FEATURE ARTICLE

Physical Chemistry of Biological Free Energy Transduction As Demonstrated by Elastic Protein-Based Polymers†

Dan W. Urry

Department of Chemical Engineering and Materials Science, University of Minnesota, Twin Cities Campus, 421 Washington Avenue SE, Minneapolis, Minnesota 55455, and the Biological Process Technology Institute, 1479 Gortner Avenue, St. Paul, Minnesota 55108

Received: July 3, 1997; In Final Form: September 4, 1997⊗

This article, on protein-based polymers comprised of repeating peptide sequences, reviews studies from the author's laboratory covering a period of more than two decades; it presents a general mechanism for protein folding and function and demonstrates the mechanism by designing model proteins capable of performing many of the energy conversions that sustain life and by designing diverse biomolecular machines and materials with promising applications for society. All polymers with the correct balance of apolar and polar moieties, including water soluble proteins and protein-based polymers, increase order by a hydrophobic folding and assembly transition as the temperature is raised above a critical onset temperature, designated as T_t. Instead of varying the temperature, however, innumerable variables lower the value of T_t from above to below the operating temperature to drive folding and function. Thus, this inverse temperature transition provides a fundamental mechanism whereby proteins fold and function and whereby the energy conversions that sustain living organisms can occur at constant temperature. Phenomenologically, this mechanism results in five axioms or principles for protein function and protein engineering whereby designed protein-based polymers interconvert six free energies interconverted by living organisms. The six intensive variables for biological free energy transduction are mechanical force, temperature, chemical potential, electrochemical potential, pressure, and electromagnetic radiation. No matter how seemingly disparate, virtually every protein function can be classified in terms of a form, or forms, of free energy transduction. Mechanistically, the design, preparation, and characterization of families of related protein-based polymers show the usually considered electrostatic charge−charge interactions not to be the physical basis for the energy conversions. Instead, presented and analyzed experimental data indicate competition for hydration between apolar and polar species to be responsible. In short, the value of T_t is determined by the amount of water of hydrophobic hydration; hydration of polar species, as required on ionization, occurs at the expense of hydrophobic hydration and raises the value of T_t, and the energy required to destructure hydrophobic hydration results in hydrophobic-induced pK_a shifts. Formalisms are outlined that describe hydrophobic-induced pK_a shifts, related positive cooperativity of acid-based titration curves, and the involved energy conversions.

"One of the principal objects of theoretical research in any department of knowledge is to find the *point of view* from which the subject appears in its *greatest simplicity*." [1]

Introduction

How living organisms access available energy and convert it into those forms necessary to sustain and propagate life is the quintessential enigma of biology! The fundamental challenge presented by living organisms, therefore, is to develop an understanding of biological free energy transduction, or, more loosely stated, biological energy conversion. Such a physicochemical perspective differs somewhat from that of biologists who commonly state, for example, that "...the question of biological organization is *the* major question in biology," [2] and that "...organization exists in the living organism, and this organization is not something fundamentally mystical and unamenable to scientific attack, but rather the basic problem confronting the biologist." [3] The description of an organized biological structure itself surely does not constitute energy conversion, but the folding and assembly of biological macromolecules resulting in the formation of an organized biological structure does represent a particular energy conversion, and changes in organization at the molecular level can be the catalyst for energy conversion.

Free Energy Transduction. As demonstrated below, changes in the folding and assembly of properly designed model proteins can, in fact, catalyze virtually all of the free energy transductions recognized in biology. Accordingly, to the physical chemist, the basic problem of biology becomes how changes in intensive variables of the free energy give rise to changes in folding and assembly that perform useful work. To wit, the performance of a particular kind of work is simply that kind of energy on display.

The Work of Organization. With the proper protein composition, it is remarkable that heating to raise the temperature of aqueous solutions can give rise to organized protein, that is, to an increase in order, or naturation, of the protein.[4−7] This occurs

† This manuscript was largely written while at the Laboratory of Molecular Biophysics, UAB School of Medicine, University of Alabama at Birmingham, but was completed after relocation to the University of Minnesota, Twin Cities Campus.

⊗ Abstract published in *Advance ACS Abstracts,* November 15, 1997.

S1089-5647(97)02167-6 CCC: $14.00

by means of hydrophobic folding and assembly, i.e., the separation of oil-like moieties of the protein from water. Perhaps most importantly for living organisms, there are many means of achieving organization by hydrophobic folding and assembly other than by raising the temperature.[7,8] Changes in chemical potential, such as raising the concentration of salt or increasing the proton concentration with a protonatable anionic side chain in the protein, can give rise to the molecular organization of structure formation. Applying an electrochemical potential, capable of reducing a redox moiety attached to the protein, can drive structure formation. Again, with the appropriate protein composition, a change in pressure can change structure, as can the absorption of electromagnetic radiation of a particular frequency. These changes in the noted intensive variables—of temperature, chemical potential, electrochemical potential, pressure, and electromagnetic radiation—function as driving forces for the mechanical work of organization, which, demonstrated for our case in what follows, is structure formation arising out of macromolecular hydrophobic folding and assembly.

Performance of Mechanical Work, e.g., Pumping Iron. The organization resulting from molecular folding and assembly constitutes a mechanical energy output from whatever energy input that produced it. When using *protein-based polymers* (polymers comprised of repeating peptide sequences) as the model proteins, mechanical work output can easily be demonstrated by the more obvious work of lifting weights, i.e., of pumping iron. Indeed, by using cross-linked elastic protein-based polymers to form dominantly entropic elastomeric bands, the organizational process of molecular folding and assembly resulting from the application of changes in the above-noted intensive variables can be displayed as macroscopic contractions capable of lifting weights that are thousands of times greater than the dry weight of the contracting elastomer. Thus, with proper design of elastic protein-based polymers, inputs of thermal, chemical, electrochemical, pressure, and light energy can each be used to perform useful mechanical work.[8]

Structural Changes That Produce Work in Addition to Mechanical Work. Not only can organization be the product of different energy inputs but a change in organization, i.e., a change in molecular folding and assembly, can be the catalyst for interconverting pairs of energies not including mechanical energy. Should the model protein contain two different functional entities, each responsive to the change in a different intensive variable, then a change in one intensive variable represents an energy input that by altering one functional group acts upon and changes the property of the second functional group as the mechanism for achieving an energy output.[9−11]

Pumping Protons. By way of an example that is to be described in more detail later in this article, consider a model protein designed to contain both a carboxylate function and an oxidized state of a redox function. With appropriate design of the protein-based polymer, electrochemical reduction of the redox function, by driving a change in molecular organization, effects a change in the pK_a of the carboxyl and causes the uptake of a proton.[10,11] The application of a reductive electrochemical potential has resulted in a change in the chemical potential of proton in the system. This is the conversion of an electrical energy input into a chemical energy output, as occurs in the electron transport system of the inner mitochondrial and thylakoid membranes. This example, and the family of free energy transductions it represents, illustrate the *principle of Le Châtelier*, as discussed below.

Phenomenology: Temperature of Hydrophobic Folding and Assembly Transitions. *The Temperature, T_t, of the Transition.* Fortunately, there is a simple unifying phenomenology, possibly in the words of Gibbs[1] a *point of view* of *greatest simplicity*, whereby the above seemingly disparate phenomena of energy conversion can be described, analyzed, and classified. It involves the temperature, T_t, at which hydrophobic folding and assembly transitions occur. As the Gibbs free energy, ΔG, is defined as $\Delta G = \Delta H - T\Delta S$ and as the change in Gibbs free energy for a transition, ΔG_t, is zero, then $\Delta H_t = T_t \Delta S_t$, and therefore, $T_t = \Delta H_t/\Delta S_t$.

For a family of model proteins wherein substitutions of but a few residues per 100 residues conserve both structure and the structural change for the hydrophobic folding and assembly transition, the ratio $\Delta H_t/\Delta S_t$ becomes dominated by differences in the interactions of the water solvent with a substituent residue of the protein-based polymer, and, as will be seen, T_t becomes a measure of the hydrophobicity of the substituent residue. Comparing the T_t values of all of the substituent amino acid residues suitably referenced in a parent model protein results in the development of a T_t-based hydrophobicity scale for proteins and related polymers.[12] Furthermore, as we shall see, T_t also becomes a relative measure of the numbers of waters of hydrophobic hydration that change to bulk water during the transition.

For convenience, T_t is taken as the readily measured temperature for the onset of turbidity, i.e., for the onset of the aggregational phenomenon of the transition on raising the temperature. Accordingly, heating to raise the temperature from below to 10 or 15° above T_t drives hydrophobic folding and assembly with the performance of mechanical work. Of more general relevance for living organisms, however, is the following. Instead of raising the temperature from below to above T_t to drive hydrophobic folding and assembly, it is possible, by innumerable ways that increase hydrophobicity or the expression of hydrophobicity, to lower T_t from above to below an operating temperature with the same result of driving hydrophobic folding and assembly.[8]

The ΔT_t-Mechanism. Lowering the value of T_t to drive hydrophobic folding and assembly in the direct performance of mechanical work, or in hydrophobically shifting the properties of functional groups associated with the protein to achieve diverse energy outputs, is descriptively called the ΔT_t-mechanism.[7,8] With knowledge of the dependence of T_t on a host of variables—chain length, polymer concentration, polymer composition, salts, organic solutes, pH and side chain charge, redox state of attached prosthetic group, pressure, chemical modification of side chains (e.g., phosphorylation), light, ion pairing, etc., it becomes possible empirically to design protein-based polymers for specific free energy transductions. This extensive list of dependencies of T_t will be briefly noted below in order to demonstrate the universality of the T_t perspective in relation to free energy transduction by polymers that exhibit hydrophobic folding and assembly transitions.

In the present review, this phenomenological description of the behavior of transductional model proteins is followed by an analysis of the physical basis for this mechanism of free energy transduction, and finally by a brief consideration of formalisms for pK_a shifts and positive cooperativity which extends into a general formalism for free energy transduction.

Mechanism: The Physical Basis for Free Energy Transduction. *Competition for Hydration between Apolar and Polar Moieties.* The initial insight for understanding the physical basis for free energy transduction, as demonstrated by cross-linked, hydrophobically folded and assembled elastic model proteins, came from the experimental observation of a stretch-induced increase in the pK_a of a carboxyl side chain.[13] Therefore,

although the water content of the elastomer increases on stretching and the mean distance between charges increases, the change in free energy required for ionization of the carboxyl to form carboxylate increases. One concludes that the water, which enters as the hydrophobically folded elastomer is forcibly unfolded and which hydrates the stretch-exposed hydrophobic groups, is not suitable for hydration of carboxylates. This analysis led to the proposal of a competition for hydration between apolar (hydrophobic) and polar (e.g., charged) residues.[7,13]

Under such circumstances, one expects, as the pH is sufficiently raised to cause the carboxyls to ionize, that the hydration of the charged species must occur in part at the expense of the water of hydrophobic hydration. The proposal was tested by a differential scanning calorimetry study where it was deduced that formation of less than two charged carboxylate residues per 100 residues of polymer destructured almost three-fourths of the special structured waters of hydrophobic hydration thought to surround hydrophobic residues when exposed to water.[14] In a recent, more definitive study using microwave dielectric relaxation to identify and characterize waters of hydrophobic hydration, the formation of 1−2 carboxylates per 100 residues of polymer was directly observed to destructure more than two-thirds of the measurable waters of hydrophobic hydration.[15]

Hydrophobic-Induced pK_a Shifts. As the carboxylates must destructure waters of hydrophobic hydration in order to achieve their own hydration, the work of doing so represents an increase in free energy of the carboxylate, which is seen as an increase in pK_a. Such increases in the pK_a of carboxyls are referred to as hydrophobic-induced pK_a shifts in contrast to increases in pK_a arising from charge−charge repulsion.[16−18] Two remarkable aspects about hydrophobic-induced pK_a shifts are that they can be so large, more than six pH units, and that they increase supralinearly as the hydrophobicity is linearly increased.[19] Another significant feature can be seen in the acid−base titration curves of carboxyls exhibiting hydrophobic-induced pK_a shifts; the curves can be very steep, i.e., positively cooperative, with the attendant increased efficiency for energy conversion.[20] On the other hand, pK_a shifts due to charge−charge repulsion are generally much smaller; they occur only with a high density of the same charge, and they are characterized by much broader (negatively cooperative) acid−base titration curves[21] with the attendant poor efficiency for energy conversion.

Formalisms for pK_a Shifts and Positive Cooperativity (Energy Conversion). The Henderson−Hasselbalch equation, which describes acid−base titration curves for dilute solutions of weak acids and bases, $pH = pK + \log[\alpha/(1 - \alpha)]$, where α is the degree of ionization, can serve as a starting point for formalisms of free energy transduction. This is because the acid−base titration curve is a plot of degree of ionization versus pH, which actually represents a plot of the fraction of completion of an energy conversion versus chemical energy in units of 2.3*RT*. During the titration of a carboxyl function with NaOH, the concentration (activity) and hence the chemical energy of the COOH moiety decrease and those of the COO^- moiety increase. By definition, the chemical potential, $\mu = RT \ln a$, where a is the activity. Since for the low proton concentration, $[H^+]$, for the range of interest throughout most titrations, $\mu_H = RT \ln[H^+] = 2.3RT \log[H^+] = -2.3RT$ pH and therefore, $pH = -\mu_H/2.3RT$. Also, since by definition the chemical potential of the proton, $\mu_H = (\partial G/\partial n_H)_{T,P,n}$, i.e., the change in Gibbs free energy, ∂G, per change in moles of protons, ∂_H, at constant temperature, T, pressure, P, and all other chemical species, n, pH is a measure of the change in Gibbs free energy per mole of protons in units of *RT* divided by the factor 2.3. Thus, the Henderson−Hasselbalch equation, with pH given as a function of α (now recognized as the fraction of completion of the energy conversion), provides a beginning formalism for considering biological free energy transduction, as each energy conversion can be analyzed and plotted in an analogous way.

Indeed, Overbeek,[22] Harris and Rice,[23] and Katchalsky and Gillis[24] have each introduced into the Henderson−Hasselbalch equation the effects of charge−charge repulsion in polyelectrolytes resulting in pK_a shifts and broadened (negatively cooperative) acid−base titration curves. As shown by Katchalsky and co-workers,[25] the relief of charge−charge repulsion, which occurs in cross-linked polyelectrolytes on decreasing α, can be used to perform the mechanical work of lifting a weight. Accordingly, the degree of protonation, $(1 - \alpha)$, becomes proportional to the potential energy of a raised weight.

Using suitably hydrophobic and cross-linked elastic protein-based polymers, we have demonstrated that protonation of one or two carboxylates in 100 residues can be used to perform the mechanical work of lifting a weight 1000 times the dry weight of the synthetic muscle.[26] Furthermore, using designed elastic protein-based polymers, we have recently introduced the effects of hydrophobic-induced pK_a shifts and positive cooperativity into an extended Henderson−Hasselbalch formalism for describing acid−base titration curves and have successfully calculated the pK_a shifts and positive cooperativity with the derived formalisms (Peng and Urry, in preparation). The approach combines cooperativity concepts from allosteric theory and multiple equilibria with direct, experimental characterization of the charge dependence of waters of hydrophobic hydration.[27]

The next step in developing a general formalism for biological free energy transduction is to replace the degree of protonation of carboxylates with the degree of reduction of an attached redox couple and to plot the degree of reduction vs the electromotive force or the applied electrical potential, suitably referenced. Completing the correlation, as with protonation, the reduction of a redox couple can also be used to perform the mechanical work of lifting a weight[28] or even to perform the chemical work of shifting a pK_a with the pickup of protons.[10] Therefore, the general formalism for free energy transduction can be given in terms of fraction of energy output as a function of energy input. There is, of course, a particular range of intensive variables over which the transition occurs to catalyze the energy conversion that is inherent in the transitional nature of hydrophobic folding and assembly.

Model Protein-Based Polymers for Free Energy Transduction

The phenomenology underlying the process of free energy transduction of discussion here is the transition of hydrophobic folding and assembly in an aqueous medium. Therefore, as will become apparent, all polymers with the correct balance of apolar and polar residues and with the correct functional groups can perform these energy conversions. The issue becomes, therefore, the polymeric system that provides the most effective insight into and utilization of this form of free energy transduction. The answer seems clearly to be polymers based on repeating amino acid sequences, as argued below.

Protein-Based Polymers. *Definition.* Protein-based polymers[29] are comprised of repeating peptide sequences, where the repeating unit can be as few as two or as many as hundreds of residues and where the repeating unit may recur but a few times or as many as hundreds of times. The diversity and control of structure possible with protein-based polymers make these unmatchable among the known polymers.

TABLE 1: List of General Advantages of Protein-Based Polymers

1. Two modes of synthesis: chemical and recombinant DNA technology: Chemical synthesis allows for relatively rapid screening of properties of numerous compositions. Once completed, slower gene construction and development of an effective expression system provides for rapid, large scale production and other advantages listed below.
2. Diversity of monomers: There is possible the selection of 20 different monomeric units when using recombinant DNA technology, and enzymatic and chemical posttranslational modifications provide further diversity.
3. Precise control of sequence: Any protein-based polymer sequence of the 20 monomers can be specified using recombinant DNA technology, allowing production of polymers that may be unfavorable from the standpoints of chemical synthesis and energetics. There may, however, be certain limitations regarding expression that can be overcome with knowledge of the principles of protein engineering.
4. Exact control of stereochemistry: This is essential for control of structure and physical properties. The occurrence of some racemization is inevitable during the chemical synthesis of a long protein-based polymer. Even a small amount (1%) of racemization can critically limit desirable polymer properties.
5. Precise chain lengths: All of the protein-based polymers from a single gene are of the same length and the whole range of chain lengths are possible up to 2000 or more residues. This allows for fine-tuning of desirable properties.
6. Capacity to mimic proteins: With an adequate understanding of protein engineering, there exists an essentially unlimited capacity to mimic complex proteins in terms of both structure and function.
7. Circumstances of protein function to guide analyses and approach: It is possible to consider functional proteins, e.g., with prosthetic groups and cofactors, to guide study of variables relating to function in developing a useful understanding of protein engineering, e.g., see Tables 2 and 3.
8. Properties and uses beyond those of known proteins: Protein-based polymers can be designed with properties and functions that go beyond what evolution has called upon proteins to do, once the rules for protein engineering have been established. Example: programmable, biodegradable plastic protein-based polymers.
9. Low cost of bioproduction: The cost of bioproduction, at least a 10 000-fold decrease from chemical synthesis, has the potential to be competitive with the cost of petroleum-based polymers.
10. Produced from renewable resources: Protein-based polymers can be produced from renewable resources and are environmentally friendly for the complete life cycle of production to disposal.
11. Axioms for protein-based polymer engineering: The axioms (listed in Table 5, developed in terms of free energy transductions performed by designed protein-based polymers, and based in part on the information in Tables 2, 3, and 4) provide the basis for the design of all polymers capable of exhibiting inverse temperature transitions.

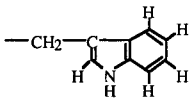
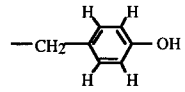
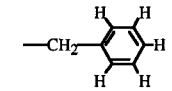
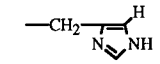
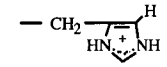
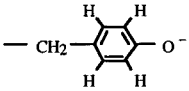
Advantages of Protein-Based Polymers. Table 1 lists 11 advantages of using protein-based polymers as model polymers for elucidating the principles of polymer-effected free energy transduction and as new materials of commercial value. As each item of the listing has an associated explanation, Table 1 should be consulted such that no further space within the text need be given to this general issue.

Desirable Properties of the Protein-Based Polymer Family for Developing Principles of Free Energy Transduction. The list of eleven items of Table 1 establish protein-based polymers as extraordinary and generally unmatchable by any other known polymers. Here we address the properties of protein-based polymers that would be particularly favorable for development of the principles of free energy transduction. The principal desirable feature is the existence of transitional behavior such that a well-defined energy input can convert the model polymer from one state to another over a finite range of the intensive variable. The transition between the two states should (a) be of low energy with the heat of the transition, ΔH_t, of the order of a fraction of a kilocalorie per mole of residue, eliminating metastable states and irreversible processes in order that the thermodynamics be continuously defined and the energy conversion reversible, (b) be accessible, for example, in the aqueous temperature range of, say, 0−80 °C, (c) be perturbable wherein application of many different intensive variables can shift properties of the transition, and (d) exhibit positive cooperativity for efficient energy conversion.

Elastic Protein-Based Polymers. On raising the temperature, elastic protein-based polymers of our experience hydrophobically fold and assemble, due to the periodicity of hydrophobic groups, in such a manner that some of the peptide moieties of the backbone are free to undergo large librational motions. As the extension of stretching occurs, these backbone torsional oscillations, or rocking motions, become progressively damped with an attendant decrease in entropy and with the result of the development of an entropic elastic restoring force. This allows for dominantly entropic elastic protein-based polymers, e.g., f_e/f ratio < 0.2 where f, the total elastomeric force, is the sum of f_e, the internal energy component, and of f_s, the entropic component, of the elastomeric force. Accordingly, such transitions can be of low energy and can be devoid of metastable states. Another element of the family of protein-based polymers utilized here is that conditions relevant to energy conversion are accessible wherein a significant proportion of the water in the system is water of hydrophobic hydration. Because of this, the water of hydrophobic hydration can be quantified and characterized as a function of those variables that control hydrophobic folding and assembly and that drive energy conversion.

Poly(GVGVP): The Parent, Elastic, Phase Transitional, Protein-Based Polymer. In addition to the list of advantages of protein-based polymers given in Table 1, there are special advantages of the parent elastic protein-based polymer, poly(GVGVP), as the model system for studying and developing the principles of free energy transduction, e.g., (i) side chain simplicity, (ii) chemical stability, (iii) exhibiting phase transitional behavior, which is accessible, perturbable, and of low energy, (iv) specifically exhibiting an inverse temperature transition, (v) exhibiting hydrophobic folding and assembly on raising the temperature or on lowering transition temperature from above to below an operating temperature, (vi) ready physical characterization and interpretation leading to molecular structure and function, (vii) substitutable position(s) without significantly altering structure and viscoelastic state, (viii) exhibiting entropic elasticity when cross-linked, (ix) providing the only hydrophobicity scale directly dependent on hydrophobic folding and assembly, (x) demonstrating stretch-induced and hydrophobic-induced pK_a shifts with attendant cooperativity, (xi) allowing direct characterization of waters of hydrophobic hydration, (xii) demonstrating apolar−polar repulsive free energy of hydration, (xiii) designable for performing the free energy transductions of living organisms, (xiv) providing the

TABLE 2: T_t-Based Hydrophobicity Scale for the Naturally Occurring Amino Acid Residues [in Order of More Hydrophobic (Low T_t) to More Polar (High T_t)][e]

Residue	R Group	Abbreviation	Letter	T_t[a]	ΔH_t kcal/mol[d] ± 0.05	ΔS_t kcal/mol[d] ± 0.05
Tryptophan		Trp	W	–90°C	2.10	7.37
Tyrosine		Tyr	Y	-55°C	1.87	6.32
Phenylalanine		Phe	F	–30°C	1.93	6.61
Histidine		His	H	–10°C		
Proline (calc.)[b]	$-CH_2CH_2CH_2-$	Pro	P	(–8°C)		
Leucine	$-CH_2CH(CH_3)_2$	Leu	L	5°C	1.51	5.03
Isoleucine	$-CH(CH_3)CH_2CH_3$	Ile	I	10°C	1.43	4.60
Methionine	$-CH_2CH_2SCH_3$	Met	M	20°C	1.00	3.29
Valine	$-CH(CH_3)_2$	Val	V	24°C	1.20	3.90
Histidine		His^+	H^+	30°C		
Glutamic Acid	$-CH_2CH_2COOH$	Glu	E	30°C	0.96	3.14
Cysteine	$-CH_2SH$	Cys	C	30°C		
Lysine	$-CH_2CH_2CH_2CH_2NH_2$	Lys^o	K^o	35°C	0.71	2.26
Proline (exptl)[c]	$-CH_2CH_2CH_2-$	Pro	P	40°C	0.92	2.98
Alanine	$-CH_3$	Ala	A	45°C	0.85	2.64
Aspartic Acid	$-CH_2COOH$	Asp	D	45°C	0.78	2.57
Threonine	$-CH(OH)CH_3$	Thr	T	50°C	0.82	2.60
Asparagine	$-CH_2CONH_2$	Asn	N	50°C	0.71	2.29
Serine	$-CH_2OH$	Ser	S	50°C	0.59	1.86
Glycine	$-H$	Gly	G	55°C	0.70	2.25
Arginine	$-CH_2CH_2CH_2NHC(NH)NH_2$	Arg	R	60°C		
Glutamine	$-CH_2CH_2CONH_2$	Gln	Q	60°C	0.55	1.76
Lysine	$-CH_2CH_2CH_2CH_2NH_3^+$	Lys	K	120°C		
Tyrosinate		Tyr^-	Y^-	120°C	0.31	0.94
Aspartate	$-CH_2COO^-$	Asp^-	D^-	120°C		
Glutamate	$-CH_2CH_2COO^-$	Glu^-	E^-	250°C		

[a] T_t is the onset temperature for the hydrophobic folding and assembly transition, i.e., inverse temperature transition, in pbs (0.15 N NaCl, 0.01 M phosphate) as determined by light scattering. The values are linearly extrapolated to $f_X = 1$ and rounded to a number divisible by 5. ΔH and ΔS are the values at $f_X = 0.2$ on the curve for a linear fit of the DSC-derived endothermic heats and entropies of the transitions for the polymers in water. [b] The calculated T_t value for Pro comes from poly(GVGVP) when the experimental values of Val and Gly are used. This hydrophobicity value of −8 °C is unique to the β-spiral structure where there is hydrophobic contact between the Val_i^1 γ-CH_3 and the adjacent Pro_i^2 δCH_2 and the interturn Pro_{i+3}^2 βCH_2 moieties. [c] The experimental value determined from poly[f_V(GVGVP),f_P(GVGPP)]. [d] Per mole of pentamer. [e] Adapted from refs 6, 8, and 74.

opportunity for generally elucidating principles of protein function and engineering, etc.

Side Chain Simplicity. The side chains for the Gly (G), Val (V), and Pro (P) residues as shown in Table 2 are either simply the hydrogen atom or an aliphatic grouping. Accordingly, the interpretation of properties exhibited by poly(GVGVP) is simplified.

Chemical Stability. The absence of any reactive side chains means that the protein-based polymer is stable over long periods of time and over many different variables. Some care nonetheless is required when preparing and characterizing these protein-based polymers. Extremes of pH can result in peptide hydrolysis, and elevated temperatures for prolonged times can result in limited but significant racemization that alters properties of the transition and decreases efficiencies of energy conversion.[30] Also great care is required during chemical synthesis to limit racemization. This, of course, is not an issue when utilizing the properly purified microbial product.

Phase Transitional Properties. The phase transitional behavior of poly(GVGVP) is demonstrated in Figure 1A, where $(GVGVP)_{251}$ is soluble in water in all proportions below 25 °C, but on raising the temperature above 25 °C aggregation occurs, followed by settling with the result of a clean phase separation. Following the turbidity of the water−$(GVGVP)_{251}$

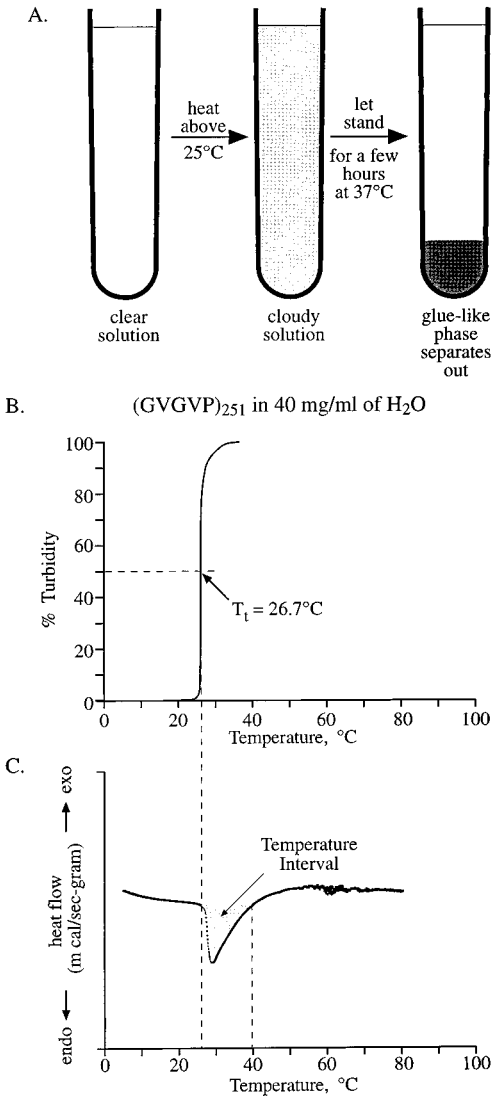


Figure 1. Behavior of a binary mixture of water and protein-based polymer, $(GVGVP)_{251}$. (A) Heat-induced phase separation. (B) Temperature profile of aggregation followed by light scattering. When at a high concentration limit of 40 mg/mL for the high molecular weight (approximately 100 000 Da) protein-based polymer, $(GVGVP)_{251}$, the midpoint of the turbidity is designated as T_t and signifies the onset of the transition. (C) Differential scanning calorimetry of the binary mixture showing the temperature interval and heat of the endothermic transition.

mixture as the temperature is raised provides a useful measurement for the onset of aggregation; the quantity T_t, the critical temperature that characterizes the transition, can be taken as the point of one-half-maximal turbidity for a high concentration limit, as shown in Figure 1B. This is analogous to the determination of the cloud point, T_{cl}, the temperature at which a plot of the temperature versus intensity of the transmitted beam vanishes, but the cloud point is not uniquely defined at low concentrations, e.g., below 7 mg/mL for poly(GVGVP).[31] Furthermore, as seen in Figure 1C, the transition is localized, and the heat of the transition is small, less than 1 kcal/mol-residue. Also, the transition is ideally situated in the 25−37 °C range. As will become apparent below, another useful feature is that the more dense viscoelastic phase of Figure 1A, which is approximately 40% protein-based polymer and 60% water by weight,[32] can be examined after removal of the overlying solution, and the transition, which now represents a rearrangement of both the structure of the protein-based polymer and of the water, can yet be observed by differential scanning calorimetry and by dielectric relaxation methods. This becomes particularly instructive for understanding the nature of elasticity and the nature and behavior of water in the function of these transductional protein-based polymers.

The structural change exhibited by the protein-based polymer, poly(GVGVP), during the transition has received much study.[32−35] The physical characterizations of the transition have included: nuclear magnetic resonance structural[36−38] and relaxation[39,40] studies, circular dichroism,[32] Raman spectroscopy,[41] X-ray crystallography,[42] light, scanning electron and transmission electron microscopies,[43,44] dielectric relaxation,[15,45,46] molecular mechanics, and dynamics calculations,[47−51] elastic and quasi-elastic light-scattering data[31,52−54] analyzed in terms of mean-field critical behavior and Flory−Huggins free energy of interactions,[55] differential scanning calorimetry,[14,56] and thermoelasticity measurements.[57]

Inverse Temperature Transition. The cyclic analogue, cyclo-$(GVGVAP)_2$, is soluble in water at low temperature, but it crystallizes (as seen in Figure 2A) on raising the temperature and redissolves on lowering the temperature.[58] The cyclopentadecapeptide of the linear poly(GVGVP), namely cyclo-$(GVGVP)_3$, also associates on raising the temperature of aqueous solutions and dissolves on lowering the temperature; its crystal structure is seen in Figure 2B.[42] The linear poly(GVGVP) associates on raising the temperature to form filamentous structures with a twisted filament substructure (see Figure 2C and the optical diffraction insert),[44] and polymers containing occasional Glu residues and others with occasional Lys residues assemble while being chemically cross-linked to form several micrometer diameter fibers that splay-out into parallel aligned fibrils which in turn are comprised of parallel aligned twisted filaments (see Figure 2D−F).[43] These crystal, X-ray structure, and light, transmission, and scanning electron microscopy data demonstrate that the elastic protein-based polymer, poly-(GVGVP), becomes more ordered on raising the temperature from below to above the temperature of the phase transition. For this reason the transition is referred to as an *inverse temperature transition*.

Hydrophobically Folded and Assembled Structure. The above increase in the order of the protein-based polymer on raising the temperature on superficial examination would seem to contradict the second law of thermodynamics that the order of a system decrease on increasing the temperature; the apparent contradiction resolves, of course, once the total system, protein-based polymer plus water, is considered. Before raising the temperature which causes the protein-based polymer to become folded and assembled as depicted in Figure 2, the nonpolar, or hydrophobic, side chains are surrounded by water that is of low entropy. So large is the decrease in entropy of the water surrounding nonpolar solutes that when this was first appreciated by Frank and Evans,[59] they referred to the water as "icebergs." As seen below, it is not a small number of water molecules constituting the immediate surface of the nonpolar solute that are rigidly fixed in position or frozen as in ice, but rather it is a surprisingly large number or a "halo" of water molecules reaching well beyond the immediate surface layer that are of an entropy less than that of bulk water, thus making up in numbers what they lack in rigidity.

Of course, when the protein-based polymer becomes folded and assembled, this "halo" of more ordered water becomes bulk water as the nonpolar side chains come into intra- and intermolecular contact. This we call hydrophobic folding and assembly, and it is in the capacity to modulate the temperature at which hydrophobic folding and assembly occurs that diverse free energy transductions can occur and can do so with positive cooperativity.

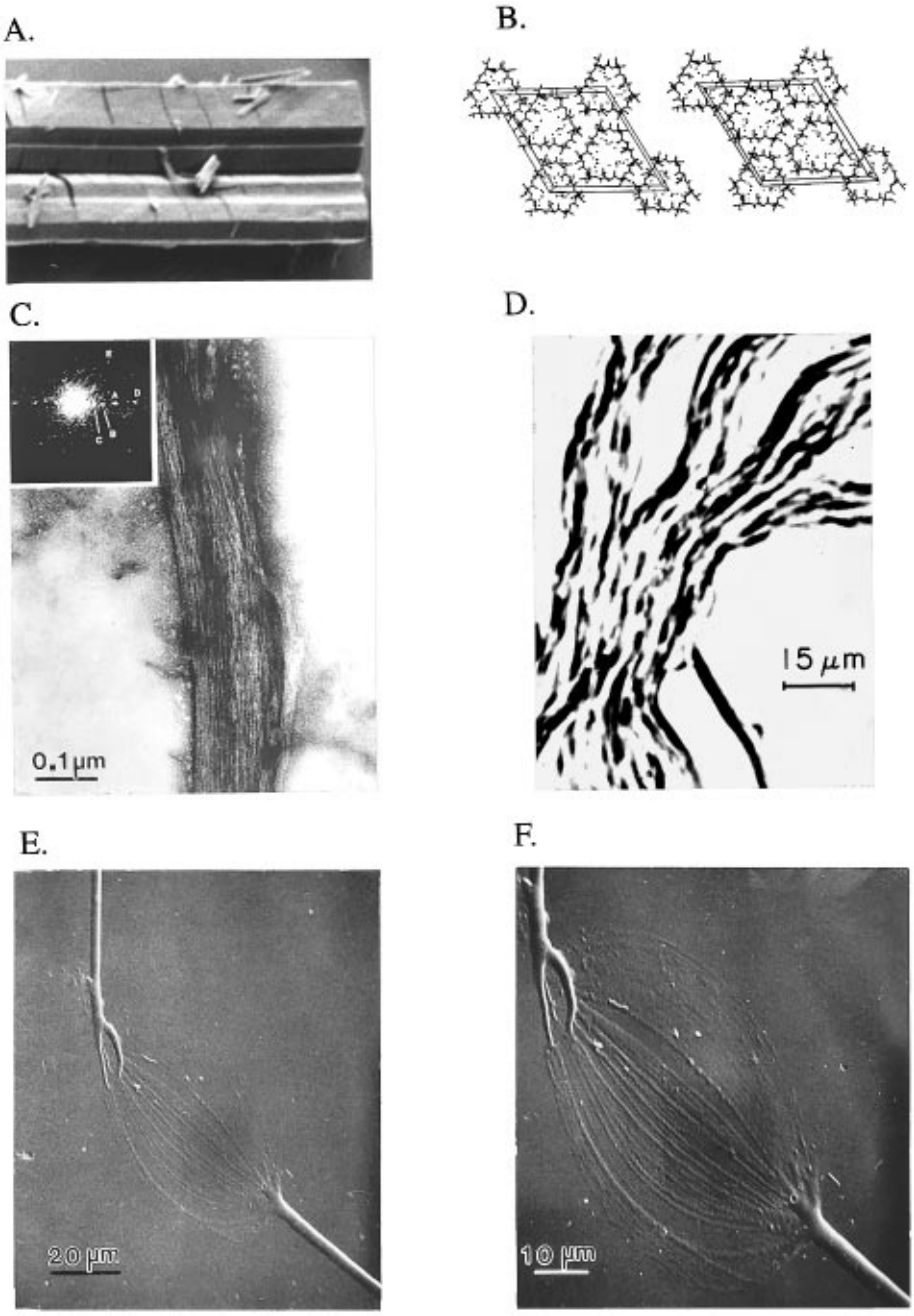


Figure 2. Temperature-elicited association of related protein-based polymers on raising the temperature. (A) Crystals of the cyclic analogue, cyclo(GVGVAP)$_2$, obtained on raising the temperature. Adapted with permission from ref 58. (B) Crystal structure of the cyclic analogue, cyclo-(GVGVP)$_3$, which also associates on raising the temperature. The molecules are seen to associate primarily by hydrophobic side chain contacts. The water is within rather than between the stacks of molecules. This is the cyclic conformational correlate of the linear poly(GVGVP).[101] Reproduced with permission of ref 42. (C) Transmission electron micrograph with negative staining of heat aggregated poly(GVGVP) showing the polymers to have self-assembled into parallel aligned filaments; the inset shows the optical diffraction of the micrograph with equatorial and off-meridional reflections indicating the lateral periodicity of the filaments and a helical twisted rope substructure. Reproduced with permission from ref 44. (D) Light micrograph showing of substituted poly(GVGVP) chains, some with Lys(K) and others with Glu(E), having self-assembled into fibers comprised of fibrils during chemical cross-linking. (E and F) Scanning electron micrograph of a cross-linked fiber splaying out into many parallel aligned fibrils that coalesce back into a common fiber. Reproduced with permission from ref 43.

Molecular Structure. The dynamic molecular structure that we have derived for the parent elastic protein-based polymer is represented in Figure 3. At the top of the figure is a schematic representation of the polymer chain as a series of β-turns inserted due to the presence of the Pro(P)−Gly(G) sequence, where the β-turn is given in detail from X-ray structure in Figure 2B.[42] Represented in Figure 3C is a schematic helical structure that results from optimizing the hydrophobic folding, and in Figure 3D the β-turns are schematically shown as spacers with hydrophobic contacts between turns of the helix.[60] A detailed plot of the helical structure, which is called a β-spiral (a helical recurrence of β-turns), is seen in stereopair in Figure 3E, and the β-spirals are seen assembled into a fragment of a twisted filament in Figure 3F.[48] The developmental stages of this molecular structure are reviewed elsewhere.[33,35,36,48,49,61]

Substitutable Positions. The structure and function of poly-(GVGVP), or alternatively poly(VPGVG), are generally maintained as long as the Gly(G) and Pro(P) residues are left intact. The substitutable positions are identified as α and β in poly-(GαGβP) or equivalently poly(βPGαG). Fortunately, every naturally occurring amino acid residue can occur in the α-position with retention of the inverse temperature transition

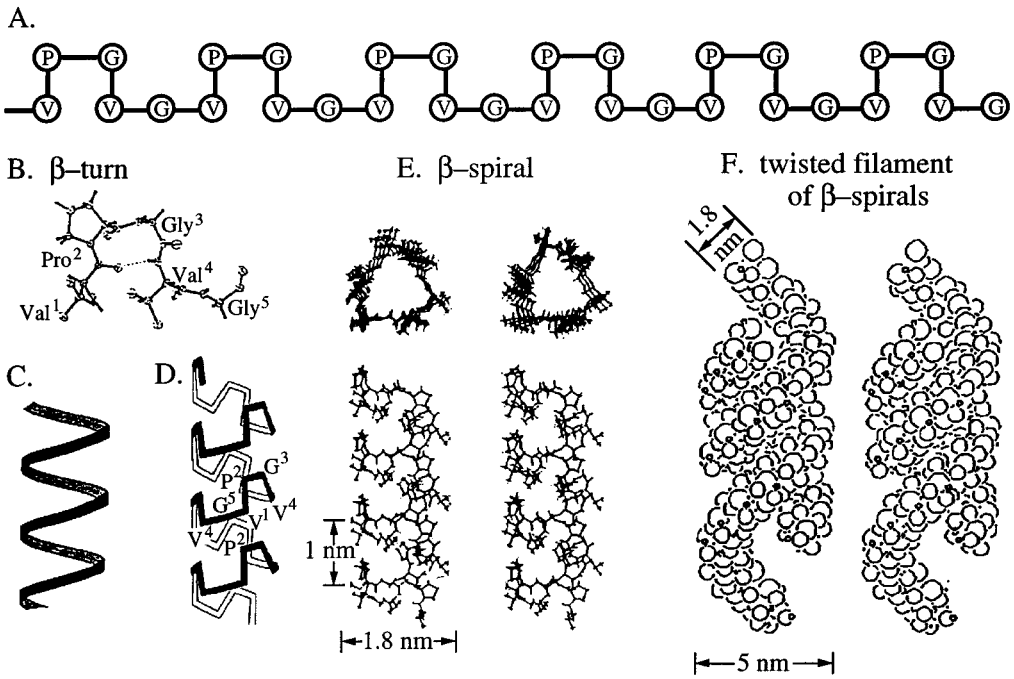


Figure 3. Working molecular structure of poly(GVGVP), or alternatively poly(VPGVG). (A) Schematic representation of the polypeptide chain with the Pro(P)−Gly(G) sequence inserting a repeating β-turn. Reproduced with permission from ref 93. (B) The repeating β-turn, as obtained from the crystal structure of cyclo(GVGVP)$_3$. Reproduced with permission from ref 42. (C and D) Helical representations of the repeating pentamer structure without and with showing the β-turns positioned as spacers between the turns of the helix. Reproduced with permission from ref 60. (E) Detailed plots in stereo (cross-eye viewing) of the helical structure of poly(GVGVP), called a β-spiral, which is a helical arrangement of β-turns. Reproduced with permission from ref 7. (F) Association of β-spirals in space-filling united residue representation giving rise to the twisted filament, or supercoiled, structures of Figure 2C. Reproduced with permission from ref 48.

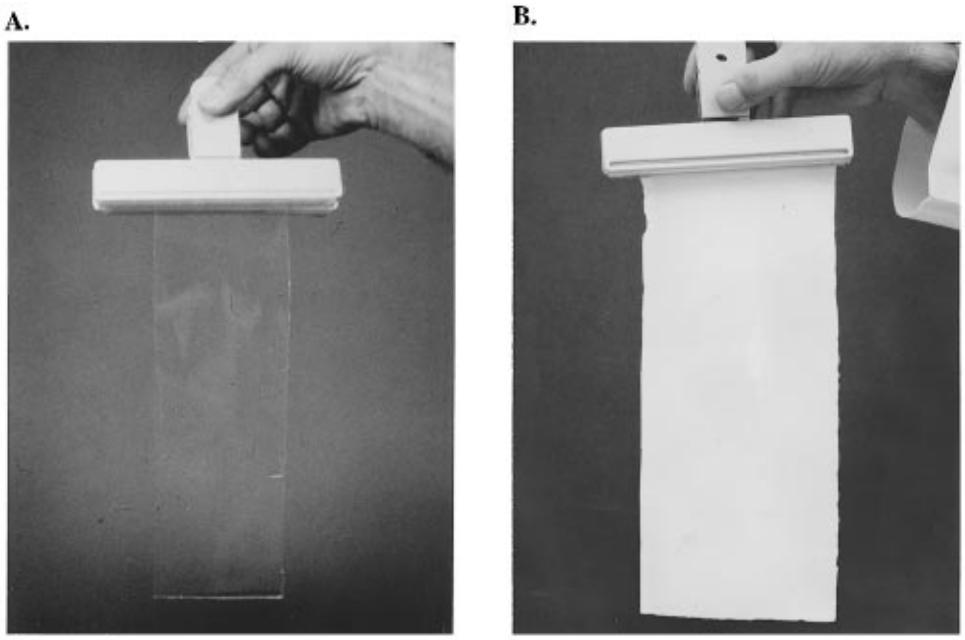


Figure 4. Sheets of γ-irradiation cross-linked elastic protein-based polymers using 20 Mrad of cobalt-60 radiation to give X^{20}-(GVGVP)$_{251}$ in (A) and X^{20}-(GVGIP)$_{260}$ in (B).

to form the viscoelastic phase. Generally the β-position can be replaced by any of the naturally occurring amino acids with retention of repeating β-turn properties and of viscoelasticity. An exception to this is poly(GVGAP), which while soluble below 25 °C undergoes its phase transition with the formation of a granular precipitate rather than a viscoelastic phase.

Elastic Matrixes. The viscoelastic phase can be formed into any desired shape and γ-irradiation cross-linked in that shape to form an elastic matrix. Commonly, a 20 Mrad cross-linking dose is used and signified by the X^{20}-prefix. Examples of large strips are seen in Figure 4A for X^{20}-(GVGVP)$_{251}$ and 4B for X^{20}-(GVGIP)$_{260}$. For X^{20}-(GVGVP)$_{251}$, the phase transition is seen as a change in dimension by a factor of 2.

Mechanism of Elasticity. The mechanism of elasticity is discussed here more for completeness of understanding the molecular model system than as an essential element for understanding free energy transduction, although in our view the proper structural mindset allowed for polymer designs resulting in magnitudes of effects not observed with random polymers. Without large effects such as structure-dependent hydrophobic-induced pK_a shifts, the arguments for the mechanism would not have been so clear, and the path for testing of

concepts by successful polymer design would not have been so direct. Nonetheless the concept of entropic elasticity that arose early in these studies was most controversial and was not well received by those who held to the random chain network perspective of entropic elasticity for protein elasticity as earlier described by Hoeve and Flory.[62,63]

To be brief, the molecular structure in Figure 3E shows suspended segments between β-turns, involving the VGV sequence, wherein the two peptide moieties can undergo large rocking motions, called librations, which contribute much entropy to the folded and assembled state. Our proposal of a peptide librational entropy mechanism of elasticity argues that the amplitudes of these rocking motions become damped on extension.[48,64] Among the molecules synthesized to test this perspective, replacement of the G residue of VGV with an L-alanine (Ala, A) residue, which even with the addition of the small CH_3 side chain in place of an H would limit peptide rocking, was found to destroy elasticity.[65] Among the physical characterization used to test this perspective, large amplitude peptide librations were sought and found to occur in the folded and assembled state with a frequency between 1 and 25 MHz, as demonstrated by carbon-13 and nitrogen-15 nuclear magnetic relaxation (T_1, T_2, and NOE)[39,40] and by dielectric relaxation measurements with a dielectric increment of 70, whereas there is no dielectric relaxation observed in the entire 1 MHz to 1GHz frequency range for the unfolded state, i.e., below the temperature of the transition.[45] Among the computational (molecular mechanics and dynamics) tests of this perspective, the large amplitudes of these relaxations were observed, representing the internal chain dynamics, and these rocking motions become damped on extension resulting in large decreases in entropy.[50,51,64] Damping of internal chain dynamics on extension, we believe, provides the decrease in entropy on stretching of X^{20}-$(GVGVP)_{251}$ that becomes the restoring force with an elastic modulus of 2×10^6 dyn/cm^2 and an f_e/f ratio of less than 0.1. This is a magnitude of elastic modulus and entropic component equivalent to those observed for the natural mammalian elastic fiber where this sequence was observed to repeat 11 times in bovine elastin without a single substitution[66] and in porcine elastin with three isomorphous substitutions of the Val positions.[67,68]

The librational entropy mechanism of elasticity should not be considered only in the context of the β-spiral structure, but rather, in our view, the librational entropy mechanism of elasticity is relevant to all polymers that hydrophobically assemble in a manner that leaves backbone segments free to undergo rocking motions of such a nature that this internal chain dynamics becomes damped on extension.

Other Advantages To Be Treated in Detail Below. The other advantages of the protein-based polymer, poly(GVGVP)—of the development of the only hydrophobicity scale based directly on the hydrophobic folding and assembly process of interest, of clearly demonstrating hydrophobic-induced pK_a shifts, of providing the opportunity for characterization of waters of hydrophobic hydration, of designability for performing the free energy transductions of living organisms, and of the opportunity for generally elucidating principles of protein function and engineering—will become clear in what follows as they represent the primary points of this review.

The T_t-Perspective: A Point of View of Greatest Simplicity for Free Energy Transduction?

Water of Hydrophobic Hydration. Recognition of the distinctive properties of water resulting from the dissolution of nonpolar (hydrophobic) solutes has its roots with the observation of John Edsall in 1935[69] of uncommonly high apparent molal heat capacities, increasing by some 20−30 cal/mol for each CH_2 moiety in series of alcohols, fatty acids, and amino acids. Shortly thereafter, in 1937, Butler[70] noted that the low solubility of nonpolar solutes in water was an entropic effect as the dissolution of nonpolar solutes was an exothermic process; i.e., nonpolar solutes exhibit an exothermic heat of dissolution yet result in large negative entropy changes on dissolution. The most descriptive characterization of water surrounding apolar solutes came from the work of Frank and Evans[59] who described "...frozen patches or microscopic icebergs around such solute molecules."

The key observations reported by Frank and Evans[59] were the following: (1) entropies of vaporization, ΔS_v, exhibited by aqueous solutions are higher, by 10 EU (cal/mol-deg), for nonpolar solutes as compared to polar solutes; (2) a plot of molal ΔS_v versus ΔH_v of vaporization for water, which reflected the effects of the solutes on water structure, was linear for small aliphatics (CH_4, C_2H_6, chloroform, and ether) and rare gases (Rn, Xe, Kr, Ar, Ne, He) with a steeper slope (0.0024/deg) as compared to a slope of 0.0019/deg for a series of polar solutes, and (3) the temperature dependence of ΔS_v became steeper as ΔS_v became larger. Frank and Evans interpreted these data in terms of "...the following physical picture. When a rare gas molecule or a nonpolar solute dissolves in water at room temperature, it modifies the water structure in the direction of greater 'crystallinity'—the water, so to speak, builds a microscopic iceberg around it.* The extent of this iceberg is greater the larger the foreign atom. This 'freezing' of water produced by the rare gas atom causes heat and entropy to be lost, beyond what would 'otherwise' have been expected." In explaining the use of the term "iceberg," which they described as "surrounding a solute molecule,"...in "some sort of quasi-solid structure," they explicitly did *not* want to imply "that the structure is exactly ice-like, ...".

The first structural insight as to the exact nature of the water surrounding nonpolar solutes was due to the studies of Stackelberger and Müller (1951)[71] who reported the crystal structure of gas hydrates, or clathrates, to be pentagonal arrangements of water molecules completely surrounding aliphatic molecules, e.g., enclosing a methane molecule within a pentagonal dodecahedron of water molecules.

The relevance of such effects to protein structure came with Kauzmann's 1959 review[5] where he made use of the term "hydrophobic bond" in relation to protein folding, wherein disruption of the waters of hydrophobic hydration resulted in hydrophobic interactions. It took yet another quarter of a century before Martha Teeter (1984)[72] directly observed pentagonal arrangements of water at the surface of hydrophobic side chains in the small protein, crambin.

Throughout the half-century following the Frank and Evans work and the 38 years since Kauzmann's contribution, there have been continuing efforts to characterize this special water in terms of its dynamic properties. The image of rigid pentagons or icebergs suggested high barriers to motion and low frequency, but evidence for such was not to be found. Instead, a large number of water molecules with a relaxation frequency just lower than that of normal bulk water has been observed to disappear on hydrophobic folding and assembly.[15]

T_t as a Characterization of Waters of Hydrophobic Hydration. The Frank and Evans[59] plot of ΔS_v versus ΔH_v, which characterized nonpolar solutes with slopes of the order of 2.4×10^{-3}/deg, suggests that the inverse ratio for a transition, $\Delta H_t/\Delta S_t$, might be used to characterize the hydrophobic nature of amino acid residues in polymers that exhibit inverse

temperature transitional behavior. The heats of the inverse temperature transitions, however, are small and challenge the sensitivity and stability of instrumentation. The result is a value of the enthalpy for the transition, ΔH_t, that is correct to within about ± 10% and that could exhibit a variably and artificially broadened transition exacerbating a calculated entropy, $\Delta S_t = \sum_{t,i} \Delta H_i/T_i$, that would also be small and would contain added experimental error. Therefore, the routine use of the ratio $\Delta H_t/\Delta S_t$ would be plagued by variability and inaccuracies, and would generally be difficult to determine.

As the Gibbs free energy for the transition is zero, i.e., $\Delta G_t = \Delta H_t - T_t\Delta S_t = 0$, then $\Delta H_t = T_t\Delta S_t$, and, therefore, $T_t = \Delta H_t/\Delta S_t$. Whereas the ratio $\Delta H_t/\Delta S_t$ would be difficult to determine accurately, T_t, the temperature of the transition, provides a more easily and accurately determined quantity with which to characterize the molecular system and with which to draw comparisons of protein-based polymers of different compositions. In general, the mean temperature of the transition might be used, but there is an experimentally more precise and readily determined quantity. It is the onset of the transition, which, while lacking information of breadth of the transition, provides the practical information as to when the transition would initiate. Using the onset of the transition for T_t puts in one's hands an easily controlled on/off switch for the transition.

Comparison of T_t and ΔS_t for the Inverse Temperature Transition. Effects of altering the number of CH_2 moieties on changing the values for T_t and ΔS_t can be assessed using the model protein-based polymer system, poly[f_V(GVGVP),f_X-(GXGVP)], where f_V and f_X are mole fractions with $f_V + f_X = 1$ and X is any of the naturally occurring amino acids and chemical modifications thereof. The T_t values extrapolated to an f_X equal to 1, i.e., to the composition of poly(GXGVP), for an X of Ala ($-CH_3$), of Val ($-CH(CH_3)_2$), and of Ile ($-CH(CH_3)CH_2CH_3$) are 45, 24, and 10 °C, respectively. As seen below in Table 2, the values of ΔS_t for the same series are 2.64, 3.90, and 4.60 cal/mol-K, respectively, that is, approximately 0.65 cal/mol-K per CH_2 moiety.[73] ΔS_t increases linearly with the number of CH_2 moieties, whereas the T_t values are not so linear. On the other hand, as will be seen below, a plot of T_t versus f_X is a linear quantity for values of f_X up to 0.5 or more. This makes it possible to develop a relative hydrophobicity scale based on the practical knowledge of T_t.

The T_t-Based Hydrophobicity Scale. For developing the T_t-based hydrophobicity scale, the chosen model protein-based polymer is poly[f_V(GVGVP),f_X(GXGVP)], where f_V and f_X are mole fractions with $f_V + f_X = 1$ and where X is any of the amino acid residues. For this effort, hundreds of polymers were synthesized, each including as the guest residue, X, one of the 20 naturally occurring amino acid residues with different values of f_X. It then becomes possible to determine the value of T_t for each protein-based polymer as a function of f_X under common solvent conditions. In general, the chosen medium is phosphate-buffered saline (0.15 N NaCl and 0.01 M phosphate) at pH 7.4 unless another pH is required for a particular state of a functional side chain.[8,12]

Figure 5A shows the temperature profiles for the onset of aggregation for a series of protein-based polymers containing different guest residues at an f_X of 0.2, and Figure 5B demonstrates linear plots of f_X versus T_t for all of the amino acid residues and a few interesting modifications thereof. If these plots are extrapolated to a common value of f_X, then a measure of the relative hydrophobicities is obtained. For convenience as indicated in Table 2,[6,8,73] the comparisons are made at the extrapolated values of $f_X = 1$. Clearly, lower values of T_t correlate with greater hydrophobicity, and higher values

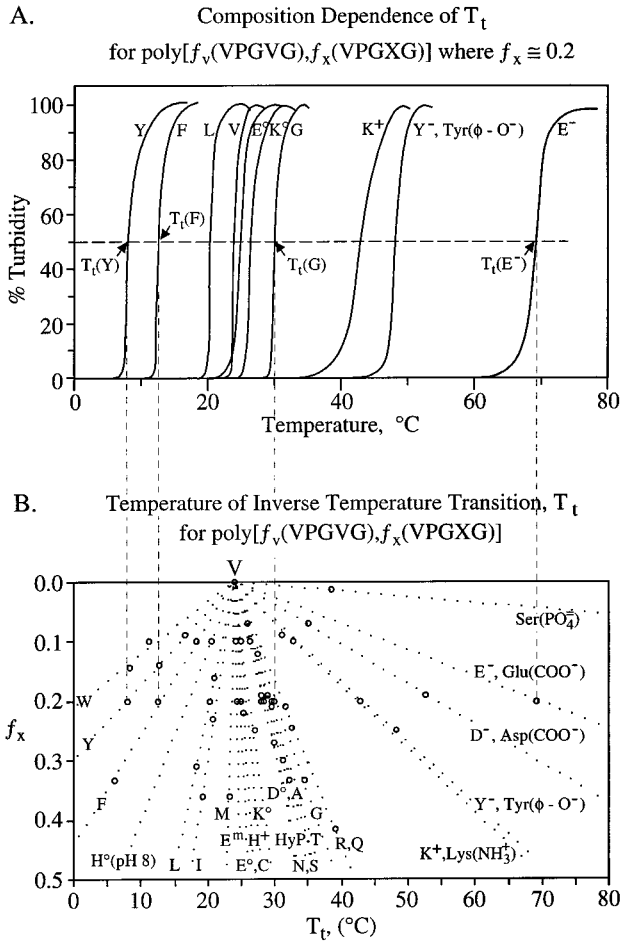


Figure 5. (A) Temperature profiles of aggregation of poly[f_V-(VPGVG),f_X(VPGXG)] for $f_X = 0.2$ with different X-residues at 40 mg/mL and high molecular weights. (B) Plots of T_t versus f_X for all of the amino acid residues and some chemical modifications thereof. This provides the data for Table 2, the T_t-based hydrophobicity scale. See text for discussion. Reproduced with permission from refs 8 and 12.

of T_t provide a measure of more polar side chains. Additional chemical modifications and prosthetic groups of relevance to biology are included in Table 3.[74,75]

Practicality of the T_t-Based Analysis. With the T_t perspective, one only need know T_t, i.e., where the temperature of the inverse temperature transition is, to know whether the protein-based polymer is hydrophobically folded and assembled or whether it is dissolved in solution, or, if cross-linked, whether the matrix is contracted or swollen, that is, one need not know several additional pieces of information and carry out a calculation before it is possible to know what a Gibbs free energy or an enthalpy or an entropy might mean with regard to the state of the system or how to move the molecular system from one state to another. With the T_t perspective, it is only necessary to know where T_t is with respect to the operating temperature and how to control the value of T_t. Perhaps the T_t perspective does provide the point of view of greatest simplicity for free energy transduction catalyzed by protein-based polymers capable of inverse temperature transitions. The following section contains a brief discussion of many ways whereby T_t can be controlled.

Dependencies of the Temperature, T_t, of the Inverse Temperature Transition. An extensive, but incomplete, list of the dependencies of T_t is given in Table 4. Most of the information given in Table 4 has been previously reviewed,[8,76] although a substantial amount of relevant data has yet to appear in print.[73] As this review focuses on free energy transduction, however, there is insufficient space for inclusion of such detail.

TABLE 3: T_t-Based Hydrophobicity Scale for Chemical Modifications and Prosthetic Groups;[a] T_t = Temperature of Inverse Temperature Transition for Poly[f_V(VPGVG),f_X(VPGXG)][e]

residue X	T_t (°C), extrapolated to $f_X = 1$
Lys(dihydro-NMeN)[b,d]	−130
Glu(NADH)[c]	−30
Lys(6-OH-tetrahydro-NMeN)[b,d]	15
Glu($FADH_2$)	25
Glu(AMP)	70
Glu(NAD)[c]	120
Lys(NMeN, oxidized)[b,d]	120
Glu(FAD)	120
Ser(PO_4^{2-})	1000

[a] The usual conditions are for 40 mg/mL polymer, 0.15 N NaCl, and 0.01 M phosphate at pH 7.4. [b] NMeN is for *N*-methyl nicotinamide pendant on a lysyl side chain, i.e., *N*-methyl nicotinate attached by amide linkage to the ϵ-NH_2 of Lys, and the most hydrophobic reduced state is *N*-methyl-1,6-dihydronicotinamide (dihydro-NMeN), and the second reduced state is *N*-methyl-1,6-OH 1,4,5,6-tetrahydronicotinamide (6-OH-tetrahydro-NMeN). [c] For the oxidized and reduced nicotinamide adenine dinucleotides, the conditions were 2.5 mg/mL polymer and 0.2 M sodium bicarbonate buffer at pH 9.2. [d] For the oxidized and reduced *N*-methyl nicotinamide, the conditions were 5.0 mg/mL polymer, 0.1 M potassium bicarbonate buffer at pH 9.5, and 0.1 M potassium chloride. [e] Reproduced with permission from ref 75.

Nonetheless, the message should be very clear; the countless variables that alter or control T_t are comprehensive.

Virtually every interaction and modification that a polymer capable of an inverse temperature transition may have can be characterized in terms of its effect on T_t, the temperature of its inverse temperature transition. This provides a unifying perspective for understanding polymer-based free energy transduction, i.e., the T_t-perspective, a proposed point of view of greatest simplicity.

The ΔT_t-Mechanism of Free Energy Transduction. *Driving Hydrophobic Folding To Produce Mechanical Work.* Simply by changing the temperature of the inverse temperature transition—by changing the value of T_t—a properly designed protein-based polymer can catalyze a change in free energy from one form to another. This ΔT_t-mechanism of free energy transduction is schematically represented in Figure 6A. The initial example was chemomechanical transduction, a proton-driven contraction.[26] The elastic matrix, X^{20}-poly[f_V(GVGVP),f_E-(GEGVP)], exhibited an apparent pK_a of 4.5, as obtained from a plot of T_t versus pH. As shown in Figure 6B, on holding this matrix at a fixed extension at a pH of 2.1, it begins developing force as the temperature is raised above 20 °C, whereas when at a pH of 4.5, the matrix does not begin to develop force until the temperature is above 40 °C. Both curves are examples of thermomechanical transduction. If the temperature is held constant, say at 37 °C, and the pH is lowered to 2, however, the elastic matrix contracts and lifts a weight, and, on raising the pH to 4.5, it relaxes and lowers the weight.[26] Increasing the chemical potential of proton results in the contraction of the matrix with the performance of mechanical work.

As represented in Figure 6A for length changes that would perform mechanical work, an extended β-spiral shortens by hydrophobic folding over a particular temperature range depending on its hydrophobicity, and any energy input that lowers T_t from above to below an operating temperature can drive hydrophobic folding, isothermally, to perform mechanical work. As the polymers become more hydrophobic, T_t becomes lower and the transition becomes sharper.

Coupling of Different Functional Groups in the Same Hydrophobic Domain. Protonation of a carboxyl increases hydrophobicity, i.e., lowers T_t, and reduction of a redox function also increases hydrophobicity and lowers T_t. The coupling of these effects is shown schematically as proposed in Figure 7A where the increase in hydrophobicity due to reduction would raise the pK_a of a carboxyl and where the increase in hydrophobicity due to protonation of a carboxylate would shift the redox potential.[9] A specific example involving the carboxyl of an aspartic acid residue and an *N*-methyl nicotinamide attached to a Lys side chain is seen in Figure 7B. Reduction of the nicotinamide lowers T_t and raises the pK_a of the carboxyl.[10,11] As the reaction is carried out at pH 9.2 for reasons of *N*-methyl nicotinamide stability and as the pK_a shifts from 8.5 to 11, at this pH reduction drives the uptake of a proton. The electrical energy input has resulted in a chemical energy output. This is electrochemical transduction.

The Hexagon: A Representation of Pairwise Interconversions of Six Energies. Schematically represented in Figure

TABLE 4: Dependencies of the Temperature, T_t, of the Inverse Temperature Transition

1. Dependence of T_t on polymer concentration[32]
2. Dependence of T_t on polymer chain length[30,32]
3. Dependence of T_t on polymer amino acid composition[8,12] (Table 2)
4. Dependence of T_t on salts, e.g., the Hofmeister (lyotropic) series[8]
5. Dependence of T_t on organic solutes and solvents[8]
6. Dependence of T_t on polymer side chain ionization[8,26]
7. Dependence of T_t on chemical modification of polymer side chains[75] (Table 3)
   Examples: phosphorylation, nitration, sulfation, and glycosylation
8. Dependence of T_t on pressure (special role of aromatic residues)[82]
   a. Clapeyron-Clausius relation and calculation of ΔV for W, F, and Y
   b. Estimation of numbers of water molecules equivalent to the ΔV
9. Dependence of T_t on redox state of prosthetic group attached to polymer[28,83]
   Examples: *N*-methyl nicotinamide (NMeN), nicotinamide adenine dinucleotide (NAD), and flavin adenine dinucleotide (FAD) (Table 3)
10. Dependence of T_t on the absorption of light by prosthetic group attached to polymer[84]
    Examples: azobenzene and cinnamide
11. Dependence of T_t on side chain charge neutralization by ion pairing
    a. Cation neutralization of anionic side chain
       (1) Na^+ salts (unpublished)
       (2) calcium ion binding at carboxylates with shifted pK_a values (unpublished)
       (3) cationic drugs binding at carboxylates with shifted pK_a values[76]
       (4) similar binding of cationic cofactors, prosthetic groups, etc. (unpublished)
    b. Anionic neutralization of cationic side chain
       (1) anions binding at amino groups with shifted pK_a values (unpublished)
       (2) anionic drugs binding at amino groups with shifted pK_a values[76]
       (3) similar binding of anionic cofactors, prosthetic groups, etc. (unpublished)
    c. Ion pairing between chains (unpublished)
       (1) anionic polymer associating with cationic polymer
       (2) pairing between chains of periodic alternating charge

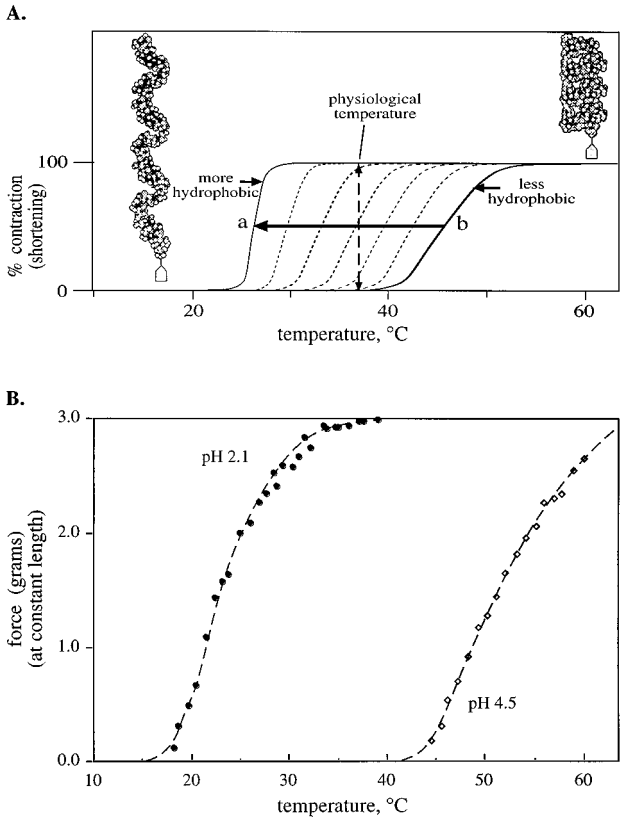


Figure 6. (A) Plots as a function of temperature of the shortening or contraction of elastomeric strips of cross-linked elastic protein-based polymers of different hydrophobicities. As the polymer becomes more hydrophobic, the contraction, which is a representation of the phase transition, occurs at lower temperatures and over a narrower temperature interval. The shortening is schematically represented as the hydrophobic folding of a single β-spiral of poly(GVGVP) doing the useful mechanical work of lifting a weight. It will be seen below in Figure 16 that this plot can be general for different free energy transductions where different intensive variables (potentials) are used in place of temperature. (B) Experimental data of the development of force in the protein-based polymer, poly[0.8(GVGVP),0.2(GEGVP)], at pH of 2.1 where all Glu residues are as carboxyls (COOH) and at pH of 4.5 where there are approximately 2 carboxylates per 100 residues. Charge formation raises the temperature at which the inverse temperature transition occurs. Therefore, at a constant temperature of 37 °C, changing the pH can drive contraction/relaxation, that is, chemomechanical transduction. Adapted with permission from ref 26.

8 are the 6 energies and the associated 15 pairwise free energy transductions that can occur by means of the inverse temperature transitions of properly designed protein-based polymers. There are many ways to refer to the representations of Figure 8; they can be discussed in terms of the energies or *Vis Vita* of Leibnitz,[77] of the forces as used by Newton or of the potentials of Gibbs. Perhaps it is most clear to use potentials or their equivalences and thereby separate the energies into the products of intensive factors or variables and extrinsic or capacity factors or variables. In this context, the six relevant intensive variables of the free energy are mechanical force, pressure, chemical potential, temperature, electrochemical potential, and electromagnetic frequency.

The 15 pairwise free energy transductions considered possible by the ΔT_t-mechanism are discussed further in terms of Axioms 2, 3, and 4. There exists a very important 16th pairwise conversion, chemo-chemical transduction, i.e., the conversion of one form of chemical energy into another. Protein-based polymers have been designed and experiments have been performed that demonstrate to date 10 out of 16 of these energy conversions.

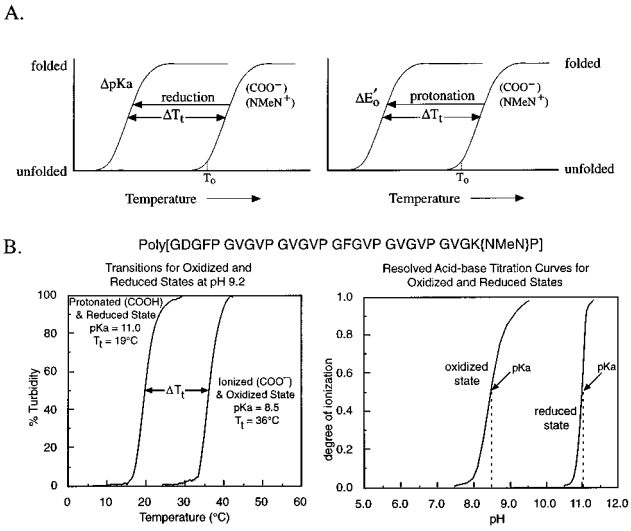


Figure 7. (A) Representation of the ΔT_t-mechanism when the elastic protein-based polymer contains two different functional groups. Either reduction lowers the value of T_t (increases the hydrophobicity) to induce a change in the pK_a of carboxyls or protonation lowers the value of T_t (increases the hydrophobicity) to induce a change in the reduction potential of a redox function. Reproduced with permission from ref 9. (B) Reduction of a particular designed elastic protein-based polymer, a polytricosapeptide containing an aspartyl residue and an *N*-methyl nicotinamide attached to a lysyl side chain, lowers the value of T_t (plot on left side of part B) and raises the pK_a of the carboxyl functional group of aspartic acid (plot on right side of part B). Adapted or reproduced with permission from refs 10 and 11.

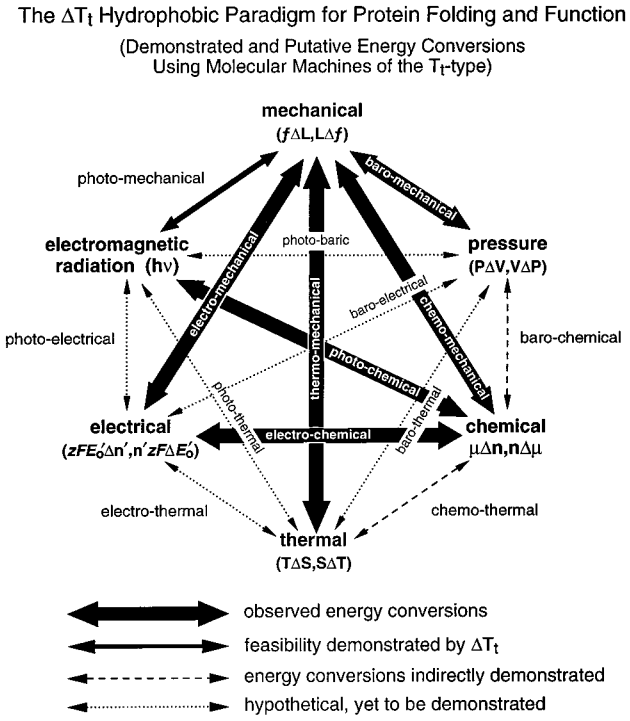


Figure 8. Hexagonal representation of the pairwise interconversions of six different energies, which appear to be possible by means of inverse temperature transitions of specifically designed protein-based polymers. The boldfaced arrows indicate observed conversions.

Five Axioms for Free Energy Transduction by Protein-Based Polymers Capable of Inverse Temperature Transitions. Listed in Table 5 are the set of five axioms that derive from these studies on free energy transduction by means of elastic protein-based polymers capable of inverse temperature transitions. The relevant axiom of Table 5 should be consulted when reading the following more detailed explanations.

TABLE 5: Five Axioms for Protein Engineering of Protein-Based Polymers Capable of Inverse Temperature Transitions of Hydrophobic Folding and Assembly

AXIOM 1: The manner in which a guest amino acid residue, or chemical modification thereof, alters the temperature, T_t, of a hydrophobic folding and/or assembly transition is a functional measure of its hydrophobicity. A decrease in T_t represents an increase in hydrophobicity, and an increase in T_t represents a decrease in hydrophobicity.
AXIOM 2: Raising the temperature above T_t results in hydrophobic folding and assembly and can be used to perform useful mechanical work, e.g., of lifting weights; this is thermomechanical transduction.
AXIOM 3: At constant temperature, lowering the value of T_t from above to below an operating temperature, i.e., increasing the hydrophobicity by any of the many variables of Table 4, also results in hydrophobic folding and assembly and can be used to perform useful mechanical work of building a structure, e.g., of lifting a weight.
AXIOM 4: Any two distinct functional groups responsive to different variables among the many of (i) temperature, (ii) pressure, (iii) changes in the concentrations of chemicals, (iv) changes in the redox state of a biological prosthetic group, and (v) light-elicited changes in chemical structure, each of which could be used to alter the value of T_t to perform mechanical work resulting from folding and assembly, can be coupled one to the other by being part of the same hydrophobic folding and assembly domain.
AXIOM 5: The above energy conversions can be demonstrated to be more efficient when carried out under the influence of more hydrophobic domains.

Axiom 1 enables design of functional protein-based polymers. The thermally elicited hydrophobic folding and assembly transitions of polymers—of the composition poly[f_V(GVGVP),f_X-(GXGVP)], where f_V and f_X are mole fractions with $f_V + f_X = 1$ and where X was each of the naturally occurring amino acids and relevant chemical modifications thereof—allowed for the development of a hydrophobicity scale based on the temperature, T_t, at which the hydrophobic folding and assembly transition occurs.[8,12] Since for a transition $\Delta G_t = \Delta H_t + T_t\Delta S_t = 0$, the value of T_t is a measure of the heat of the transition, ΔH_t, divided by the entropy change for the transition, ΔS_t, i.e., $T_t \approx \Delta H_t/\Delta S_t$. As noted previously, a decrease in T_t represents an increase in hydrophobicity, and an increase in T_t represents a decrease in hydrophobicity. The T_t-based hydrophobicity scale becomes a particularly practical scale. With warm blooded animals or with any other constant temperature condition, it matters less what the heat of the transition is but critically whether the value of T_t is above or below the operating temperature.

Axiom 2 demonstrates the performance of mechanical work by raising the temperature. As an engine is a machine capable of performing useful mechanical work, protein-based polymers capable of performing useful mechanical work can be referred to as molecular engines. As the work performed is a direct result of the inverse temperature transition of hydrophobic folding and assembly, such molecular engines are referred to as *T_t-type molecular machines of the first kind.* This is *thermomechanical transduction.*[78,79]

Axiom 3 entails the performance of mechanical work by changing T_t, and it includes the energy conversions of *chemomechanical*,[13,20,26,80] *baromechanical*,[81,82] *electromechanical*[28,83] and *photomechanical*[84] *transduction*. Protein-based polymers capable of performing useful mechanical work due to a change in T_t, i.e., a ΔT_t, are also *T_t-type molecular machines of the first kind* as the hydrophobic folding and assembly directly performs mechanical work. A video has been made of appropriately designed and cross-linked elastic protein-based polymers pumping iron on the basis of *Axioms 2* and *3*.

Axiom 4 utilizes two different functional groups as part of the same hydrophobic domain. These elastic protein-based polymers are called *T_t-based molecular machines of the second kind* (molecular machines involved in the performance of other than mechanical work). The set of relevant free energy transductions are: *electrochemical*,[10] *electrothermal*, *baroelectrical*, *photovoltaic*, *thermochemical*, *photothermal*, *barothermal*, *barochemical*, *photobaric*, *photochemical*, and *chemochemical transduction*.

This axiom includes the very important biological energy conversions of electrochemical transduction (the redox-driven proton concentration gradient of the inner mitochondrial and thylakoid membranes) and chemochemical transduction of oxidative phosphorylation (the proton gradient-driven phosphorylation of ADP to form ATP, the universal biological energy currency). With properly designed elastic protein-based polymers, electrochemical transduction has been demonstrated utilizing chemically synthesized Asp and *N*-methyl nicotinamide-Lys residues,[10] and designed polymers using recombinant DNA technology containing Glu and Lys with *N*-methyl nicotinamide chemically attached have also demonstrated electrochemical transduction. The use of the inverse temperature transition of model proteins to achieve coupling of functional groups differs substantially from the more commonly described energy coupling in chemical reactions previously considered relevant to living organisms.[77]

The Principle of Le Châtelier. With regard to axioms 3 and 4 the Principle of Le Châtelier may be stated as follows: for any system capable of an inverse temperature transition and at rest (at equilibrium), the introduction of a stress such as a particular energy input that changes the temperature of the inverse temperature transition from one side to the other of the operating temperature causes the system to react in such a way as to relieve the stress as a particular energy output. It is the design of the polymer, of course, that determines what the input and output energies are to be. The question now becomes one of the mechanism whereby the stress is sensed and the particular condition wherein the stress can be relieved during an inverse temperature transition of hydrophobic folding and/or assembly.

Axiom 5 is the efficiency axiom.[9,19,20] The basis for it is given below in Figures 9, 12, and 14, wherein it is demonstrated for systems capable of inverse temperature transitions that more efficient energy conversion occurs when under the influence of more hydrophobic environments or domains.

Physical Basis for Free Energy Transduction

At a superficial glance, there exists a parallel between the charge−charge repulsion mechanism for chemomechanical transduction where extension occurs with increased ionization[25] and the inverse temperature transition related mechanism where ionization also results in extension,[26] and incautious observers might leap to conclude a common underlying mechanism. The initial response to such a view, of course, includes the argument of the paucity of charge, only 2 or 3 charges per 300 backbone atoms for the T_t-driven polymer results in full extension, whereas 50 or more charges per 200 backbone atoms are required in water for an electrostatic repulsion based polymer such as poly(methacrylic acid) to result in full extension. The next level in the exchange introduces the argument for a low dielectric constant in the T_t-driven polymer leading to charge−charge repulsion at greater distances, but at the macroscopic level for

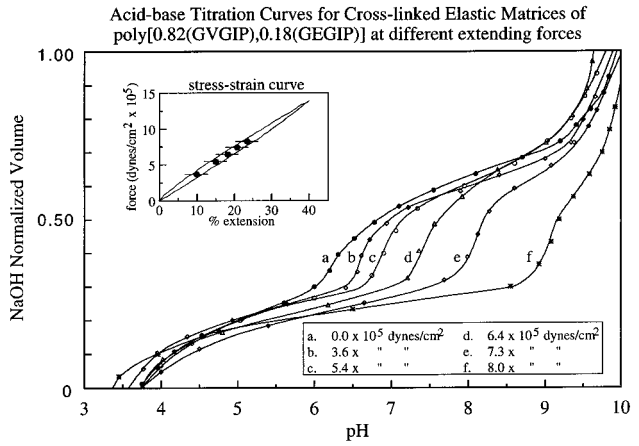


Figure 9. Series of titration curves for cross-linked poly[0.82-(GVGIP),0.18(GEGVP)] for different extensions. As the mechanical force is applied, the pK_a increases. This is stretch-induced pK_a shifts with a striking nonlinearity between mechanical energy input and chemical energy output, as plotted below in Figure 14B. See text for discussion. Reproduced with permission from ref 20.

the state of the highest possible density, there is a measured dielectric constant of 65 in a particular case in point[46] rather than a value of 5 or less as required for the electrostatic repulsion view to obtain.[18] At this stage the electrostatic proponent turns to the microscopic or molecular dimension to say that a dielectric constant of 5 would be relevant at the condensed molecular level. As difficult as this is to accept for the phase separated state that contains a finely interspersed 50% water by weight, the definitive response for the new perspective is that the underlying mechanism can be observed while the polymer remains in the fully hydrated and unfolded state, and that changes in the degree of ionization at temperatures below T_t, without passing through the inverse temperature transition, critically change the nature of the important hydrophobic hydration.[15] A further discrepancy is that the titration curves for polymers wherein the charge−charge repulsion mechanism is operative exhibit negative cooperativity[21] whereas those of the polymers capable of inverse temperature transition exhibit positive cooperativity (see, for example, Figure 15 below). Fortunately, as described below, several sets of experimental data made possible in part by the many advantages of these elastic protein-based polymers, perhaps even more definitively, require replacement of the familiar perspectives of direct charge−charge interaction with perhaps an equally facile understanding of competition between apolar (hydrophobic) and polar (charged) residues for hydration.

Stretch-Induced pK_a Shifts, the Initial Physical Insight. *Delineation of Mechanism by $(\partial\mu/\partial f)_n$.* The charge−charge repulsion mechanism for chemomechanical transduction can be eliminated as the mechanism underlying free energy transduction catalyzed by elastic protein-based polymers capable of inverse temperature transitions. This delineation is achieved quite simply by determination of the pK_a of the cross-linked protein-based polymer matrix, X[20]-poly[0.82(GVGIP),0.18(GXGIP)], as a function of the extension force.[13,20] The elastomeric band is held at a fixed extension force, and an acid−base titration is carried out, as shown in Figure 9. On increasing the force, Δf is positive, yet as a result the concentration of proton, $[H^+]$, required to be at the pK_a, i.e., at a degree of ionization $\alpha = 0.5$, is decreased, that is, $\Delta\mu$ is negative where $\mu = RT \ln[H^+]$. Therefore, for the elastomeric protein-based polymer of Figure 9, $(\partial\mu/\partial f)_{n=\alpha=0.5} < 0$; that is, the partial of the chemical potential with respect to the force at constant composition ($n = \alpha = 0.5$) is negative. In short, stretching causes an uptake of protons. Just the opposite situation is the case for the charge−charge repulsion mechanism, $(\partial\mu/\partial f)_{n=\alpha=0.5} > 0$;[25] on extension of cross-linked poly(methacrylic acid), the pK_a decreases; the distance between charges increases allowing for more charges to form with the result that stretching causes the release of protons. Clearly, the mechanisms are different!

Hydrophobic Hydration Unsuited for Hydration of Charged Species. The increase in pK_a on stretching of the hydrophobically folded elastomeric protein-based polymer means that the free energy of the carboxylate increases; this is remarkable, because on stretching the elastomer takes up water in an exothermic reaction.[62,67] Before extension, the elastomeric matrix is hydrophobically folded and assembled. On stretching, the hydrophobic groups become forcibly unfolded, and this exposure to water results in an exothermic hydrophobic hydration, as discussed above. Surprisingly, even with addition of water to the matrix, the free energy of the carboxylate becomes less favorable. Why should the free energy of the charged carboxylate moiety increase on extension? One might expect that the increase in water, by hydrating the matrix including the charged group, would favor carboxylate formation. We are brought to the viewpoint that the form of the water in the matrix, the water of hydrophobic hydration, is not suited for hydration of charged groups.

A Proposed Apolar−Polar Repulsive Free Energy of Hydration. *Competition for Hydration between Charged and Hydrophobic Species.* We propose, as the answer to the above question, that there exists a competition for hydration between apolar (i.e., hydrophobic) and polar (e.g., charged) groups. It was concluded above, in relation to the T_t-based hydrophobicity scale of Tables 2 and 3, that more hydrophobic side chains lowered and that more polar side chains raised the temperature of the inverse temperature transition. The corollary is that increased water of hydrophobic hydration lowers T_t and that decreased hydrophobic hydration raises the value of T_t. Since formation of charged groups increases T_t, as occurs on ionization of carboxylates, it follows that formation of carboxylates would decrease the water of hydrophobic hydration. Since the ratio $\Delta H_t/\Delta S_t$, which is T_t, is an inverse measure of the relative amounts of hydrophobic hydration, it is to be expected, therefore, that T_t increases because in order to achieve its own hydration the charged species must take water from the hydrophobic hydration.

It is proposed, therefore, that the molecular mechanism underlying the Principle of Le Châtelier, as described above in relation to free energy transduction by elastic protein-based polymers capable of inverse temperature transitions, is the competition for hydration between apolar and polar species. In other words, there occurs a repulsive free energy of hydration between hydrophobic and charged species constrained to exist as components along a chain polymer. The next issue is whether experimental approaches exist that can provide a more direct observation of this competition.

Differential Scanning Calorimetry as a Function of Degree of Ionization. *Relevance of ΔH_t to the Amount of Hydrophobic Hydration.* Again it is noted that the dissolution of hydrophobic groups in water is an exothermic reaction resulting from the formation of structured water of hydrophobic hydration.[59,70] If the formation of structured waters surrounding hydrophobic groups is an exothermic reaction, then the inverse temperature transition of hydrophobic folding and assembly should be an endothermic reaction in order to provide the heat required to destructure the water of hydrophobic hydration.

If there is a competition for hydration between apolar and polar groups distributed along a chain molecule, then the

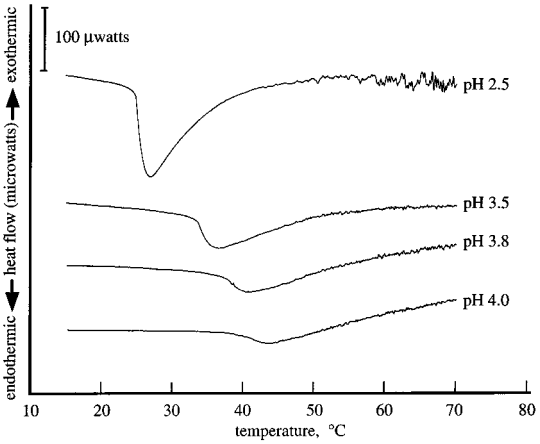


Figure 10. Series of differential scanning calorimetry curves for poly-[0.8(GVGVP),0.2(GEGVP)] at different pH values. As the polymer becomes charged the heat of the inverse temperature transition decreases. See text for discussion. Reproduced with permission from ref 14.

formation of charged species should decrease the amount of structured water of hydrophobic hydration. In terms of calorimetry data for the inverse temperature transition, the formation of charged species should, therefore, decrease the heat required to destructure the remaining water of hydrophobic hydration and result in a decreased ΔH_t.

The differential scanning calorimetry (DSC) data of Figure 10 reflects the above expectation.[14] As there are four glutamic acid (Glu, E) residues per 100 residues in the polymer, poly-[0.8(GVGVP),0.2(GEGVP)], of the DSC study and as the pK_a is 4.3, by the pH of 4.0 of the lower, fourth curve of Figure 10, fewer than two carboxyls have ionized to form carboxylates and the heat of the transition is reduced almost to one-fourth. *Therefore, the formation of less than two charges per 100 residues would appear to be sufficient to destructure three-quarters of the thermodynamically measured water of hydrophobic hydration.* As will be seen below, this same result is obtained when directly observing the water of hydrophobic hydration by means of microwave dielectric relaxation.

Direct Characterization of Water of Hydrophobic Hydration, N_{hh}. *An Intense Relaxation near 5 GHz.* Previous dielectric relaxation studies in the 1 MHz to 1 GHz range of several protein-based polymers suggested that there would be a relaxation just above 1 GHz with an intensity that would be dependent on hydrophobicity.[45,46] Using microwave dielectric relaxation in the 0.05−26.5 GHz range, the relaxation spectrum of pure water was compared with the two-component system of water plus protein-based polymer. At the polymer concentration of 1672 mg $(GVGIP)_{260}$ in four mL of pure water and at 7 °C, when plotted as the absorbance analogue of the imaginary part of the dielectric permittivity, a resolved relaxation with a maximum near 5 GHz is observed to be more intense than the water peak that occurs just above 10 GHz at this temperature.[15] This peak clearly must arise from water interacting with protein-based polymer. The issue becomes one of determining the nature of this interaction. Accordingly, the variables of concentration, hydrophobicity, temperature, and pH were used to characterize this water using four different protein-based polymer compositions.[15]

Concentration, Hydrophocicity, and Temperature Dependence of the 5 GHz Peak.[15] On a per pentamer basis the amount of interacting water increases with dilution, as seen in Figure 11A, and, as required for water of hydrophobic hydration, there is more water per pentamer for the more hydrophobic $(GVGIP)_{260}$ than for $(GVGVP)_{251}$. The most informative data for the identification of the nature of the interacting water is seen in Figure 11B. On raising the temperature above 7 °C, the water per pentamer drops to near zero exactly as the temperature passes above 10 °C for $(GVGIP)_{260}$ and above 25 °C for $(GVGVP)_{251}$. These are the temperatures for hydrophobic folding and assembly transitions for the respective polymers. The interacting water disappears as the polymers hydrophobically fold and assemble. *The conclusion is that the water with a relaxation nominally at 5 GHz is water of hydrophobic hydration!* This water of hydrophobic hydration is now referred to as N_{hh}.

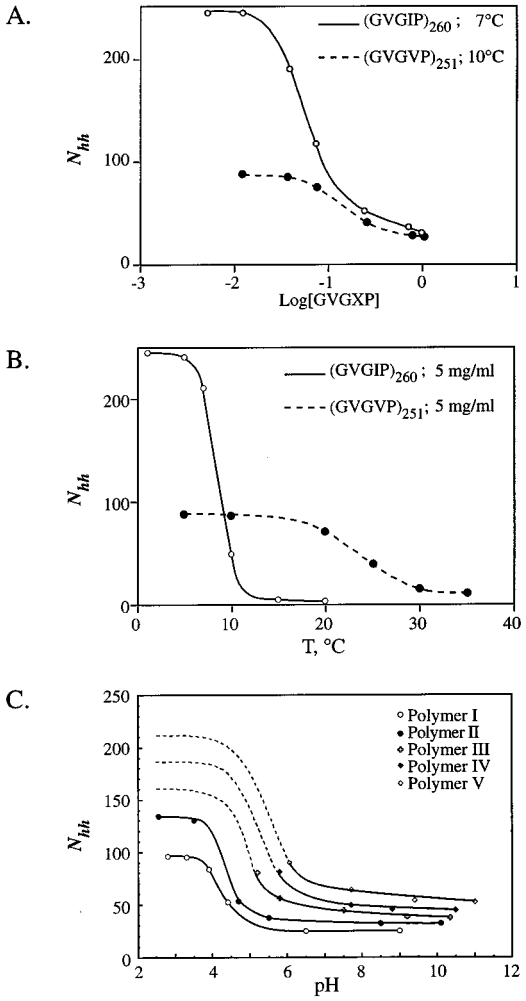


Figure 11. Microwave dielectric relaxation data used to estimate the amount of water of hydrophobic hydration, N_{hh}. See text for discussion. Reproduced with permission from refs 15 for (A) and (B) and 74 for (C).

While water near this relaxation frequency had been previously observed for several proteins,[85−88] such water had not been directly correlated with the hydrophobic folding and assembly process. Also using microwave dielectric relaxation, a weakly restrained water has recently been identified in the same frequency range for cytochrome *c*, myoglobin, ovalbumin, bovine serum albumin, and hemoglobin by Suzuki and coworkers,[89] and most recently for the myosin S1 fragment where correlation was made between the weakly restrained water and numbers of CH_2 moieties[90] on the surface, of a crystal structure of the S1 subfragment.[91] The data of Figure 11A,B provide the foundation that they may indeed be observing water of hydrophobic hydration in this much more complex system.

The somewhat surprising feature of the data of Figure 11 is the magnitude of N_{hh} per pentamer. While the method of approximating N_{hh} is subject to some error, we believe less than

20%, the estimation of 100 and more molecules per pentamer indicates that the hydrophobically perturbed water extends well beyond the surface layer of previously observed pentagonally arranged waters seen in crystal structures.

Thus, instead of a few more rigidly held water molecules from the envisioned "icebergs" of Frank and Evans[59] *undergoing large entropy and heat changes to become bulk water, it would appear, during the inverse temperature transition, that a larger number of more dynamic water molecules undergo smaller entropy and heat changes per molecule to become bulk water.*

pH Dependence of the 5 GHz Relaxation, i.e., of N_{hh}. For concentrations of 30 mg/mL and at 1 °C, the pH dependence of the estimated waters of hydrophobic hydration, $N_{hh,}$ is given in Figure 11C for the following five polytricosapeptides:[74]

I (GVGVP GVGVP G<u>E</u>GVP GVGVP GVGVP GVGVP)$_n$(GVGVP) where $n = 36$

II (GVGVP GVG**F**P G<u>E</u>G**F**P GVGVP GVGVP GVGVP)$_n$ where $\langle n \rangle = 40$

III (GVGVP GVGVP G<u>E</u>GVP GVGVP GVG**F**P G**F**G**F**P)$_n$(GVGVP) where $n = 39$

IV (GVGVP GVG**F**P **G**<u>**E**</u>**GFP** GVGVP GVG**F**P GVG**F**P)$_n$(GVGVP) where $n = 15$

V (GVGVP GVG**F**P **G**<u>**E**</u>**GFP** GVGVP GVG**F**P G**F**G**F**P)$_n$(GVGVP) where $n = 42$

First, it is noted that polymer II containing two more hydrophobic Phe (F) residues per 30-mer than polymer I exhibits an increase in N_{hh}. The increase can be independently estimated using the T_t-based hydrophobicity scale[8,15] and the N_{hh} values for $(GVGVP)_{251}$ and $(GVGIP)_{260}$. Most significantly, approximately two-thirds of the waters of hydrophobic hydration are lost by the point at which the pH reaches the pK_a, that is, by the point at which there are 1.7 charged carboxylates, COO^-, per 100 residues. Recall that the calorimetry data of Figure 10 was similarly interpreted and that a competition for hydration had been the interpretation of the stretch-induced pK_a shifts of Figure 9. For the first time in Figure 11C, therefore, *the proposed competition for hydration between hydrophobic and charged moieties is directly observed; on formation of charged species water of hydrophobic hydration is destructured.*

Competition for Hydration without a Change in Phase. It should be emphasized that at the temperature (1 °C) of the pH titration of Figure 11C there is no phase separation for polymers I and II; the T_t values for these polymers are above 1 °C for both the uncharged (COOH) and ionized (COO^-) states. Accordingly, the difference in pK_a values for the two polymers cannot be due to an assumed change in the dielectric constant of a condensed phase. Furthermore, as the polymers are made more hydrophobic and T_t for the COOH state moves below 0 °C, as for polymers III, IV, and V, there exists a smooth progression in the increase in pK_a (note Figure 14 below). It would seem clear that the change in pK_a does not arise from the changed dielectric constant on going to a condensed phase but rather the pK_a shift is the result of the competition for hydration in the hydrated state.

The Virtual Nature of the Fully Hydrophobically Hydrated State for T_t below 0 °C. When a sufficiently hydrophobic protein-based polymer is hydrophobically unfolded due to the ionized state, as the protonation proceeds, the polymer hydrophobically folds and assembles without ever having formed the totally hydrated state because the value of T_t for the fully hydrophobically hydrated state is below 0 °C. This is the case for polymers III, IV, and V of Figure 11C. It is only necessary that the hydrophobic hydration progress to the point where the value of T_t moves some 10−15° below the operating temperature to achieve complete folding and assembly. Under many circumstances for natural proteins, the fully hydrophobically hydrated state is not experimentally accessible, and, therefore, it is not quite so obvious that the competition for hydration is the underlying mechanism. This is one of the significant advantages of research using protein-based polymers with the stepwise design capacities.

Hydrophobic-Induced pK_a Shifts. In addition to the stretch-induced pK_a shifts of Figure 9, three studies using different model protein compositions and experimental variables have systematically varied hydrophobicity and thereby demonstrated hydrophobic-induced pK_a shifts: (1) dilution of a recurrent ionizable residue in a poly(5-mer) from 1 per 5 residues to 1.2 per 100 residues by stepwise replacement with an apolar (hydrophobic) residue,[16−18] (2) maintenance of the composition of a poly(30-mer) but rearranging the proximity of more hydrophobic residues to the ionizable residue,[93] and (3) increasing systematically the hydrophobic residues within a 30-mer while holding the ionizable residue of the poly(30-mer) constant at 1 per 30 residues.[19,20]

The Poly[f_V(IPGVG),f_X(IPGXG)] Model System with f_X Varied from 1 to 0.06. Using the polypentapeptide model system, poly-[f_V(IPGVG),f_X(IPGXG)], where f_V and f_X are mole fractions with $f_V + f_X = 1$, where X is a residue with an ionizable function such as the carboxyl of an aspartic acid (Asp, D) or a glutamic acid (Glu, E) residue or the amino function of a lysine (Lys, K) residue, the quantity f_X is varied from 1 to 0.06.[16−18] The pK_a is then determined and plotted as a function of f_X. As seen in Figure 12A for Glu in water at $f_X = 1$, the pK_a is shifted somewhat higher than the minimal pK_a at an intermediate value of f_X near 0.7. On further dilution, the pK_a increases again even more dramatically; in fact, the pK_a increases asymptotically as f_X approaches zero.

Significantly, the increase in pK_a as f_X approaches 1 from 0.7 is removed on addition of 0.15 N NaCl; this is consistent with the increase in pK_a at high values of f_X being due to an electrostatic effect, i.e., charge−charge repulsion, that is screened by an ionic atmosphere. Similar effects of salt are seen in Figure 12, however, at low values of f_X, but only a small part of the pK_a shift is relaxed. Decreasing the number of polar, ionizable residues while increasing the number of hydrophobic residues appears to be a more effective way to induce pK_a shifts in water than increasing the number of charged residues. Clearly, the free energy of the carboxylate anion increases as the hydrophobicity increases.

Limitations of the Born Expression for Estimating These pK_a Shifts. In Figure 12B, it is the free energy of the NH_3^+ of the lysine residue that increases with charge−charge repulsion at f_X values greater than 0.75 and also with increased hydrophobicity for f_X values less than 0.75 such that the pK_a decreases with increased charge density and with increased hydrophobicity. Calculations for Glu-, Asp-, and Lys-containing polymers have been carried out using the Born expression for the free energy of solvation, $\Delta G(\mathrm{SE}) = [(ze)^2/2r](1 - \epsilon^{-1})$,[92] where the charge on the ion is z, the unit electron charge is e, the dielectric constant of the surroundings of the charge is ϵ, and the radius of the ion is r. In particular, the change in the solvation energy, $\Delta\Delta G(\mathrm{SE})$, due to a change in the dielectric constant from ϵ_i to ϵ_{i-1} becomes $[(ze)^2/2r][(\epsilon_{i-1} - \epsilon_i)/\epsilon_{i-1}\epsilon_i]$. The elements of the calculation are presented elsewhere,[18] and the calculated curves using different values for the minimal dielectric constant are plotted in Figure 12B. Only with the unreasonable dielectric constant of 5 or less can the experimental curve be approximated. It was already demonstrated above, however, that the pK_a shifts begin without formation of the phase separated state and continue in a uniform manner without discontinuity

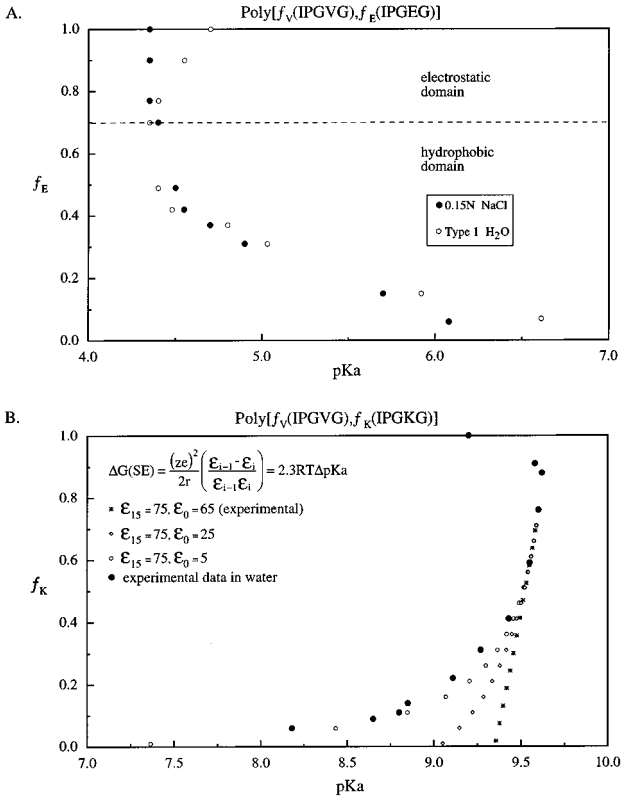


Figure 12. Plots of the pK_a dependence on the mole fraction, f_X, for the protein-based polymer, poly[f_V(IPGVG),f_X(IPGXG)] for both X = E in part A and X = K in part B. See text for discussion. Reproduced with permission from refs 16 and 18.

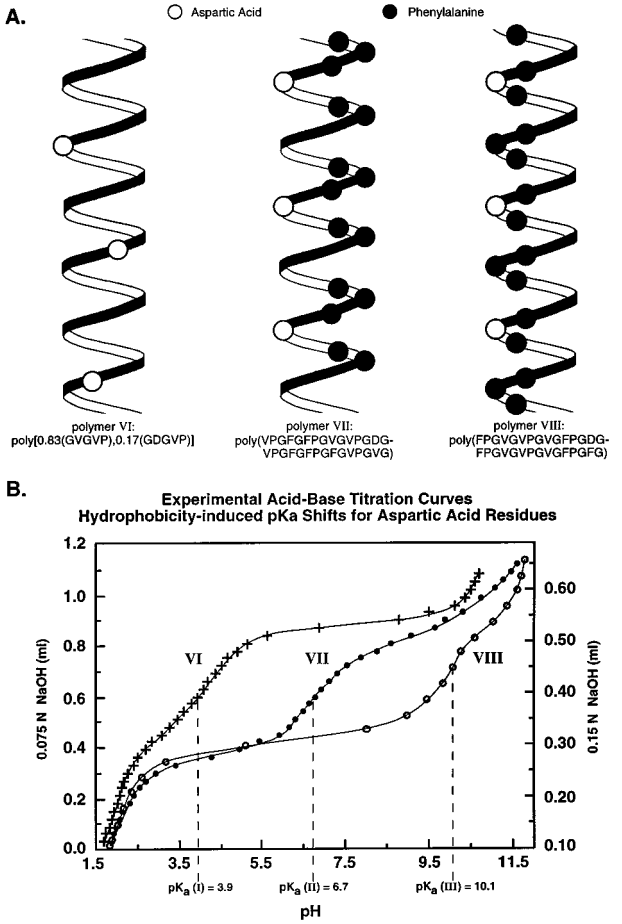


Figure 13. Structures in part A and corresponding acid−base titration curves in part B. See text for discussion. Reproduced with permission from ref 93.

as the hydrophobicity increases and the T_t progresses further and further below 0 °C.

The results of Figure 12 demonstrate that both electrostatic and hydrophobic ranges of f_X occur for inducing pK_a shifts, that in these polypentapeptides the more dilute the charge the larger the pK_a shift, and that the larger pK_a shifts result as the more polar residue is systematically replaced by a more hydrophobic residue. With poly-L-glutamic acid where there is maximal charge−charge repulsion for a polypeptide having 100 carboxyls for every 300 backbone atoms, the pK_a shift is no more than observed in this more hydrophobic system with only 1.2 carboxyls per 300 backbone atoms.[94] In short, it appears that hydrophobic-induced pK_a shifts can be larger in aqueous systems than electrostatic-induced pK_a shifts. This is demonstrated even more dramatically in the results reviewed below.

Poly(30-mers) with Five Phe(F) Residues Distal and Then Proximal to an Aspartyl Carboxyl. The β-spiral has approximately three pentamers per turn; this is the basis for the sequential periodicities considered below of one charge and of five distal or five proximal Phe residues per 30-mer. For simplicity the representation of Figure 3C will be used to indicate the location of subsequent substitutions, but it might be noted that this is a schematic representation for the structure of Figure 3E, which is itself a simplified picture for the twisted filament resulting from the supercoiling of several β-spirals as depicted in Figure 3F.

A set of Asp(D)-containing polymers that contributes to our understanding of designing for optimal hydrophobic-induced pK_a shifts[93] are: polymer VI, poly[0.83(GVGVP),0.17(GDGVP)]; polymer VII, poly(GDGVP GFGFP GFGVP GVGVP GFGFP GVGVP); polymer VIII, poly(GDGFP GVGVP GVGFP GFGFP GVGVP GVGFP) of Figure 13 with schematic structural representations in (A) and acid−base titrations in (B). The replacement of five Val(V) by five Phe(F) residues at positions *distal* to the ionizable carboxyl function of an aspartic acid residue as in polymer VII gives a substantial pK shift of 2.8 pH units. Even more striking, however, is the 6.2 pH unit shift exhibited by polymer VIII when the five Phe(F) residues are moved as *proximal* to the carboxyl function as possible given the above-described structure as represented in Figures 3C and 13A.

Even with poly(methacrylic acid), [−CH_3CCOOH−CH_2−]$_n$, where there is a carboxyl on every other backbone atom, i.e., 50 carboxyls per 100 backbone atoms, the pK_a shift is only about one-half the magnitude observed here[21] when there is only 1 carboxyl per 90 backbone atoms in close structural proximity to the more hydrophobic Phe residues.

The remarkable results of Figure 13 allow the conclusions that the same composition can result in a different pK_a shift depending on the proximity of the more hydrophobic residues to the ionizable group and that large hydrophobic-induced pK shifts of greater than 6 pH units can be obtained.

Poly(30-mers) of Increasing Hydrophobicity Due to Phe(F) Replacing Val(V). Using a series of polymers such as those of Figure 13, a plot of the observed pK_a shifts that result as the number of *proximal* Phe residues increases from 0 to 2 to 3 to 4 and finally to 5 is given in Figure 14A for both the Asp- and Glu-containing polymers.[19] What is observed is a very nonlinear hydrophobic-induced pK_a shift. The increase in hydrophobicity from 0 to 2 Phe residues per 30 residues causes only a ΔpK_a of 0.4 for the Asp residue and 0.3 for the Glu residue. On the other hand, the same increase of 2 Phe residues per 30-mer from 3 to 5 causes a ΔpK_a of 5.4 for the Asp residue and 3.2 for the Glu residue. The ΔpK_a is 10-fold greater when the same

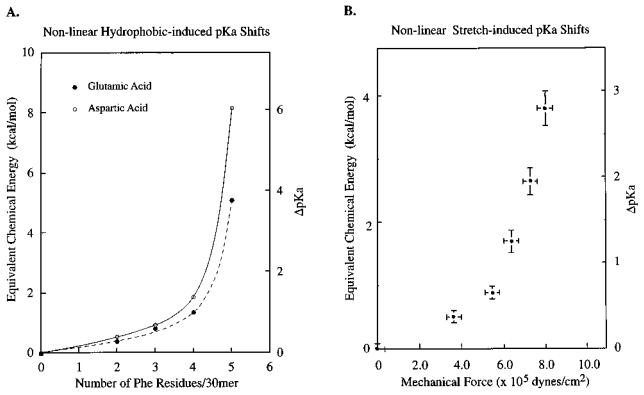


Figure 14. Nonlinear hydrophobic-induced (in part A) and analogous stretch-induced pK_a shifts (in part B). See text for discussion. Reproduced with permission from refs 19 and 20.

hydrophobicity change occurs while under the influence of a more hydrophobic domain.

Poising, Axiom 5. The data of Figure 14A[19] has striking implications with respect to the efficiency of energy conversion as does the similar curve of Figure 14B,[20] which is a plot of the data of Figure 9. If the particular energy input is the reduction of a nicotinamide moiety, for example, which constitutes a particular increase in hydrophobicity, the magnitude of the resulting ΔpK_a would be greater if there were several Phe residues present per 30 mer than if there were no Phe residues per 30 mer. As a change in pK_a can be equated to a change in the chemical energy of picking up or releasing a proton, the electrochemical energy input of reduction of a nicotinamide would result in the uptake of more protons, that is, would be more efficiently converted to chemical energy, when the hydrophobicity of the model protein is greater. This concept of more efficient energy conversion due to the presence of a more hydrophobic domain is called "poising" and is the basis for axiom 5.

Hydrophobic-Induced pK_a Shifts with Associated Positive Cooperativity. The acid−base titration curves for polymers I, II, III, and V given in Figure 15 demonstrate the remarkable and progressive increase in steepness or positive cooperativity as the hydrophobicity increases and as the pK_a shift increases. In fact, the same change in Gibbs free energy reflected in the pK_a shift is found in the increased cooperativity.[13,27] As formation of a charged species must destructure water of hydrophobic hydration, the extent of work performed must be reflected in the increase in pK_a of carboxylates; that is, the pK_a shifts can be used to measure the apolar−polar repulsive free energy of hydration. Furthermore, the competition for hydration, wherein the forming carboxylates cooperate to destructure the water of hydrophobic hydration, provides a basis for the positive cooperativity. On the other hand, the opposite effect obtains for charge−charge repulsion where a negative cooperativity arises[21] because the presence of charge raises the free energy required for formation of the next charge.

Formalisms for pK_a Shifts, Cooperativity, and Energy Conversions

Henderson−Hasselbalch Equation. For dilute weak acids and for an isolated polypeptide containing one ionizable residue such as an aspartic or glutamic acid residue without significant hydrophobicity in the remaining residues, the well-known Henderson−Hasselbalch equation applies, i.e.,

$$\mathrm{pH} = \mathrm{p}K_o + \log[\alpha/(1-\alpha)] \qquad (1)$$

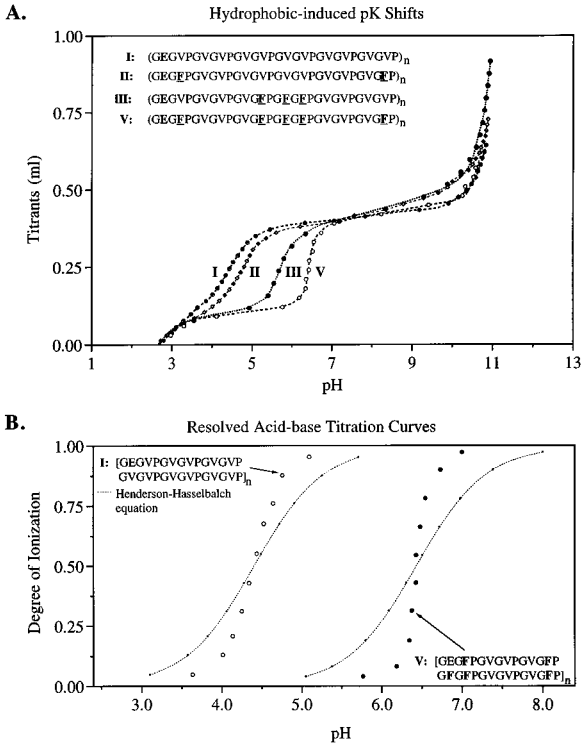


Figure 15. Acid−base titrations of a series of polytricosapeptides with increasing hydrophobicity (in part A) and a pair of resolved curves (in part B) plotted in comparison with the curve for the Henderson−Hasselbalch equation. Note the dramatic difference in the steepness of the curves. See text for discussion. Reproduced with permission from ref 74.

where pK_o is an unperturbed pK and where $\alpha = [COO^-]/\{[COOH] + [COO^-]\}$ and $(1-\alpha) = [COOH]/\{[COOH] + [COO^-]\}$.

Introduction of Hill Coefficients and the Limitation of the Wyman Equation. For a protein-based polymer containing widely dispersed aspartic or glutamic acid residues and with significant intervening hydrophobic residues

$$\mathrm{pH} = \mathrm{p}K + (1/n)\log\{[(COO^-)_n]/[(COOH)_n]\} \qquad (2)$$

where n is the number of sparse ionizable residues in a single protein-based polymer chain. Due to the cooperative actions of carboxylates while under the influence of substantial hydrophobic hydration, p$K \neq$ pK_o, but rather pK = pK_o + ΔpK. Furthermore, on the assumption that when one chain starts to ionize, due to the positive cooperativity, it completely ionizes before the next chain starts, to an adequate approximation, the experimentally measurable degree of ionization, $\alpha \approx [(COO^-)_n]/[(COOH)_n]$ and n is replaced by a phenomenological *n* to correspond with this assumption. Equation 2 can now be restated as

$$\mathrm{pH} = \mathrm{p}K_o + \Delta\mathrm{p}K + (1/n)\log[\alpha/(1-\alpha)] \qquad (3)$$

This provides a Henderson−Hasselbalch-like equation which has been generalized phenomenologically to introduce cooperativity into the acid−base titration theory in a manner analogous to the phenomenological introduction of cooperativity into the equilibrium constant or saturation function describing the oxygenation of hemoglobin.[95] The quantity *n* is equivalent to a Hill coefficient,[96] and the ΔpK ($= \Delta\Delta G_o/2.3RT$) can be estimated by evaluating the Wyman equation, $\Delta\Delta G_o = RT(1 - 1/n)/\alpha(1-\alpha)$, at $\alpha = 0.5$, i.e., $\Delta\mathrm{p}K = (1 - 1/n)/0.58$. This result makes apparent the relationship between the increased

steepness of the sigmoidal acid−base titration curve and the pK shift. The Wyman equation,[97] however, is valid only for relatively small values of ΔpK with a maximal ΔpK_a of 1.7. Using the acid base titration curves of Figure 15, the Hill coefficient is of the order of 8 which by the Wyman equation the ΔpK_a would calculate to be 1.5. The experimental pK_a shift is 2.0 from a curve with a value of n of 1.3 which, on back-calculating with the Wyman equation in its valid range, would give a total pK_a shift of 2.4. As seen in Figures 13 and 14A, the magnitude of the hydrophobic-induced pK_a shifts can be as large as 6 or more, which is entirely outside of the approximation underlying the derivation of the Wyman equation.

A more complete mathematical analysis of cooperativity has been presented by Monod, Wyman, and Changeux,[98] but it is purely phenomenological, lacking the physical basis that would enable polymer design based on the description. A cooperativity treatment has been derived on the basis of the above demonstrated competition for hydration between apolar and polar moieties in the Ph.D. thesis of S. Q. Peng where the pH dependence of N_{hh} in Figure 11C proves sufficient to calculate the pK_a shifts and the attending cooperativity observed in the acid−base titration curves of polymers I and II (Peng and Urry, in preparation).

Generalized Henderson−Hasselbalch Equation. Since $K = e^{-\Delta G/RT} = 10^{-\Delta G/2.3RT}$, then $\log K_o = -\Delta G_o/2.3RT = -pK_o$, and eq 3 can be rewritten in terms of changes in Gibbs free energy, ΔG, to give

$$pH = \Delta G_o/2.3RT + \Delta\Delta G_o/2.3RT + (1/n)\log[\alpha/(1-\alpha)] \quad (4)$$

Also following Harris and Rice,[23] the last term of eq 4 can be effectively restated as $\log[\alpha/(1-\alpha)] + (\partial\Delta G/\partial\alpha)_T/2.3RT$, where $(\partial\Delta G/\partial\alpha)_T = 0$ at $\alpha = 0.5$ to give

$$pH = pK_o + \Delta pK + \log[\alpha/(1-\alpha)] + (\partial\Delta G/\partial\alpha)_T/2.3RT \quad (5)$$

that is, the last term of eq 5 provides for the change in steepness of the sigmoidal curve from that given by the simple Henderson−Hasselbalch equation, as shown in Figure 15B.

As we have seen in Figure 12 and the associated discussion, there are two primary mechanisms in aqueous dominated media for pK shifts and changes in the steepness of the experimental sigmoidal titration curve. These are referred to as electrostatic-induced, principally charge−charge repulsion (c−c), and as hydrophobic-induced, that is, the apolar−polar repulsive free energy of hydration (a−p). The general expression can now be written specifically to include these two primary interactions as

$$pH = pK_o + \Delta pK_{c-c} + \Delta pK_{a-p} + \log[\alpha/(1-\alpha)] + \{[(\partial\Delta G/\partial\alpha)_T]_{c-c} + [(\partial\Delta G/\partial\alpha)_T]_{a-p}\}/2.3RT \quad (6)$$

While the ΔpK_{c-c} and ΔpK_{a-p} values are of the same sign, the partial derivatives are of the opposite sign, that is, $[(\partial\Delta G/\partial\alpha)_T]_{c-c}$ results in negative cooperativity with a sigmoidal curve, which is broader than given by the Henderson−Hasselbalch equation, and $[(\partial\Delta G/\partial\alpha)_T]_{a-p}$ results in positive cooperativity with a sigmoidal curve that is steeper than given by the Henderson−Hasselbalch equation. This allows for the analysis of protein titration data with the possibility of separation of the extent of charge−charge repulsion from apolar−polar repulsion with regard to the ΔpK_a.

General Relationships and Plots for Diverse Energy Conversions. The plot of α versus pH is actually a plot of the fraction of completion of energy conversion versus a normalized free energy, i.e., $pH = \Delta G/2.3RT$, or the availability of protons as in Figure 16A. An analogous plot for electrochemical reduction would be fraction of, or percent, reduction versus the electrochemical potential, i.e., the availability of electrons as in Figure 16B. Therefore, the steepness of the sigmoidal curve becomes a measure of the efficiency of the energy conversion because a smaller energy step along the x-axis is required to achieve complete energy conversion for the steeper (positively cooperative) than for the broader (negatively cooperative) curve. Accordingly, the charge−charge repulsion mechanism results in smaller pK_a shifts combined with a negative cooperativity, which makes it an *inefficient* mechanism for energy conversion. On the other hand, the apolar−polar hydration repulsion mechanism results in larger pK_a shifts combined with a positive cooperativity, which makes it an *efficient* mechanism for energy conversion. It is perhaps to be expected that the mechanism based on the apolar−polar repulsive free energy of hydration would become a dominant mechanism in the evolution of living organisms.

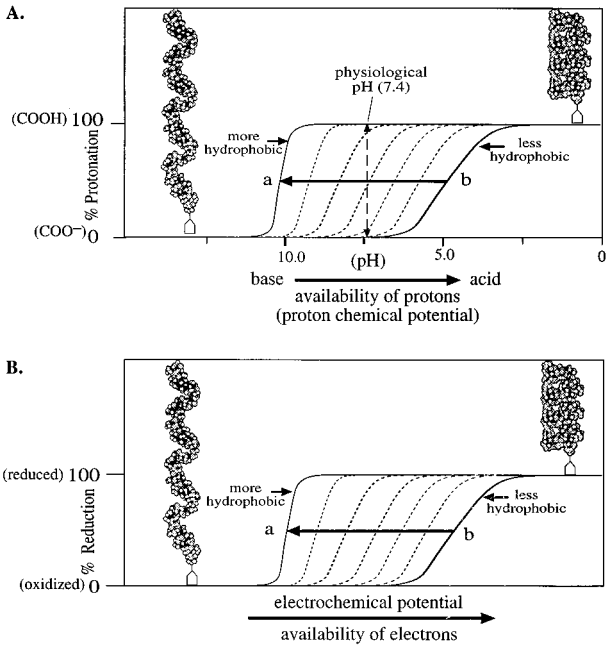


Figure 16. Schematic representations of chemomechanical transduction, e.g., protonation-driven contraction (in part A), and electromechanical transduction, e.g., reduction-driven contraction (in part B), where the percent of length change has been replaced by the equivalent percent protonation or reduction. Simply by interchanging the ordinates, the plots represent electrochemical transduction. In particular, part A represents a replotting of the data of Figure 7B, right-hand side, with the pH reversed to coincide with the form of the plot of Figure 6A. Accordingly, there emerges a general way of considering the whole range of free energy transductions possible by means of protein-based polymers capable of inverse temperature transitions.

Comparison of the schematic curves of parts A and B of Figure 16 demonstrates their equivalence. Indeed, the equivalence is such that one could exchange ordinates resulting in the effect of reduction being equivalent to the effect of protonation. With such an interchange the plots represent electrochemical transduction. In this way, it becomes possible graphically to envision a generalized formalism for free energy transduction that is presently under development.

Conclusions

Free Energy Transduction by Inverse Temperature Transition. Proteins are the catalysts of biological energy conversion. Using as model proteins a particular family of protein-

based polymers, many energy conversions of living organisms can be demonstrated by controlling hydrophobic folding and assembly transitions. Because the hydrophobic folding and assembly transition leads to an increase in the order of the protein component of the system on raising the temperature, it is called an inverse temperature transition.

Hydrophobic Hydration, N_{hh}. The central figure in this tale of free energy transduction by inverse temperature transition is the water that surrounds hydrophobic groups, its unique thermodynamic properties (exothermic on formation but of large negative entropy change thereby limiting solubility) and yet the facility with which this special structured water, with an entropy less than that of bulk water, can be destructured. In particular, the amount of this water of hydrophobic hydration, which determines the temperature ($T_t \approx \Delta H_t/\Delta S_t$) at which the inverse temperature transition occurs, can be controlled by the presence of adequately proximal polar groups.

This understanding is consistent with the findings of Levitt and Sharon[99] who estimated a change of binding energy of −0.27 kcal/mol on going from bulk water to hydrophobic hydration. Thus, waters of hydrophobic hydration, experimentally delineated from bulk water with reorientation correlation times only about twice as long as those of bulk water[15] but with hundreds of hydrophobic hydration molecules per pentamer, exert their significant thermodynamic effect by being so many in number rather than by being so different in physical properties from bulk water.

Delineation of Electrostatic-from Hydrophobic-Induced pK_a Shifts. *Effect of Stretching.* On stretching of the cross-linked and hydrophobically folded and assembled state of these elastic protein-based polymers, the mean distance between charges increases and the matrix takes up water. Both of these effects, based on electrostatic theory, should relax the pK_a shifts. In fact, just the opposite occurs; stretching increases the pK_a shifts. This delineation can be simply stated in terms of the partial of the proton chemical potential, $\partial\mu_H$, taken with respect to the force applied to achieve extension, ∂f, at constant polymer composition, i.e., at the pK_a where the degree of ionization, α, equals 0.5: for the electrostatic charge−charge repulsion case $(\partial\mu_H/\partial f)_{\alpha=0.5} > 0$,[25] whereas for the apolar−polar repulsion case $(\partial\mu_H/\partial f)_{\alpha} < 0$.[13,20]

Effect of Dilution of Ionizable Sites. Stepwise decreases in the number of ionizable sites from 1 in 10 residues to 1.2 in 100 residues by replacement of Glu(E), Asp(D), or Lys(K) residues by Val(V) residues causes increasingly greater shifts in the pK_a values, whereas by charge−charge repulsion of electrostatic theory, decreasing the number of ionizable sites by the modestly hydrophobic Val residue should relax the pK_a shifts.

Effect of Increases in Hydrophobicity at Constant Concentration of Ionizable Sites. Maintaining the number of ionizable sites at 1 in 30 residues and stepwise increasing hydrophobicity by sequentially replacing Val(V) residues by 2, 3, 4, and 5 of the much more hydrophobic Phe(F) residues per 30-mer results in nonlinear and ultimately large pK_a shifts, up to over 6 pH units. By comparison, the magnitudes in the Westheimer and Shookhof[100] compilation of reported dicarboxylic acid pK_a shifts,[100] which exemplified the electrostatic theory of pK_a shifts,[101−104] were no more than a few pH units or less. There was one exception; it was a ΔpK_a of 5.36 for the severely structurally constrained *cis*-caronic acid, which reduces to a ΔpK_a of 0.9 for *trans*-caronic acid. The caronic acid circumstance, however, is not relevant to an elastic polymer with barriers to structural rearrangement of less than 1.5 kcal/mol-residue.[39,40,45] Any chance *cis*-caronic acid-like juxtaposition of charge in the hydrophobically assembled state of elastic protein-based polymers would simply result in the structure elastically relaxing rather than maintaining an 8+ kcal/mol repulsive juxtaposition as required to give rise to pK_a shifts of 6 or greater. This is particularly so when there are only 3.4 carboxyls per 100 residues and more than 60 flexible torsion angles between carboxyls as is the case for the polymers of Figure 13.

Inverse Correlation of Increasing pK_a Shifts and Changes in Cooperativity. When considering polymers, a very clear distinction can be drawn between the predictions of the electrostatic theory of pK_a shifts due to charge−charge repulsion and those of pK_a shifts resulting from an apolar−polar repulsive free energy of interaction. The electrostatic theory of pK_a shifts in polyelectrolytes clearly associates increased ΔpK_a values due to charge−charge repulsion with increasingly broadened (negatively cooperative) acid−base titration curves.[21−24] The data are exactly the opposite for elastic protein-based polymers capable of hydrophobic folding and assembly transitions. The increasing pK_a shifts of Figures 9, 13, and 15 are associated with increasingly steeper (positively cooperative) acid−base titration curves.

The Work of Destruction of Hydrophobic Hydration. In Figure 11C, ionization of carboxyls to form charged carboxylates destructures hydrophobic hydration. This occurs at a temperature of 1 °C, which is below the value of T_t for both polymers; that is, both polymers I and II remain in solution throughout the titration. Therefore, in our view, the free energy of the pK_a shift must come from the work required to destructure hydrophobic hydration. The pK_a shift cannot result from a phase transition leading to a possible change in dielectric constant in the vicinity of the carboxylate, as such does not occur for either polymer.

On the basis of the above analysis of data, we see no experimental basis for supporting the electrostatic theory for the pK_a shifts exhibited by elastic protein-based polymers capable of hydrophobic folding and assembly transitions.

Apolar−Polar Competition for Hydration. The physical process underlying this mechanism of free energy transduction is the competition for hydration that exists between hydrophobic and polar (especially charged) moieties that are constrained by sequence to be sufficiently proximal such that they compete for the same water molecules for hydration. Thus, a particular hydrophobic group can be fully exposed to water but not have its full complement of structured water of hydrophobic hydration because a charged group that may be 10 Å or more removed has reoriented the water molecules for its own hydration. This is called an apolar−polar repulsive free energy of hydration which can cause polar and apolar residues to be physically repulsed to the extent allowed by the intrinsic energetics of the intervening covalent structure. That this is a dominant force controlling protein folding and function has yet to be generally appreciated. On the basis of our studies and in our view, *the apolar−polar repulsive free energy of hydration is the physical process underlying the "hydrophobic effect" and is fundamental to diverse biological free energy transduction.*

The T_t-Trigger or Switch: The ΔT_t-Mechanism. Any change in the polarity or hydrophobicity of a substituent of the protein-based polymer changes the temperature, T_t, of the inverse temperature transition of hydrophobic folding and assembly by means of having changed the amount of water of hydrophobic hydration. Obviously, the temperature can be raised from below to above T_t to drive folding and assembly. Of greatest relevance, however, are the innumerable ways of increasing the functional water of hydrophobic hydration to lower T_t from above to below an operating temperature with the result of hydrophobic folding and assembly. This makes T_t an on/off trigger or switch for free energy transduction. By this means, energy inputs, which

alter the polarity of the polar species that in turn shifts T_t by changing the amount of hydrophobic hydration, become transduced into energy outputs by effecting the hydrophobic folding and assembly transition. Thus, from experimental and design perspectives, the T_t point of view provides a simple analytical means with which to achieve protein-based polymers of new and interesting capacities.

***T_t*-type Molecular Machines.** Conceptually, the simplest energy output is the performance of useful mechanical work that occurs as a direct result of hydrophobic folding and assembly; such polymeric systems are molecular engines, specifically called *T_t-type molecular machines of the first kind*. More diverse are *T_t-type molecular machines of the second kind*, in which an energy input increases the amount of water of hydrophobic hydration which in turn shifts the properties of a functional group in competition for hydration such as the change in pK_a of an ionizable group, the change in the electrical potential[105] of an attached redox group, or the change in property of any other functional group that is capable of existing in two or more functional states of differing hydrophobicity. The hydrophobic-induced change in the property, such as a pK_a, of a functional group provides a minimal measure of the apolar−polar repulsive free energy of hydration.

The Usual Virtual Nature of Full Hydrophobic Hydration. The steps in free energy transduction by inverse temperature transition are: an energy input alters N_{hh}, the amount of water of hydrophobic hydration; this in turn either alters the structure with the performance of mechanical work as the output or alters, by the competition for hydration, the physical property of a coupled functional group, such as the pK_a or E_h,[105] as a chemical energy or electrical energy output. It should be appreciated that in the usual circumstance when T_t is shifted from above to below the operating temperature, folding and/or assembly occur as N_{hh} begins to build yet before the full complement of hydrophobic hydration, N_{hh}, ever occurs. Only when studies can be carried out a temperature below T_t for both the more and less hydrophobic states of a functional group can the full N_{hh} be observed. Thus, there is a virtual nature to the fully hydrophobically hydrated state that has made its earlier detection more difficult to realize, and it is only a *residual* N_{hh} that would normally be observed,[106] as in the higher pH range of Figure 11C.

The series of five elastic protein-based polymers of Figure 11C provided the unique opportunity to progress stepwise in hydrophobicity from polymers I and II with T_t values above 4 °C and for which it was, therefore, possible at 1 °C directly to measure N_{hh} over the entire pH range of interest and to continue with polymers III, IV, and V as T_t values progressively shift below 0 °C and for which it was, therefore, only possible to measure a *residual* N_{hh} even at 2 °C.

Concepts Introduced during Development of Elastic Protein-Based Polymers for Free Energy Transduction. The conclusions of this article can also be given in terms of the following chronological listing of the concepts introduced during the development of elastic protein-based polymers for free energy transduction: (1) the concept of the damping of internal chain dynamics on extension as the source of entropic elastomeric force, called the librational entropy mechanism of elasticity, (2) the concept of T_t, the temperature of the hydrophobic folding and assembly transition, being used as the fundamental measure of hydrophobicity and providing a practical on/off switching capacity, (3) the generally obvious concept that raising the temperature from below to above T_t is a means of performing mechanical work by cross-linked elastic protein-based polymers, (4) the concept of the ΔT_t-mechanism wherein the value of T_t is changed, rather than the temperature, as a means of achieving free energy transduction, (5) the concept of energy conversion by means of the coupling of different functional moieties by being part of the same hydrophobic folding and assembly domain arising out of, for example, (a) hydrophobic-induced pK_a shifts, (b) hydrophobic-induced shift in redox potential, and (c) demonstrated coupling of carboxyl and redox functions to result in electrochemical transduction, (6) the concept of the competition for hydration between apolar (hydrophobic) and polar (e.g., charged) moieties to give rise to pK_a shifts and positive cooperativity, (7) the concept of "poising" for achieving higher efficiencies, (8) essential equivalence of the inverse temperature transition of a phase separation and the intramolecular phase separation of the hydrophobic folding of a globular protein or assembly of the protomer subunits to form a multisubunit globular protein such as phosphofructose kinase, that is, the extension to globular proteins, and (9) extension to all polymers where, however, the degree of expression of the above effects is limited due to the lack of the many advantages of protein-based polymers of Table 1.

Acknowledgment. The author thanks the previous and present members of the Laboratory of Molecular Biophysics for their contributions to the work reviewed here and gratefully acknowledges the support of the Department of the Navy, Office of Naval Research, Grant Number N00014-89-J-1970 without which the major part of the reported progress would not have occurred.

References and Notes

(1) J. Willard Gibbs in an 1881 letter to the American Academy of Arts and Sciences: Wheeler, L. P. *Josiah Willard Gibbs, the History of a Great Mind*; Yale University Press: New Haven, CT, and London, 1952; pp 88−89.
(2) Ingber, D. J. *Cell Sci.* **1993**, *104*, 613−627.
(3) Needham, J. Terry, Lectures, Yale University: New Haven, CT, 1936; quoted in ref 2.
(4) Privalov, P. L. *Crit. Rev. Biochem. Mol. Biol.* **1990**, *25*, 281−305.
(5) Kauzmann, W. *Adv. Protein Chem.* **1959**, *14*, 1−63.
(6) Urry, D. W.; Luan, C.-H. *Bioelectrochemistry: Principles and Practice*; Birkhäuser Verlag AG: Basel, Switzerland, 1995; pp 105−182.
(7) Urry, D. W. *Prog. Biophys. Mol. Biol.* **1992**, *57*, 23−57.
(8) Urry, D. W. *Angew. Chem.* (German) **1993**, *105*, 859−883; *Angew. Chem., Int. Ed. Engl.* **1993**, *32*, 819−841.
(9) Urry, D. W. *Int. J. Quantum Chem.: Quantum Biol. Symp.* **1994**, *21*, 3−15.
(10) Urry, D. W.; Hayes, L. C.; Gowda, D. C.; Peng, S.-Q.; Jing, N. *Biochem. Biophys. Res. Commun.* **1995**, *210*, 1031−1039.
(11) Urry, D. W.; Hayes, L. C.; Peng, S.-Q. SPIE-The International Society for Optical Engineering Smart Structures and Materials 1996−Smart Materials Technologies and Biomimetics, San Diego, CA, 1996; Vol. 2716, pp 343−346.
(12) Urry, D. W.; Gowda, D. C.; Parker, T. M.; Luan, C.-H.; Reid, M. C.; Harris, C. M.; Pattanaik, A.; Harris, R. D. *Biopolymers* **1992**, *32*, 1243−1250.
(13) Urry, D. W.; Peng, S.-Q., Hayes, L. C.; Jaggard, J.; Harris, R. D. *Biopolymers* **1990**, *30*, 215−218.
(14) Urry, D. W.; Luan, C.-H.; Harris, R. D.; Prasad, K. U. *Polym. Prepr. Am. Chem. Soc., Div. Polym. Chem.* **1990,** *31*, 188−189.
(15) Urry, D. W.; Peng, S.-Q.; Xu, J.; McPherson, D. T. *J. Am. Chem. Soc.* **1997**, *119*, 1161−1162.
(16) Urry, D. W.; Peng, S.-Q.; Parker, T. M. *J. Am. Chem. Soc.* **1993**, *115*, 7509−7510.
(17) Urry, D. W.; Peng, S.-Q.; Parker, T. M.; Gowda, D. C.; Harris, R. D. *Angew. Chem.* (German) **1993**, *105*, 1523−1525; *Angew. Chem., Int. Ed. Engl.* **1993**, *32*, 1440−1442.
(18) Urry, D. W.; Peng, S.-Q.; Gowda, D. C.; Parker, T. M.; Harris, R. D. *Chem. Phys Lett.* **1994**, *225*, 97−103.
(19) Urry, D. W.; Gowda, D. C.; Peng, S.-Q.; Parker, T. M. *Chem. Phys. Lett.* **1995**, *239*, 67−74.
(20) Urry, D. W.; Peng, S.-Q. *J. Am. Chem. Soc.* **1995**, *117*, 8478−8479.
(21) Katchalsky, A. *J. Polym. Sci.* **1951**, *7*, 393−412.
(22) Overbeek, J. T. G. *Bull. Soc. Chim. Belg.* **1948**, *57*, 252.
(23) Harris, F. E.; Rice, S. A. *J. Phys. Chem.* **1954**, *58*, 725−732.
(24) Katchalsky, A.; Gillis, J. *Recl. Trav. Chim. Pays-Bas* **1949**, *678*, 879.
(25) Katchalsky, A.; Lifson, S.; Michaeli, I.; Zwick, M. *Size & Shape of Contractile Polymers: Conversion of Chemical & Mechanical Energy*; Pergamon Press: New York, 1960; pp 1−40.

(26) Urry, D. W.; Haynes, B.; Zhang, H.; Harris, R. D.; Prasad, K. U. *Proc. Natl. Acad. Sci. U.S.A.* **1988**, *85*, 3407−3411.

(27) Peng, S. Q. Ph.D. Thesis, University of Alabama at Birmingham, 1997.

(28) Urry, D. W.; Hayes, L. C.; Gowda, D. C. *Biochem. Biophys. Res. Commun.* **1994**, *204*, 230−237.

(29) The first use of the name, protein-based polymers, occurred as the Symposium on Bioengineering of Protein-based Polymers of the April 23−29, 1990 National American Chemical Society meeting, which was organized by D. Kaplan, M. Marron, and D. Tirrell, who developed the term.

(30) Urry, D. W.; McPherson, D. T.; Urry, D. W.; Xu, J.; Daniell, H.; Guda, C.; Gowda, D. C.; Jing, N.; Parker, T. M. *The Polymeric Materials Encyclopedia: Synthesis, Properties and Applications*; CRC Press: Boca Raton, FL, 1996; pp 7263−7279.

(31) Sciortino, F.; Palma, M. U.; Urry, D. W.; Prasad, K. U. *Biochem. Biophys. Res. Commun.* **1988**, *157*, 1061−1066.

(32) Urry, D. W.; Trapane, T. L.; Prasad, K. U. *Biopolymers* **1985**, *24*, 2345−2356.

(33) Urry, D. W. *Methods in Enzymology*; Academic Press, Inc.: New York, 1982; pp 673−716.

(34) Urry, D. W. *Biomolecular Stereodynamics III*; Adenine Press: Guilderland: NY, 1985; pp 173−196.

(35) Urry, D. W. *Mol. Conformation and Biol. Interactions*, Indian Academy of Science: Bangalore, India, 1991; pp 555−583.

(36) Urry, D. W.; Long, M. M. *CRC Crit. Rev. Biochem.* **1976**, *4*, 1−45.

(37) Urry, D. W.; Trapane, T. L.; Sugano, H.; Prasad, K. U. *J. Am. Chem. Soc.* **1981**, *103*, 2080−2089.

(38) Urry, D. W.; Chang, D. K.; Krishna, R.; Huang, D. H.; Trapane, T. L.; Prasad, K. U. *Biopolymers* **1989**, *28*, 819−833.

(39) Urry, D. W.; Trapane, T. L.; Iqbal, M.; Venkatachalam, C. M.; Prasad, K. U. *Biochemistry* **1985**, *24*, 5182−5189.

(40) Urry, D. W.; Trapane, T. L.; McMichens, R. B.; Iqbal, M.; Harris, R. D.; Prasad, K. U. *Biopolymers* **1986** *25*, S209−S228.

(41) Thomas, G. J., Jr.; Prescott, B.; Urry, D. W. *Biopolymers* **1987**, *26*, 921−934.

(42) Cook, W. J.; Einspahr, H. M.; Trapane, T. L.; Urry, D. W.; Bugg, C. E. *J. Am. Chem. Soc.* **1980**, *102*, 5502−5505.

(43) Urry, D. W.; Okamoto, K.; Harris, R. D.; Hendrix, C. F.; Long, M. M. *Biochemistry* **1976**, *15*, 4083−4089.

(44) Volpin, D.; Urry, D. W.; Pasquali-Ronchetti, I.; Gotte, L. *Micron* **1976**, *7*, 193−198.

(45) Henze, R.; Urry, D. W. *J. Am. Chem. Soc.* **1985**, *107*, 2991−2993.

(46) Buchet, R.; Luan, C.-H.; Prasad, K. U.; Harris, R. D.; Urry, D. W. *J. Phys. Chem.* **1988**, *92*, 511−517.

(47) Venkatachalam, C. M.; Urry, D. W. *Macromolecules* **1981**, *14*, 1225−1229.

(48) Urry, D. W.; Venkatachalam, C. M.; Long, M. M.; Prasad, K. U. *Conformation in Biol*, G. N. Ramachandran Festschrift Volume; Adenine Press: Guilderland, NY, 1982; pp 11−27.

(49) Chang, D. K.; Venkatachalam, C. M.; Prasad, K. U.; Urry, D. W. *J. Biomol. Struct. Dyn.* **1989**, *6*, 851−858.

(50) Chang, D. K.; Urry, D. W. *J. Comput. Chem.* **1989**, *10*, 850−855.

(51) Wasserman, Z. R.; Salemme, F. R. *Biopolymers* **1990**, *29*, 1613−1631.

(52) San Biagio, P. L.; Madonia, F.; Trapane, T. L.; Urry, D. W. *Chem. Phys. Lett.* **1988**, *145*, 571−574.

(53) Sciortino, F.; Prasad, K. U.; Urry, D. W.; Palma, M. U. *Chem. Phys. Lett.* **1988**, *153*, 557−559.

(54) Sciortino, F.; Urry, D. W.; Palma, M. U.; Prasad, K. U. *Biopolymers* **1990**, *29*, 1401−1407.

(55) Sciortino, F.; Prasad, K. U.; Urry, D. W.; Palma, M. U. *Biopolymers* **1993**, *33*, 743−52.

(56) Luan, C.-H.; Parker, T. M.; Prasad, K. U.; Urry, D. W. *Biopolymers* **1991**, *31*, 465−475.

(57) Luan, C.-H.; Jaggard, J.; Harris, R. D; Urry, D. W. *Int. J. of Quantum Chem.: Quantum Biol. Symp.* **1989**, *16*, 235−244.

(58) Urry, D. W.; Long, M. M.; Sugano, H. *J. Biol. Chem.* **1978**, *253*, 6301−6302.

(59) Frank, H. S.; Evans, M. W. *J. Chem. Phys.* **1945**, *13*, 507−532.

(60) Urry, D. W. *Ultrastruct. Pathol.* **1983**, *4*, 227−251.

(61) Urry, D. W. *J. Protein Chem.* **1984**, *3*, 403−436.

(62) Hoeve, C. A. J.; Flory, P. J. *Biopolymers* **1974**, *13*, 677−683.

(63) Hoeve, C. A. J.; Flory, P. J. *J. Am. Chem. Soc.* **1958**, *80*, 6523−6526.

(64) Urry, D. W.; Venkatachalam, C. M. *Int. J. Quantum Chem.: Quantum Biol. Symp.* **1983**, *10*, 81−93.

(65) Urry, D. W.; Trapane, T. L.; Long; M. M.; Prasad, K. U. *J. Chem. Soc., Faraday Trans. 1* **1983**, *79*, 853−868.

(66) Yeh, H.; Ornstein-Goldstein, N.; Indik, Z.; Sheppard, P.; Anderson, N.; Rosenbloom, J. C.; Cicila, G.; Zana, R.; Tondre, C. *J. Phys. Chem.* **1972**, *76*, 1737−1743.

(67) Sandberg, L. B.; Soskel, N. T.; Leslie, J. B. N. *Engl. J. Med.* **1981**, *304*, 566−579.

(68) Sandberg, L. B.; Leslie, J. G.; Leach, C. T.; Torres, V. L.; Smith, A. R.; Smith, D. W. *Pathol. Biol.* **1985**, *33*, 266−274.

(69) Edsall, J. T. *J. Am. Chem. Soc.* **1935**, *57*, 1506−1507.

(70) Quoted in Frank, H. S.; Evans, M. W. *J. Chem. Phys.* **1945**, *13*, 507−532.

(71) Stackelberg, M. V.; Müller, H. R. *Naturwissenschaften* **1951**, *38*, 456.

(72) Teeter, M. M. *Proc. Natl. Acad. Sci. U.S.A.* **1984**, *81*, 6014−6018

(73) Luan, C.-H.; Urry, D. W. Invited article for the *Polymer Data Handbook*; Oxford Press: New York, 1997, in press.

(74) Urry, D. W. Biotechnol. Bioeng., 1997, in press.

(75) Pattanaik, A.; Gowda, D. C; Urry, D. W. *Biochem. Biophys. Res. Commun.* **1991**, *178*, 539−545.

(76) Urry, D. W.; Harris, C. M.; Luan, C.-X.; Luan, C.-H.; Gowda, D. C.; Parker, T. M.; Peng, S.-Q.; Xu, J. *Controlled Drug Delivery: The Next Generation*; American Chemical Society: Washington, DC, 1997; pp 405−437.

(77) Blumenfeld, L. A.; Tikhonov, A. N. *Biophysical Thermodynamics of Intracellular Processes, Molecular Machines of the Living Cell*; Springer-Verlag: New York, 1994; Chapter 2.

(78) Urry, D. W.; Long, M. M.; Harris, R. D.; Prasad, K. U. *Biopolymers* **1986**, *25*, 1939−1953.

(79) Urry, D. W.; Harris, R. D.; Long, M. M.; Prasad, K. U. *Int. J. Pept. Protein Res.* **1986**, *28*, 649−660.

(80) Urry, D. W.; Harris, R. D.; Prasad, K. U. *J. Am. Chem. Soc.* **1988**, *110*, 3303−3305.

(81) Urry, D. W.; Hayes, L. C.; Gowda, D. C.; Parker, T. M. *Chem. Phys. Lett.* **1991**, *182*, 101−106.

(82) Urry, D. W.; Hayes, L. C.; Parker, T. M.; Harris, R. D. *Chem. Phys. Lett.* **1993**, *201*, 336−340.

(83) Urry, D. W.; Hayes, L. C.; Gowda, D. C.; Harris, C. M.; Harris, R. D. *Biochem. Biophys. Res. Commun.* **1992**, *188*, 611−617.

(84) Strzegowski, L. A.; Martinez, M.B.; Gowda, D. C.; Urry, D. W.; Tirrell, D. A. *J. Am. Chem. Soc.* **1994**, *116*, 813−814.

(85) Grant, E. A.; McLean, V. E. R.; Nightingale, N. R. V.; Sheppard, J. R.; Chapman, M. J. *Bioelectromagnetics* **1986**, *7*, 151−162.

(86) Pethig, R. *Annu. Rev. Phys. Chem.* **1992**, *43*, 177−205.

(87) Harvey, S. C.; Hoekstra, P. *J. Phys. Chem.* **1972**, *76*, 2987−2994.

(88) Grigera, J. R.; Vericat, F.; Hallenga, K.; Berendsen, H. J. C. *Biopolymers* **1979**, *18*, 35−45.

(89) Suzuki, M.; Shigamatsu, J.; Kodama, T. *J. Phys. Chem.* **1996**, *100*, 7279−7282.

(90) Suzuki, M.; Shigamatsu, J.; Fukunishi, Y.; Harada, Y.; Yanagida, T.; Kodama, T. *Biophys. J.* **1997**, *72*, 18−23.

(91) Rayment, I.; Holden, H. M.; Whittaker, M.; Yohn, C. B.; Lorenz, M.; Holmes, K. C.; Milligan, R. A. *Science* **1993**, *261*, 50−58.

(92) Gurney, R. W. *Ions in solution*; Cambridge University Press: London, 1936 (Dover Publications Inc.: New York, reprinted 1960); Chapter 1.

(93) Urry, D. W.; Gowda, D. C.; Peng, S.-Q.; Parker, T. M.; Jing, N.; Harris, R. D. *Biopolymers* **1994**, *34*, 889−896.

(94) Urry, D. W. *Annu. Rev. Phys. Chem.* **1968**, *19*, 477−530.

(95) Stryer, L. *Biochemistry*, 3rd ed.; W. H. Freeman & Company, New York, 1988; pp 154−155.

(96) Levitzki, A. *Molecular Biology, Biochemistry and Biophysics, Vol. 28, Quantitative Aspects of Allosteric Mechanisms*; Springer-Verlag: New York, 1978; Chapter 3.

(97) Wyman, J. *Cold Spring Harbor Symp. Quant. Biol.* **1963**, *28*, 483−489.

(98) Monod, J.; Wyman, J.; Changeux, J.−P. *J. Mol. Biol.* **1965**, *12*, 88−118.

(99) Levitt, M.; Sharon, R. *Proc. Natl. Acad. Sci. U.S.A.* **1988**, *85*, 7557−7561.

(100) Westheimer, F.H.; Shookhof, M.W. *J. Am. Chem. Soc.* **1939**, *61*, 555−560.

(101) Kirkwood, J. G.; Weistheimer, F. H. *J. Chem. Phys.* **1938**, *6*, 506−512.

(102) Weistheimer, F. H.; Kirkwood, J. G. *J. Chem. Phys.* **1938**, *6*, 513−517.

(103) Bjerrum, N. *Z. Phys. Chem.* **1923**, *106*, 219−242.

(104) Greenspan, J. *Z. Phys. Chem.* **1933**, *12*, 339−361.

(105) Clark, W. *Mansfield Oxidation−reduction Potentials of Organic Systems*; Williams & Wilkins Company: Baltimore, MD, 1960; Chapter 2.

(106) A recent example of observed changes in what we would term residual hydrophobic hydration, N_{hh}, has been demonstrated to occur during hydrolysis of ATP by the myosin motor domain, the myosin subfragment, S1, in ref 89.

(107) Urry, D. W.; Trapane, T. L.; Sugano, H.; Prasad, K. U. *J. Am. Chem. Soc.* **1981**, *103*, 2080−2089.