the hemoglobin with an energetic implication every bit as meaningful as the binding of oxygen itself. The shift in loading curves, $\partial N_{O_2}/\partial \mu_w\,|_{\mu_{O_2}}$, integrated



americ hemoglobin A.

$[1 - (N_s/N_w)(n_w/n_s)]$ asso-

$$\Big|_{\mu_w}. \qquad (9)$$

vo forms of hemoglobin, or
en here. That is all that pure
brains to decide which kind
he combined acts of adding
obin. From the comparative
t of water or solute "bound"
ry bit as meaningful as the
$\partial N_{O_2}/\partial \mu_w \,|_{\mu_{O_2}}$, integrated

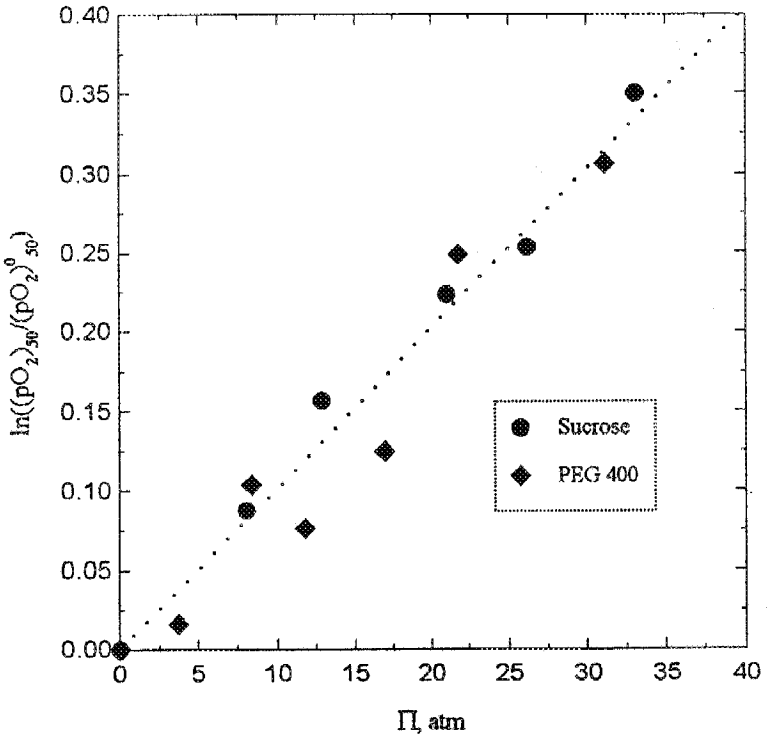


FIG. 10 Osmotic stress-induced shift in $p_{50}(O_2)$, the oxygen pressure required to half-load hemoglobin. Equivalent effects of two different osmotic agents.

over the full range of oxygen pressures, gives

$$N_{ew}^{oxy} - N_{ew}^{deoxy} = \int_{\mu_{O_2}=0}^{\mu_{O_2}=\text{“}\infty\text{”}} \frac{\partial N_{ew}}{\partial \mu_{O_2}}\bigg|_{\mu_w} d\mu_{O_2}$$

$$= \int_{\mu_{O_2}=0}^{\mu_{O_2}=\text{“}\infty\text{”}} \frac{\partial N_{O_2}}{\partial \mu_w}\bigg|_{\mu_{O_2}} d\mu_{O_2} \sim 65 \text{ waters}. \qquad (10)$$

The same number emerges from the shift in $(pO_2)_{50}$, the O_2 pressure at which hemoglobin is 50% loaded with oxygen. In the spirit of allostery (Wyman and Gill, 1990), we imagine a step-like addition Δn_{O_2} of four oxygen molecules at this half-way point $(pO_2)_{50}$ (Fig. 10). The change in $d\mu_{O_2}^{50} = d\left[kT \ln (p_{O_2}^{50})\right]$ with change $d\mu_w = -\bar{v}_w \, d\Pi$ in water activity looks like a Clausius–Clapeyron equation,

$$\frac{d\mu_{O_2}^{50}}{d\mu_w} = \frac{\Delta n_w}{\Delta n_{O_2}}, \qquad (11)$$

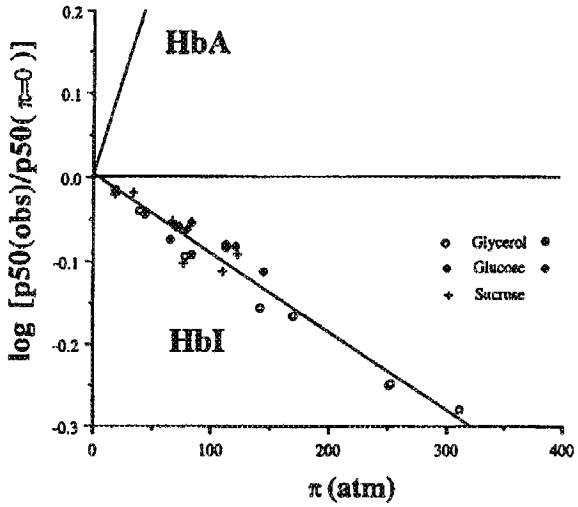


FIG. 11 Osmotic stress aids loading of oxygen by HbI, a dimeric form of hemoglobin whose response is dominated by a decrease in the size of the solute-excluding cavity upon oxygenation. (From Royer *et al.*, 1996.)

The measured slope $d\mu_{O_2}^{50}/d\mu_w$ and the known Δn_{O_2} give the change in excess waters,

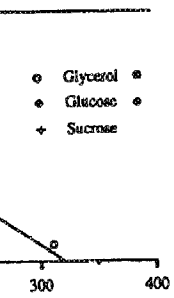
$$\Delta n_w = \frac{d\mu_{O_2}^{50}}{d\mu_w}\Delta n_{O_2}. \tag{12}$$

Further analysis suggests that this gain in waters with oxygenation is a combination of two kinds of events. Hemoglobin has a cavity that is *smaller* in the oxy form; if osmotic sensitivity were due to this cavity, the oxy form would have fewer rather than more waters. Royer *et al.* (1996) have examined the osmotic sensitivity of a dimeric HbI form of hemoglobin that preserves this cavity but does not show any of the hydration changes that go with changes in tetrameric packing (Fig. 11). They nicely confirm that HbI loads oxygen more easily when it is subjected to osmotic stress. There is a decrease of ~6.2 waters per dimer upon oxygenation.

The net change of ~65 waters for the tetramer is likely due to a positive term from increased surfaces, as in the BSA immersion measurements, offset by a cavity term, similar to the solute exclusion seen in channels.

IV. Intermolecular Forces: Forces of Solvation

From the perspective of work on single molecules, it is almost too easy to proceed to the idea of doing work on configurations of molecules from which one



c form of hemoglobin whose response
avity upon oxygenation. (From Royer

n_{O_2} give the change in excess

(12)

s with oxygenation is a combi-
avity that is *smaller* in the oxy
the oxy form would have fewer
xamined the osmotic sensitivity
es this cavity but does not show
in tetrameric packing (Fig. 11).
easily when it is subjected to
per dimer upon oxygenation.
is likely due to a positive term
neasurements, offset by a cavity
els.

olvation

es, it is almost too easy to pro-
s of molecules from which one

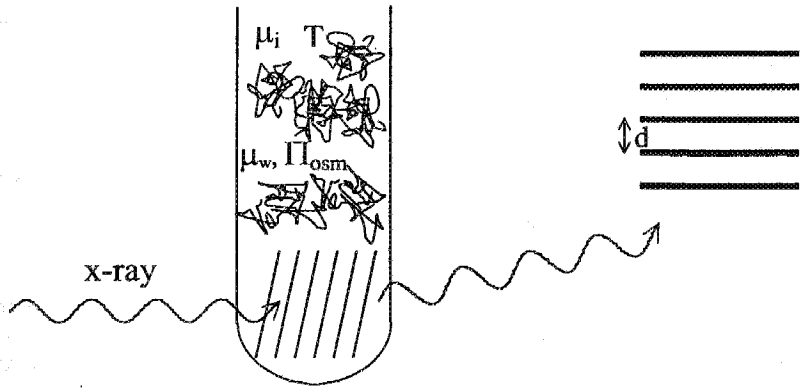


FIG. 12 Scheme of an array of macromolecules under stress of excluded polymer. The aggregate acts as a single unit in a reservoir of solutes i and water w of varied chemical potential μ_i and μ_w or $\Pi_{osmotic}$.

particular solute is completely excluded. Large polymers cannot enter the small spaces between molecules in the lamellar arrays of membranes, the hexagonal arrays of DNA, or any of a great number of macromolecular phases.

Here, the system observed is the array as a whole rather than a single molecule (Fig. 12). It responds to the varied stress of excluded polymers (as opposed to small solutes), to the osmotic stress of competition for water, as well as to all conditions in the bathing reservoir. The change in chemical potential of water is singled out for special consideration as the operative parameter for force measurement. A change $d\mu_w$ equals a change $d\Pi_{\text{osmotic}}$ except for a difference in sign convention and for the factor of molecular (or molar) volume of water $\bar{v}_w$,

$$d\mu_w = -\bar{v}_w\, d\Pi_{\text{osmotic}},\ \bar{v}_w\, N_w = V_w, \tag{13}$$

where V_w is the total volume of water in the array, and N_w is the number of molecules or moles of water:

$$dG\,(T,\ p,\ \mu_w,\ \{\mu_i\}\,,..) = -SdT + Vdp + V_w\, d\Pi_{\text{osmotic}} - \sum_i N_i\, d\mu_i. \tag{14}$$

X-ray scattering gives the spacing and the arrangement in the array as a function of the forces exerted by external polymer as well as of the chemical potentials of all species that can exchange between the array and the sample. Usually the measured spacings d can be immediately connected with the volume of aqueous solution, which is mostly the volume of water. The applied osmotic stress Π_{osmotic} coupled with d can often be interpreted in terms of the intermolecular force with which the array fights back against the compression of stress (Parsegian *et al.*, 1986). The forces that have been measured have been summarized in several places (e.g., Leikin *et al.*, 1993; Podgornik *et al.*, 1998).

The pressure or force per area between bilayers of lipids such as egg phosphatidylcholine bilayers revealed an exponentially varying pressure vs distance (see Peter Rand, this volume). This exponentially varying repulsion can span four or five decades of pressure over a separation from ~3 to ~20 Å, with a decay distance ~3 Å and a strength that reaches the magnitude of 1000 atmospheres. Measurements between DNA double helices (Podgornik *et al.*, 1998) and between charged and uncharged polysaccharide molecules (Rau and Parsegian, 1990) in ordered arrays show similar repulsive forces. Again we have a stubborn ~3-Å exponential, with a coefficient that changes with the kind of counterion, over a range of 4 to 10 Å between molecular surfaces. It does not seem to matter whether the molecular surfaces are neutral, charged, or zwitterionic. Similar decays can be seen in ~2 M salt solution and in distilled water (Leikin *et al.*, 1993). The commonality of exponential forces in so many different systems leads one to think in terms of H-bonding and water structuring. It appears as though a law of nature has been given us: *exponentially varying forces dominate over the important last 10 Å where molecules come into contact* (Fig. 13).

Proteins are more interesting. Structured molecular surfaces such as those of collagen also interact exponentially but with a decay constant that combines the ~3-Å λ_{hyd} of water with the natural lengths of the structured surface (Leikin *et al.*, 1995). For example, because of structural contributions, collagen triple helices show an exponential repulsion with a decay length of $\lambda_{expII} = 0.65$ Å (Fig. 14), a distance that can be rationalized as a coupling between natural lengths of water and of collagen (Leikin *et al.*, 1995). The disturbance of water near a protein has now been seen in X-ray diffraction by insulin (Badger and Caspar, 1991; Yu and Caspar, 1998).

And a new idea has emerged. Variation in the ordering of water near large molecules goes with spatial variation in the ability of that water to dissolve salts and neutral solutes. Recent work on polysaccharides and on DNA suggests that solutes often distribute near macromolecules with an exponential variation in concentration $\sim e^{-r/\lambda}$ whose λ is similar to that seen with hydration forces. The salt concentration gradient generated by the hydroxypropylcellulose (HPC) surface itself appears to be an exponential function with the same 3- to 4-Å decay length as the hydration force (Bonnet-Gonnet *et al.*, 2000). The implication is that salt interacts with HPC through hydration or water structuring forces. Having seen this partitioning of NaCl in progressively ordered water, we are now in a position to ask how solutes redistribute between all kinds of molecular surfaces including those of proteins. Such redistributions themselves create forces between molecules (Appendix 5).

Again, we are working with thermodynamic forces, spatial derivatives of free energy. The temperature dependence of forces automatically measures entropy vs separation. When such a measurement has been possible on cleanly ordered systems, the entropy that emerges is itself exponential with an ~3-Å exponential

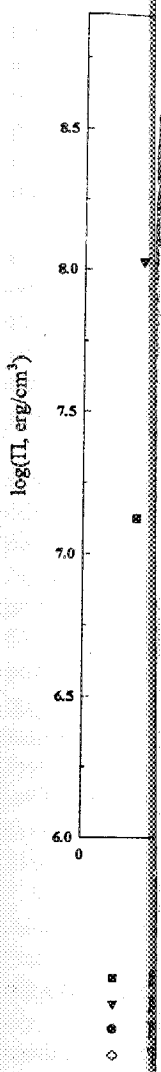


FIG. 13 Stress v
surfaces. (From L

decay (Leikin
higher temper
increases as th
possibility for
ordered water

of lipids such as egg phos-
varying pressure vs distance
rying repulsion can span four
~3 to ~20 Å, with a decay
gnitude of 1000 atmospheres.
rnik *et al.*, 1998) and between
(Rau and Parsegian, 1990) in
in we have a stubborn ~3-Å
he kind of counterion, over a
es not seem to matter whether
itterionic. Similar decays can
ter (Leikin *et al.*, 1993). The
rent systems leads one to think
ears as though a law of nature
minate over the important last

ular surfaces such as those of
ay constant that combines the
tructured surface (Leikin *et al.*,
utions, collagen triple helices
n of $\lambda_{exptl} = 0.65$ Å (Fig. 14),
tween natural lengths of water
nce of water near a protein has
dger and Caspar, 1991; Yu and

e ordering of water near large
y of that water to dissolve salts
des and on DNA suggests that
an exponential variation in con-
with hydration forces. The salt
ypropylcellulose (HPC) surface
he same 3- to 4-Å decay length
00). The implication is that salt
structuring forces. Having seen
water, we are now in a position
of molecular surfaces including
create forces between molecules

orces, spatial derivatives of free
automatically measures entropy
een possible on cleanly ordered
ential with an ~3-Å exponential

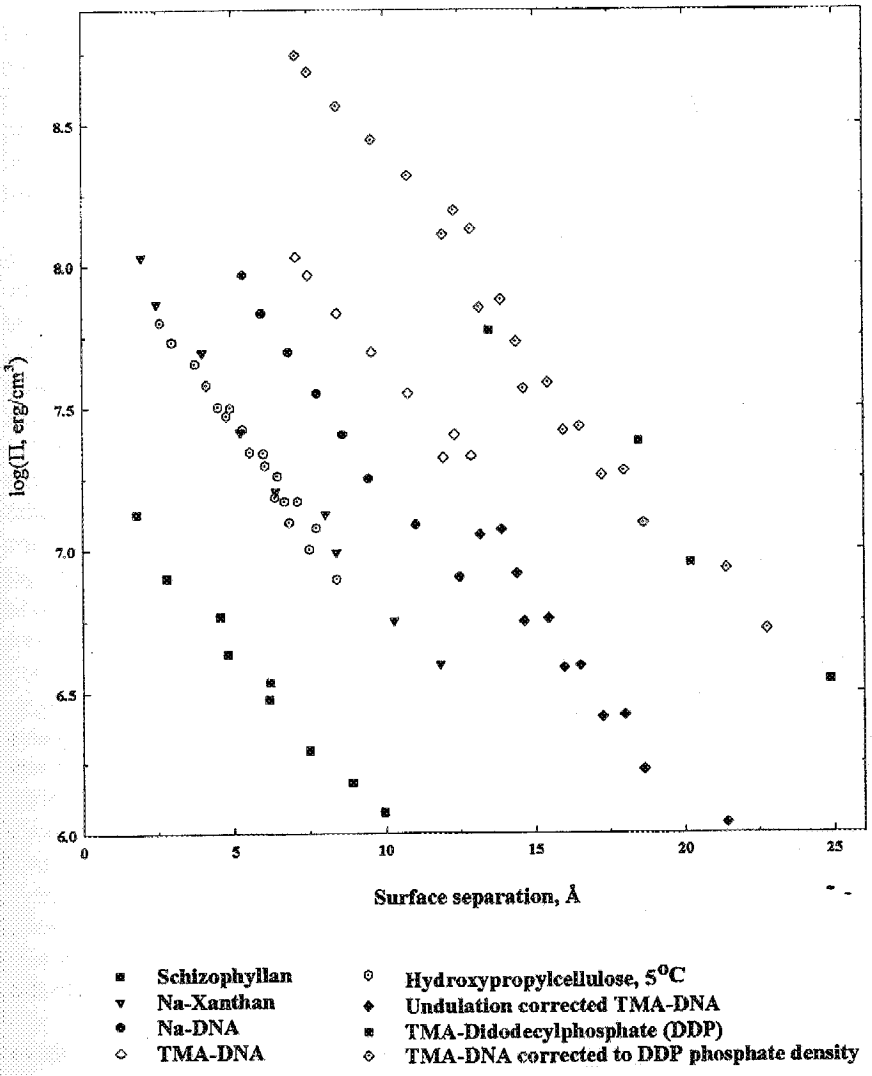


FIG. 13 Stress vs separation between macromolecules or membranes at different distances between surfaces. (From Leikin *et al.*, 1993.)

decay (Leikin *et al.*, 1991). For example, it is easier to push together HPC at higher temperatures; the entropy of the total system—HPC + bathing solution—increases as the molecules are brought together (Bonnet-Gonnet *et al.*, 2000). One possibility for the source of this entropy is the progressive release of increasingly ordered water as macromolecules are pushed together.

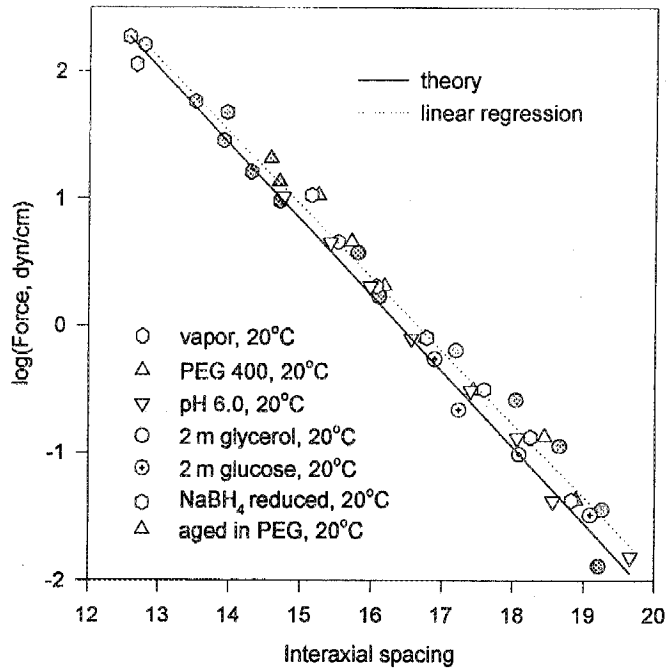


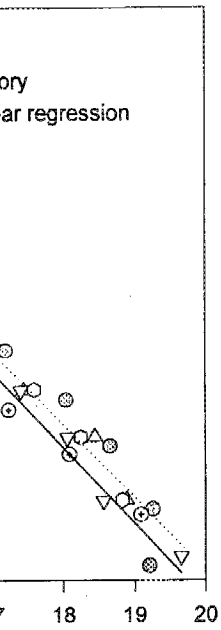
FIG. 14 Exponential force between collagen triple helices. In this case, the force extends over 5 decades. It is robust in the presence of a wide variety of solutes and conditions. The theory used to rationalize this exponential force combines the natural lengths of the water hydration force seen between unstructured surfaces and the natural length in the repeat period of the collagen triple helix.

V. Unity

What kinds of lessons emerge from looking at the ways proteins respond to the activity of water?

From the start, it is clear that the same kinds of thermodynamic forces drive water transport over all length scales. More difficult to see is that a protein is large enough that its attraction or repulsion of solutes can be spoken of in the same terms as attraction and repulsion of solutes by a macroscopic surface. This macroscopic-equivalence feature immediately liberates us to use classical concepts from the thermodynamics of surfaces. It also disciplines us to learn how to make the conceptual transition between smooth surfaces and molecular bodies.

The mind craves the certainty of structure, eschews disorder. Yet disorderly, continuously changing, diffusive molecules surround proteins and surfaces. The protein "surface" itself is rough and disorderly on the scale of molecular structure and atomic motion. We often speak of protein solvation in the geometric sense,



In this case, the force extends over
utes and conditions. The theory used
ths of the water hydration force seen
at period of the collagen triple helix.

ways proteins respond to the

f thermodynamic forces drive
cult to see is that a protein is
lutes can be spoken of in the
y a macroscopic surface. This
ates us to use classical concepts
plines us to learn how to make
and molecular bodies.
hews disorder. Yet disorderly,
nd proteins and surfaces. The
he scale of molecular structure
vation in the geometric sense,

for example, the numbers of water molecules that could coat the solvent-accessible area of a crystalline protein structure. In contrast, thermodynamics begs most structural questions, but it allows us to think about the cost of creation. It gives us procedures to ask, What composes this collection of waters and solutes that dwell in the protein's space? How do solutes and solvent drive proteins between different functionally distinct states?

In principle, a protein is in touch with everything around it. In practice, the protein's sensitivity to the price, or chemical potential, of all species depends only on the number of each component compared to the number that would be in that space were the protein not there. The cost of putting a protein into the solution or the cost of moving the protein between different forms depends on time-average, relative numbers of solutes, and solvent. Singular focus on structure misses essential mixing and averaging.

When it comes to numbers, at 55 M, water is the acknowledged winner. By any measure, there are more water molecules around a protein than any of the other small molecules in the solution. Numerical supremacy matters. The energetic consequence of a bathing species is proportional to its numerical excess or deficit (again compared with what would be there if there were no protein). That excess or deficit, multiplied by a change in chemical potential, gives the change in protein free energy. From this numbers perspective, too many labs still neglect the motive powers of water. With careful attention rightfully paid to the chemical potential of protons, ligands, small solutes, salts, etc., there is still relatively little attention given to the chemical potential of the most numerous "ligand," water. By intellectualizing first rather than looking at water effects in the same spirit as responses to pH or temperature or ligands, people have created unnecessary biases and headaches. Computation and discussion based on rigid structures miss the averaging that a living protein uses to respond to water and solute activity.

Several examples in this chapter and elsewhere in this book show that we can think profitably of water effects as well as of the effects of other solutes. Given that we vary the chemical potential of water by adding solutes, are we playing language games? Certainly there is some freedom in choice of language. We can say, "There are 55-glycerol-excluding waters" and speak of the effect of these waters in the context of free energy of solution. We can also say "There are 10 glycerols fewer than the number of glycerols that would be around the protein if it were in a freely mixing 1 molal glycerol solution." The 55 waters remains a pleasingly constant number for proteins in 0 to 1 molal solution. That number is the constant coefficient for sensitivity of protein immersion to water activity over that same range of added solute.

In any case, an ability to focus alternately on solute or solvent as the active agent presents an opportunity. Though it requires linguistic agility to speak alternatively in terms of solute or solvent action, we can escape the mental confines of either language. With this freedom to speak in the most general terms about protein–water interactions, we can go beyond thinking in terms of rigid models. We can

see the dynamics of flexible proteins in solution not only in terms of energetics of contact with water but also in terms of a protein's ongoing competition for the water in the course its activities. We might see, too, how the energies that have been revealed in hydration forces can drive protein function.

Glossary

μ_i chemical potential of species i

n_i total number of molecules of species i

w water, species #1 in 1–2–3 notation

p protein, species #2

s small solute, species #3

$\bar{v}_w$ molecular (or molar) volume of water

Π, osmotic pressure or stress, sometimes written Π_{osmotic} for emphasis; $\bar{v}_w\, d\Pi = -n_w\, d\mu_w$.

$m_3 \equiv m_s$, molality of small solutes, moles per kilogram of water, proportional to $n_3/n_1 = n_s/n_w$.

N_{cw}, number of waters in excess of what there would be were there no preferential interaction of solute and water with protein. $N_{\text{cw}} \equiv 0$ for no net redistribution of solute and water. ΔN_{cw} or $\Delta N_{\text{cw}}^{\text{ab}} = N_{\text{cw}}^{\text{b}} - N_{\text{cw}}^{\text{a}}$, difference in the number of excess waters for two different states a, b of a protein.

N_{es}, ΔN_{es} or $\Delta N_{\text{cs}}^{\text{ab}} = N_{\text{es}}^{\text{b}} - N_{\text{es}}^{\text{a}}$, the same excesses and differences for a small solute s.

$V_{\text{ew}} = N_{\text{ew}}\, \bar{v}_w$, volume corresponding to excess number of water molecules;

$\Delta V_{\text{ew}} = (V_{\text{ew}}^{\text{b}} - V_{\text{ew}}^{\text{a}}) = N_{\text{cw}}^{\text{ab}} \bar{v}_w$, difference in volume of excess or small-solute-inaccessible water between forms a and b.

$\Delta G_{\text{ab}} = G_b - G_a$, free energy difference or work necessary for an individual macromolecule to go between the two forms or states, a, b.

$K_{\text{eq}} = e^{-\Delta G_{\text{ab}}/kT} = \frac{[\text{b}]}{[\text{a}]} = \frac{\Pr(\text{b})}{\Pr(\text{a})}$, concentration ratio or probability ratio of b and a.

Also see Appendix 3.

Appendix 1

How to Connect the Change in Solution Osmolality by Immersion of a Protein with the Change in the Work of Protein Immersion as a Function of Solution Osmolality

Think numbers and number densities. Think of a solution consisting of n_w molecules of water and n_s molecules of a small solute s (glycerol, trehalose, proline, betaine, tetramethylamine N-oxide (TMAO), K^+ glutamate). Its osmolality

is proportional to the ratio n_s/n_w. (Though osmolality is measured as moles solute per kilogram of mass solvent, it is essentially a ratio of numbers.) Next imagine that immersion of one protein molecule, the first protein added, withdraws N_{ew} water molecules from the solution. The operational reality of N_{ew} will be spelled out below. For now, imagine s being repelled from the protein relative to water or, the competitive equivalent, water attracted to protein relative to solute s.

After the protein removes N_{ew} waters from the original solution, there are $(n_w - N_{ew})$ waters available to mix with the n_s solutes. The new osmolality is proportional $n_s/(n_w - N_{ew})$; the change in osmolality is proportional to $\frac{n_s}{n_w - N_{ew}} - \frac{n_s}{n_w}$. In an effectively infinite bath, $n_w >>> N_{ew}$, the change in osmolality of the remaining solution, after the first protein's immersion, is $\frac{n_s}{n_w - N_{ew}} - \frac{n_s}{n_w} = \frac{n_s}{n_w}\frac{N_{ew}}{n_w}$. Two protein molecules will withdraw $2N_{ew}$ waters and change osmolality by $\frac{n_s}{n_w}\frac{2N_{ew}}{n_w}$. As long as the solution is dilute in the number of proteins, n_p ("dilute" defined as $n_p N_{ew} <<< n_w$ and n_s), added proteins will withdraw $n_p N_{ew}$ waters and the solution osmolality will go as $\frac{n_s}{n_w}\frac{n_w}{n_w}n_p$, i.e., proportional to the number density of proteins.

More strictly speaking, we should say that the result of adding proteins is to create solutions whose vapor pressure, or chemical potential of water, is that which is measured for a solution of s and w consisting of n_s small-solute molecules and $n_w - n_p N_{ew}$ water molecules. It is this vapor pressure that is measured in the exacting Courtenay *et al.* (2000) measurements.

The osmolar consequence of added protein is linear in the original osmolality n_s/n_w. It is also reportedly, almost surprisingly, effectively linear in the number of added proteins n_p, or equivalently, linear in n_p/n_w. In other words, these two proportionalities are what is seen in $\frac{n_s}{n_w}\frac{N_{ew}}{n_w}n_p = \frac{n_s}{n_w}\frac{n_p}{n_w}N_{ew}$. The linear master plot, the effect on osmolality of adding a given amount of protein, vs original osmolality n_s/n_w immediately gives the numbers of excluding waters N_{ew}. Its slope is $\frac{n_p}{n_w}N_{ew}$; the data are for a given, known n_p/n_w. Voila N_{ew}!

Appendix 2

What Does It Mean to a Protein That It Sequesters N_{ew} Excess Waters When Immersed into the n_s, n_w Solution?

For learning purposes only, just for the next few paragraphs, imagine a spherical protein of radius a plunged into an effectively infinite bath. Its push/pull on waters and solutes creates local number densities $\nu_s(r)$ and $\nu_w(r)$. At large enough radial position r, far from the protein, these number densities go to those of the bathing solution. But let us not restrict ourselves *a priori* to saying how far is "far." In principle, the domain of perturbation can extend indefinitely, and we can think of the protein and an absurdly infinite number of neighbors, $\mathbf{N}_w$ and $\mathbf{N}_s$ (for which I

have chosen an absurdly shaped $\mathbb{N}$),

$$\mathbb{N}_w = \int_a^\infty \nu_w(r) 4\pi r^2 dr,\ \mathbb{N}_s = \int_a^\infty \nu_s(r) 4\pi r^2 dr. \tag{A2.1}$$

From the protein's point of view, not only is it situated among an infinite number of s's and w's, but also these s's and w's come at a price. Recall that the chemical potential μ_s or μ_w is the energy it takes to add a molecule of s or a molecule of w to the original bathing solution. An increase in μ_s means it takes more work to add a molecule of s *to* the bathing solution; an increase in μ_w, more work to add a molecule of water to the bathing solution.

If we think of the protein and its surrounding molecules as an entity, if we think of these neighbors as belonging to the dissolved protein, then raising the chemical potential μ_s or μ_w in which the protein is bathed makes it easier for the protein to acquire s or w *from* the bath.

It is as though the protein "owns" $\mathbb{N}_w$ waters and $\mathbb{N}_s$ solutes. If μ_w goes up, it is harder to transfer w to the bathing solution, easier to move w to the protein. It is as though the protein has paid less for its collection of magnitude $\mathbb{N}_w$. Ditto with an increase of μ_s and the protein's possession of $\mathbb{N}_s$ solutes. The act of protein immersion would change by an amount $-\mathbb{N}_w\, d\mu_w$ and $-\mathbb{N}_s\, d\mu_s$:

$$dG^{\text{immersion}}_{\text{protein}} = -\mathbb{N}_w d\mu_w - \mathbb{N}_s d\mu_s. \tag{A2.2}$$

The reason for the minus sign should be obvious. The greater the number $\mathbb{N}_s$ or $\mathbb{N}_w$ in the domain of the protein, the less the free energy of collecting those w's and s's with an increase in μ_s or μ_w in the surrounding solution.

Now we can eliminate the absurdity of infinitely many $\mathbb{N}_w$ waters and $\mathbb{N}_s$ solutes. Think of two different immersions into two different solutions whose chemical potentials differ slightly, by $d\mu_w$ and $d\mu_s$. From the Gibbs–Duhem relation, any change in chemical potentials suffers the constraint

$$n_w d\mu_w + n_s d\mu_s = 0. \tag{A2.3}$$

Chemical potentials cannot change independently.[3] We can say

$$d\mu_w = -(n_s/n_w) d\mu_s \text{ or } d\mu_s = -(n_w/n_s) d\mu_w \tag{A2.4}$$

to allow us to say

$$dG^{\text{immersion}}_{\text{protein}} = -[\mathbb{N}_w - (n_w/n_s)\mathbb{N}_s] d\mu_w$$

or

$$dG^{\text{immersion}}_{\text{protein}} = -[\mathbb{N}_s - (n_s/n_w)\mathbb{N}_w] d\mu_s. \tag{A2.5}$$

The energy of protein immersion changes with chemical potential of the bath only to the extent that the ratio of s's to w's, $\mathbb{N}_s/\mathbb{N}_w$, differs from their ratio (n_s/n_w)

[3]Recall that, for $n_s << n_w$, this $n_w d\mu_w + n_s d\mu_s = 0$ condition gives the van't Hoff law, osmotic pressure due to s added to w: $d\mu_s = d(kT \ln(n_s/n_w)$, $d\mu_w = -\nu_w\, d\,\Pi_{\text{osmotic}}$, where ν_w is the volume of a water molecule.

in the original solution. If the protein did nothing to the distribution of s and w, then there would be no consequence of changing the amount of s compared to w in the bathing solution.

- If the protein accumulates s's relative to w's, if $\mathbf{N}_s/\mathbf{N}_w > n_s/n_w$, then increased μ_s (or decreased μ_w) lowers the work to put the protein into the solution.
- If the protein accumulates w's relative to s's, if $\mathbf{N}_s/\mathbf{N}_w < n_s/n_w$, then increased μ_s (decreased μ_w) increases the work of putting the protein into the solution. With more s in the bath relative to w, it is harder to get the desired w's out of the n_s, n_w bath.

Return now to the distributions $\nu_s(r)$ and $\nu_w(r)$ where we had

$$dG^{\text{immersion}}_{\text{protein}} = -\mathbf{N}_w d\mu_w - \mathbf{N}_s d\mu_s = \int_a^\infty [-\nu_w(r)d\mu_w - \nu_s(r)d\mu_s]\,4\pi r^2 dr, \tag{A2.6}$$

but add the necessary constraint between $d\mu_w$ and $d\mu_s$,

$$dG^{\text{immersion}}_{\text{protein}} = -\left\{\int_a^\infty [\nu_w(r) - \nu_s(r)\,(n_w/n_s)]\,4\pi r^2 dr\right\} d\mu_w \equiv -N_{\text{ew}} d\mu_w \tag{A2.7}$$

or, equivalently,

$$dG^{\text{immersion}}_{\text{protein}} = -\left\{\int_a^\infty [\nu_s(r) - \nu_w(r)\,(n_s/n_w)]\,4\pi r^2 dr\right\} d\mu_s \equiv -N_{\text{es}} d\mu_s. \tag{A2.8}$$

Now the numbers multiplying the $d\mu_w$ and $d\mu_s$ are reasonable quantities. Despite the integration to "infinity," N_{ew} and N_{es} are finite numbers. The local number density $\nu_w(r)$ is reduced by a factor $[1 - (\nu_s(r)/\nu_w(r))(n_w/n_s)]$ that goes to zero at large enough distances from the protein (where $\nu_w(r)/\nu_s(r) \to (n_w/n_s)$); similarly $\nu_s(r)$.

The excess numbers of waters and solutes are then

$$N_{\text{ew}} = \int_a^\infty [\nu_w(r) - \nu_s(r)\,(n_w/n_s)]\,4\pi r^2 dr \tag{A2.9a}$$

and

$$N_{\text{es}} = \int_a^\infty [\nu_s(r) - \nu_w(r)\,(n_s/n_w)]\,4\pi r^2 dr. \tag{A2.9b}$$

Departing from the fantasy of a spherically symmetric protein, we may write these excesses as general integrals over the entire volume. Speak of a vector position $\mathbf{r}$ and integrals over all locations:

$$N_{\text{ew}} = \int_{\text{Volume}} [\nu_w(\mathbf{r}) - \nu_s(\mathbf{r})\,(n_w/n_s)]\,d\mathbf{r},$$

$$N_{\text{es}} = \int_{\text{Volume}} [\nu_s(\mathbf{r}) - \nu_w(\mathbf{r})\,(n_s/n_w)]\,d\mathbf{r}. \tag{A2.10}$$

Because

$$dG_{\text{protein}}^{\text{immersion}} = -N_{\text{ew}} d\mu_w = -N_{\text{es}} d\mu_s \tag{A2.11}$$

and

$$n_w d\mu_w + n_s d\mu_s = 0, \tag{A2.3}$$

we can write

$$N_{\text{ew}} = -N_{\text{es}}(n_w/n_s) \text{ or } N_{\text{es}} = -N_{\text{ew}}(n_s/n_w). \tag{A2.12}$$

An excess in one species is equivalent to a deficit in the other, evaluated in exact proportion to the n_s/n_w ratio in the bathing solution.

Appendix 3

1–2–3 and w–p–s, Translations

Notation can be a problem. Solute–water–protein interactions are frequently described in a language of "preferential interaction coefficients." These coefficients are based on the same thermodynamics as those of osmotic stress analyses that directly look at the effects of water on proteins and other macromolecules. Because of occasional confusion in the literature (Timasheff, 1998), it is worth pointing out explicitly the connection between the two kinds of notation (Parsegian *et al.*, 2000). The single most difficult feature of preferential interaction coefficients is to remember that

1 = water = w
2 = protein = p
3 = small solute or "cosolvent" = s

For a three-component solution, the Gibbs–Duhem relation is

$$n_1 d\mu_1 + n_2 d\mu_2 + n_3 d\mu_3 = 0 \text{ or } n_w d\mu_w + n_p d\mu_p + n_s d\mu_s = 0, \tag{A3.1}$$

where, by definition, the n_i are the total numbers of molecules of each component. To create the language of osmolalities in the 1–2–3 notation, the numbers of each species are divided by an amount of substance 1 (water) to create the requisite "per 1 kg of water[4]."

$$m_1 d\mu_1 + m_2 d\mu_2 + m_3 d\mu_3 = 0. \tag{A3.2}$$

Preferential interaction coefficients are defined in terms of changes that take place under imposed constant-chemical-potential conditions. For a constant water

[4]But for the historical convention of units "per kg water" rather than "per water molecule", m_1 would be a number ratio, $n_1/n_1 = n_w/n_w = 1$; $m_2 = n_2/n_1 = n_p/n_w$; $m_3 = n_3/n_1 = n_s/n_w$.

$_{es}d\mu_s$ (A2.11)

(A2.3)

$_v(n_s/n_w)$. (A2.12)

he other, evaluated in exact

eractions are frequently de-
ficients." These coefficients
notic stress analyses that di-
r macromolecules. Because
1998), it is worth pointing
f notation (Parsegian *et al.*,
l interaction coefficients is

lation is

$_p + n_s d\mu_s = 0,$ (A3.1)

lecules of each component.
tation, the numbers of each
) to create the requisite "per

$= 0.$ (A3.2)

terms of changes that take
litions. For a constant water

er than "per water molecule", m_1
n_w; $m_3 = n_3/n_1 = n_s/n_w$.

chemical potential, define

$$\Gamma_{\mu_1} \equiv \left.\frac{\partial m_3}{\partial m_2}\right|_{T,p,\mu_1}. \tag{A3.3}$$

The idea is, "If I add protein '2' to a solution while holding constant the chemical potential of water '1,' how does the molality of '3' (small solute molecules) change?" Conceptually, the equation assumes that the change in the number of small solutes can be measured while the water chemical potential is held fixed. At least in principle, one can imagine equilibrium dialysis between a protein-containing solution and a small-solute solution. In that spirit, it is possible to speak of the small-solute molality $m_3{}^{\Delta}$ on the two-component side of the membrane under the constraint that the water chemical potential is the same on both sides of the membrane. The difference in small-solute population because of the presence of protein "2" is then a key to the "iso-osmolal" preferential interaction coefficient,

$$\Gamma_{\mu_1} \equiv \left.\frac{\partial m_3}{\partial m_2}\right|_{T,p,\mu_1} \cong \frac{m_3 - m_3{}^{\Delta}}{m_2}. \tag{A3.4}$$

A similar coefficient can be constructed using the small-solute molality m_3^* under the constraint of constant chemical potential of small solute "3",

$$\Gamma_{\mu_3} \equiv \left.\frac{\partial m_3}{\partial m_2}\right|_{T,p,\mu_3} \cong \frac{m_3 - m_3^*}{m_2}. \tag{A3.5}$$

And, of course, there must be a derivative for the practical case of immersion into a reservoir, or between a three-component 1–2–3 solution separated by a dialysis membrane from a 1–3 solution, wherein both μ_1 and μ_3 are held fixed by virtue of an effectively infinite bath:

$$\Gamma_{\mu_1\mu_3} \equiv \left.\frac{\partial m_3}{\partial m_2}\right|_{T,p,\mu_1,\mu_3} \cong \frac{m_3 - m_3'}{m_2}. \tag{A3.6}$$

Imagine a protein that repels solute 3 compared to its repulsion of water 1 (or attracts water more than it does small solute). Add a protein to one side of a dialysis membrane dividing two solutions: m_3 the amount of solute 3 per amount of water 1 on the protein side becomes less than m_3^* or $m_3{}^{\Delta}$ or m'_3 the amount of 3 per amount of 1 on the protein-free side. In fact, in a two-component solution, a constant chemical potential of one component automatically enforces a constant chemical potential of the other, $m_1 d\mu_1 + m_3 d\mu_3 = 0$!

The last preferential interaction coefficient expression pleasingly transforms to the N_{ew} expressions used in Appendix 1. The protein-free side of the dialysis bag is, in the actual experiments, the infinite bath whose reservoir-power keeps fixed the chemical potentials of solute and water despite the intrusion of the protein. Better, recognize that in well-controlled lab experiments, chemical potentials are enforced

in the form of stipulated pH, salts, temperature, pressure, etc. Those constraints are the *de facto* reservoir.

In the language of Appendix 1, m^*_3 or $m_3{}^{\Delta}$ or m'_3 equals $\frac{n_s}{n_w}$ without the protein and $m_3 = \frac{n_s}{n_w - n_p N_{ew}}$ in the three-component solution.

The requisite derivative of immersion, tantamount to the preferential interaction coefficient in the dilute limit, is

$$\left.\frac{\partial m_3}{\partial m_2}\right|_{T,p,n_1,n_3} = \partial\left(\frac{n_s}{n_w - n_p N_{ew}}\right) \Big/ \partial\left(\frac{n_p}{n_w}\right)\Bigg|_{T,p,n_1,n_3}$$

$$= \frac{n_s}{\left(n_w - n_p N_{ew}\right)^2} n_w N_{ew}. \tag{A3.6a}$$

Experimentally, in the dilute-protein, linear limit of the Courtenay *et al.* (2000) experiments, where $n_w >>> n_p N_{ew}$, this preferential coefficient reduces to

$$\left.\frac{\partial m_3}{\partial m_2}\right|_{T,p,n_1,n_3} = \frac{n_s}{n_w} N_{ew}. \tag{A3.6b}$$

The change in $\left.\frac{\partial m_3}{\partial m_2}\right|_{T,p,n_1,n_3}$ with original osmolality $\frac{n_s}{n_w}$ immediately gives N_{ew}. There is thus happy equivalence in languages.

Appendix 4

An Example of Water- and Solute-Counting

To see how a spatially varying distribution of solute and solvent density converts into excess quantities, think in a language where there is an energy $E_s(\mathbf{r})$ to put a molecule s at a position $\mathbf{r}$ relative to an energy of the same kind of molecule s at an infinite distance from the protein. As a consequence of this interaction, there are spatially varying number densities $\nu_s(\mathbf{r})$ of solute and $\nu_w(\mathbf{r})$ of water. Specifically, there are $\nu_s(\mathbf{r})$ solutes per unit volume and $\nu_w(\mathbf{r})$ waters per unit volume at a position $\mathbf{r}$. If the volume of a solute is $\bar{v}_s$ and volume of a water is $\bar{v}_w$, then by definition of number densities (per unit volume) and molecular volumes,

$$\nu_w(x)\bar{v}_w + \nu_s(x)\bar{v}_s = 1. \tag{A4.1}$$

The Boltzmann density distribution in solute number density $\nu_s(\mathbf{r}) = \nu_s^{\infty} e^{-E_s(\mathbf{r})/kT}$, then automatically enforces a water density distribution

$$\nu_w(\mathbf{r}) = \left(1 - \nu_s(\mathbf{r})\bar{v}_s\right)/\bar{v}_w. \tag{A4.2}$$

Note the consistency with the total volume of a solution containing n_s solutes of volume $\bar{v}_s$ and n_w waters of volume $\bar{v}_w$ is $n_w\bar{v}_w + n_s\bar{v}_s$. The number density of solutes in the infinite bathing reservoir is $\nu_s^\infty = \frac{n_s}{n_w\bar{v}_w+n_s\bar{v}_s}$ and of waters $\nu_w^\infty = \frac{n_w}{n_w\bar{v}_w+n_s\bar{v}_s}$, so that $\nu_w^\infty\bar{v}_w + \nu_s^\infty\bar{v}_s = 1$.

For the excess number of solutes, recall (Appendix 2)

$$N_{\text{es}} = \int_{\text{Volume}} [\nu_s(\mathbf{r}) - \nu_w(\mathbf{r})\,(n_s/n_w)]\,d\mathbf{r}. \tag{A4.3}$$

Introducing $\nu_w(\mathbf{r}) = (1 - \nu_s(\mathbf{r})\bar{v}_s)\,/\bar{v}_w$ gives [5]

$$\nu_s(\mathbf{r}) - \nu_w(\mathbf{r})\,(n_s/n_w) = \left(e^{-E_s(\mathbf{r})/kT} - 1\right)(n_s/\bar{v}_w n_w)\,. \tag{A4.4}$$

Then

$$N_s = (n_s/\bar{v}_w n_w)\int_{\text{Volume}} \left(e^{-E_s(\mathbf{r})/kT} - 1\right) d\mathbf{r}. \tag{A4.5}$$

For simplicity in illustration, consider a flat surface. This is a one-dimensional situation wherein the excess number is really an excess number per unit area. We will use the symbol Γ_s to preserve the essential connection with the Gibbs adsorption isotherm. The total number N_s will then be Area x $\Gamma_s = A\Gamma_s$, where

$$N_{\text{es}} = \int_{x=0}^{\infty} [\nu_s(x) - \nu_w(x)(n_s/n_w)]dx = \int_{x=0}^{\infty} [\nu_s(x) - \nu_w(x)(\nu_s^\infty/\nu_w^\infty)]dx$$

$$= (n_s/\bar{v}_w n_w)A \int_{x=0}^{\infty} \left(e^{-E_s(x)/kT} - 1\right) dx. \tag{A4.6}$$

This is a flat surface that interacts with solute and water in such a way that there is an exponentially varying change in energy to place a solute molecule a distance x from the surface. $E_s(x) = E\,e^{-x/\lambda}$. For a weak interaction, $E \ll kT$, $e^{-E_s(x)/kT} \approx 1 - E_s(x)/kT$ so that

$$N_{\text{es}} = -(n_s/\bar{v}_w n_w)\,A(E/kT)\int_{x=0}^{\infty} e^{-x/\lambda}dx = -(n_s/\bar{v}_w n_w)\,A(E/kT)\lambda. \tag{A4.7}$$

For a dilute solution, $n_s \ll n_w$, $(n_s/\bar{v}_w n_w)$ is the number density of solute. If solutes are repelled relative to water, then $E > 0$. The solute deficit is the bulk solution concentration times a volume $A\lambda(E/kT)$. Note that this deficit is linear in solute concentration. At the same time, the excess N_{ew} of water is (Appendix 2)

$$N_{\text{ew}} = -N_{\text{es}}(n_w/n_s) = (n_w/n_s)(n_s/\bar{v}_w n_w)\,A(E/kT)\lambda$$

$$= (A\lambda/\bar{v}_w)(E/kT). \tag{A4.8}$$

This N_{ew} is independent of solute concentration.

[5] $\nu_s(\mathbf{r}) - \nu_w(\mathbf{r})\,(n_s/n_w) = \nu_s(\mathbf{r}) - \nu_w(\mathbf{r})(\nu_s^\infty/\nu_w^\infty) = \nu_s(\mathbf{r}) - \bar{v}_w\nu_w(\mathbf{r})(\nu_s^\infty/\bar{v}_w\nu_w^\infty) = \nu_s(\mathbf{r}) - (1 - \bar{v}_s\nu_s(\mathbf{r}))(\nu_s^\infty/\bar{v}_w\nu_w^\infty) = \nu_s(\mathbf{r})(1 + (\bar{v}_s\nu_s^\infty/\bar{v}_w\nu_w^\infty)) - (\nu_s^\infty/\bar{v}_w\nu_w^\infty) = e^{-E(\mathbf{r})/kT}(\nu_s^\infty/\bar{v}_w\nu_w^\infty) - (\nu_s^\infty/\bar{v}_w\nu_w^\infty) = (e^{-E(\mathbf{r})/kT} - 1)(\nu_s^\infty/\bar{v}_w\nu_w^\infty) = (e^{-E(\mathbf{r})/kT} - 1)(n_s/\bar{v}_w n_w)$.

Appendix 5

Pushing Proteins

What if two solute-excluding surfaces come near? Even as the free energy of one such surface changes with the concentration of excluded solute, so will the strength of interaction between two surfaces. For simplicity, consider an energy of exclusion that decays exponentially with distance from both surfaces. $E_s(x) = E\,e^{-x/\lambda}$ for an isolated surface (Appendix 4) becomes

$$E_s(x) = E\left(e^{-x/\lambda} + e^{-(D-x)/\lambda}\right) \tag{A5.1}$$

between two flat parallel surfaces a distance D apart. Now the exclusion of s between two surfaces of area A is

$$\begin{aligned} N_{es} &= \int_{x=0}^{D} [\nu_s(x) - \nu_w(x)(n_s/n_w)]dx \\ &= (n_s/\bar{v}_w n_w)\, A \int_{x=0}^{D} \left(e^{-E_s(x)/kT} - 1\right) dx. \end{aligned} \tag{A5.2}$$

For a weak interaction, $E \ll kT$, $e^{-E_s(x)/kT} \approx 1 - E_s(x)/kT$ so that

$$\begin{aligned} N_{es} &= -(n_s/\bar{v}_w n_w)\, A(E/kT) \int_{x=0}^{D} [e^{-x/\lambda} + e^{-(D-x)/\lambda}]dx \\ &= -2(n_s/\bar{v}_w n_w)\, A(E/kT)\,\lambda\left[1 - e^{-D/\lambda}\right], \end{aligned} \tag{A5.3}$$

which varies with separation as

$$\frac{\partial N_{es}(D, \mu_s)}{\partial D}|_{\mu_s} = -2(n_s/\bar{v}_w n_w)\, A(E/kT)e^{-D/\lambda}. \tag{A5.4}$$

Alternatively, recalling that $N_{ew} = -N_{es}\,(n_w/n_s)$ (Appendix 4),

$$\begin{aligned} N_{ew} &= (2/\bar{v}_w)\, A(E/kT)\,\lambda[1 - e^{-D/\lambda}] \text{ and} \frac{\partial N_{ew}(D, \mu_s)}{\partial D}|_{\mu_s} \\ &= (2/\bar{v}_w)\, A(E/kT)e^{-D/\lambda}. \end{aligned} \tag{A5.5}$$

N_{ew} is independent of solute concentration, but N_{es} is not.

At infinite separation, N_{es} and N_{ew} are simply twice those of two isolated surfaces. At contact, all the w's and s's have been expelled so that there is no "excess" or deficit of one over the other. And in between? N_{es} and N_{ew} vary exponentially. The change in interaction energy with added solute has an exponentially varying coefficient. We can speak of energy of interaction that depends on changes in

separation D and on changes in solute chemical potential $d\mu_s = kT\, d\ln(n_s)$,

$$dG(D, \mu_s) = \left.\frac{\partial G}{\partial \mu_s}\right|_D d\mu_s + \left.\frac{\partial G}{\partial D}\right|_{\mu_s} dD = -N_{es}(D, \mu_s)\, d\mu_s - P(D, \mu_s)\, A dD. \tag{A5.6}$$

Because $\dfrac{\partial^2 G}{\partial \mu_s\, \partial D} = \dfrac{\partial^2 G}{\partial D\, \partial \mu_s}$,

$$\left.\frac{\partial N_{es}(D, \mu_s)}{\partial D}\right|_{\mu_s} = A\left.\frac{\partial P(D, \mu_s)}{\partial \mu_s}\right|_D = -2(n_s/\bar{v}_w n_w)\, A(E/kT)e^{-D/\lambda}. \tag{A5.7}$$

The change in pressure $P(D, \mu_s)$ between two parallel flat surfaces, or the change in force $AP(D, \mu_s)$ between two flat parallel faces of area A, equals the change in solute excess vs separation. In this weak perturbation example, sensitivity to solute is linear in solute concentration and exponentially varying in separation.

We can also see how the interaction free energy changes with solute concentration,

$$G(D, \mu_s) - G(D = \infty, \mu_s) = -\int_\infty^D P(D, \mu_s)\, A dD, \tag{A5.8}$$

so that

$$\left.\frac{\partial(G(D, \mu_s) - G(D = \infty, \mu_s))}{\partial \mu_s}\right|_D = -\int_\infty^D A\left.\frac{\partial P(D, \mu_s)}{\partial \mu_s}\right|_D dD$$

$$= -2(n_s/\bar{v}_w n_w)\, A\lambda(E/kT)e^{-D/\lambda}. \tag{A5.9}$$

The change in the work to bring the two surfaces to contact is

$$\frac{\partial(G(D = 0, \mu_s) - G(D = \infty, \mu_s))}{\partial \mu_s}|_D = -2(n_s/\bar{v}_w n_w)\, A\lambda(E/kT), \tag{A5.10}$$

exactly twice the change in the energy of an isolated surface.
Expressed in terms of the change in water chemical potential,

$$\frac{\partial(G(D, \mu_w) - G(D = \infty, \mu_w))}{\partial \mu_w}|_D = -(2/\bar{v}_w)\, A\lambda(E/kT)e^{-D/\lambda}, \tag{A5.11}$$

these results can be converted into the interaction between oppositely curved surfaces. According to the Derjaguin approximation (Verwey and Overbeek 1999), the interaction force F_{ss} between two spheres of radius R near contact connects with the free energy per area G_{pp} between parallel planes of the same material by

$$F_{ss} = \pi R G_{pp}, \tag{A5.12}$$

where G_{pp} is given the reference value $G_{pp}(D \to \infty) = 0$. The change in sphere–sphere force with change in solute- or water chemical potential is

$$\left.\frac{\partial F_{ss}(D, \mu_s)}{\partial \mu_s}\right|_D = -2\pi R(n_s/\bar{v}_w n_w)\lambda(E/kT)e^{-D/\lambda} \tag{A5.13}$$

and

$$\left.\frac{\partial F_{ss}(D, \mu_w)}{\partial \mu_w}\right|_D = -(2\pi R/\bar{v}_w)\lambda(E/kT)e^{-D/\lambda}. \tag{A5.14}$$

Excluded solute, $E > 0$, creates an added attraction between spheres.

Acknowledgments

Sergey Bezrukov, Per Lyngs Hansen, Jack Kornblatt, Rudi Podgornik, Peter Rand, and Don Rau have contributed their reliably perceptive thoughts. I also thank Elizabeth Courtenay and Thomas Record for generous access to their preprints, for instructive correspondence, and for providing me with a copy of a figure.

References

Badger, J., and Caspar, D. L. D. (1991). Water-structure in cubic insulin crystals. *Proc. Natl. Acad. Sci. USA* **88,** 622–626.

Bezrukov, S. M., and Vodyanoy, I. (1993). Probing alamethicin channels with water-soluble polymer: Effect on conductance of channel states. *Biophys. J.* **64,** 16–25.

Bonnet-Gonnet, C., Leikin, S. L., Chi, S., Rau, D. C., and Parsegian, V. A. (2000). Measurement of forces between hydroxypropylcellulose polymers: Temperature favored assembly and salt exclusion. *J. Phys. Chem. B* **105**(9), 1877–1886.

Colombo, M. F., Rau, D. C., and Parsegian, V. A. (1992). Protein solvation in allosteric regulation: A water effect in hemoglobin. *Science* **256,** 655–659.

Courtenay, E., Capp, M. W., Anderson, C. F., and Record, M. T. (2000). Vapor pressure osmometry studies of osmolytes–protein interactions: Implications for the action of osmoprotectants in vivo and for the interpretations of "osmotic stress" experiments in vitro. *Biochemistry* **39,** 4455–4471.

Leikin, S., Rau, D. C., and Parsegian, V. A. (1991). Measured entropy and enthalpy of hydration as a function of distance between DNA double helices. *Phys. Rev. A* **44,** 1156–1162.

Leikin, S., Parsegian, V. A., Rau, D. C., and Rand, R. P. (1993). Hydration forces. *Annu. Rev. Phys. Chem.* **44,** 369–395.

Leikin, S., Rau, D. C., and Parsegian, V. A. (1995). Temperature-favored assembly of collagen is driven by hydrophilic not hydrophobic interactions. *Nature Struct. Biol.* **2**(3), 205–210.

Parsegian, V. A., Rand, R. P., Fuller, N. L., and Rau, D. C. (1986). Osmotic stress for the direct measurement of intermolecular forces. *In* "Methods in Enzymology; Biomembranes: Protons and Water: Structure and Translocation" (L. Packer, Ed.), pp. 400–416. Academic Press, New York.

Parsegian, V. A., Rand, R. P., and Rau, D. C. (1995). *In* "Methods in Enzymology" (M. L. Johnson and G. K. Ackers, Eds.), Vol. **259,** pp. 43–94. Academic Press, New York.

Parsegian, V. A., Rand, R. P., and Rau, D. C. (2000). Osmotic stress, crowding, preferential hydration, and binding. A comparison of perspectives. *Proc. Natl. Acad. Sci. USA* **97**(8), 3987–3992.

0. The change in sphere–
otential is

$$)e^{-D/\lambda} \qquad \text{(A5.13)}$$

$$\Gamma)e^{-D/\lambda}. \qquad \text{(A5.14)}$$

veen spheres.

Peter Rand, and Don Rau have
Courtenay and Thomas Record
nd for providing me with a copy

ulin crystals. *Proc. Natl. Acad.*

els with water-soluble polymer:

V. A. (2000). Measurement of
red assembly and salt exclusion.

ation in allosteric regulation: A

00). Vapor pressure osmometry
of osmoprotectants in vivo and
hemistry **39,** 4455–4471.
and enthalpy of hydration as a
1156–1162.
ration forces. *Annu. Rev. Phys.*

d assembly of collagen is driven
3), 205–210.
Osmotic stress for the direct
y; Biomembranes: Protons and
Academic Press, New York.
zymology" (M. L. Johnson and
k.
owding, preferential hydration,
SA **97**(8), 3987–3992.

Podgornik, R., Strey, H. H., and Parsegian, V. A. (1998). Colloidal DNA. *Curr. Opin. Colloid Interface Sci.* **3,** 534–539.

Rau, D. C., and Parsegian, V. A. (1990). Direct measurement of forces between linear polysaccharides xanthan and schizophyllan. *Science* **249,** 1278–1281.

Royer, W. E., Jr., Pardanani, A., Gibson, Q. H., Peterson, E. S., and Friedman, J. M. (1996). Ordered water molecules as key allosteric mediators in a cooperative dimeric hemoglobin. *Proc. Natl. Acad. Sci. USA* **93,** 14,526–14,531.

Selected osmotic stress data, http://aqueous.labs.brocku.ca/osfile.html and http://dir.nichd.nih.gov/Lpsb/docs/osmdata/osmdata.html.

Timasheff, S. N. (1998). In disperse solution, "osmotic stress" is a restricted case of preferential interactions. *Proc. Natl. Acad. Sci. USA* **95,** 7363–7367.

Verwey, E. J. W., and Overbeek, J. Th. G. (1999). "Theory of the Stability of Lyophobic Colloids." Dover Publications, Mineola, NY (originally published by Elsevier, 1948).

Vodyanoy, I., Bezrukov, S. M., and Parsegian, V. A. (1993). Probing alamethicin channels with water-soluble polymers. Size modulated osmotic action. Biophys. *J.* **65,** 2097–2105.

Wyman, J., and Gill, S. J. (1990). "Binding and Linkage, Functional Chemistry of Biological Macromolecules." University Science, Mill Valley, CA.

Yu, B., and Caspar, D. L. D. (1998). Structure of cubic insulin crystals in glucose solutions. *Biophys. J.* **74,** 616–622.

Zimmerberg, J. J., and Parsegian, V. A. (1986). Polymer inaccessible volume changes during opening and closing of a voltage-dependent ionic channel. *Nature* **323,** 36–39.