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13. ABSTRACT *(Maximum 200 words)* Using a diverse set of experimental observations on a family of elastomeric protein-based polymers, five axioms have been developed which govern protein funtion and engineering by means of hydrophobic folding and assembly. Furthermore, the underlying physical process has also been experimentally demonstrated to be competition for hydration between hydrophobic and polar moieties. These developments represent the nuts and bolts of amphiphilic polymer structure formation, function and engineering by means of hydrophobic folding and assembly.

Basically, amphiphilic polymers, including proteins and protein-based polymers, exhibit hydrophobic folding and assembly (phase) transitions with the onset ocurring as the temperature is raised above a value designated as Tt. The value of Tt is determined by the amount of hydrophobic hydration, the more hydrophobic hydration the lower the value of Tt and the less hydrophobic hydration the higher the value of Tt.

This mechanism of apolar-polar repulsive free energy of hydration, which controls hydrophobic folding and assembly, can be argued to be a more efficient mechanism for free energy transduction than the more commonly considered charge-charge interaction mechanism. Therefore, the axioms and the underlying physical process represent fundamental developments in the capacity to design amphiphilic polymers for diverse funtions, generally categorizable as free energy transduction.

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FINAL REPORT

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PRINCIPAL INVESTIGATOR: Dr. Dan W. Urry

INSTITUTION: The University of Alabama at Birmingham

GRANT TITLE: Development of Elastomeric Polypeptide Biomaterials

AWARD PERIOD: 1 June 1989 - 30 September 1997 (8 and 1/3 years)

OBJECTIVE: To design elastomeric polypeptide biomaterials in order to achieve diverse forms of free energy transduction by these water-miscible hydrophobic folding and assembling macromolecules, thereby to elucidate the underlying mechanisms and principles, and therefrom to make possible new materials and applications.

APPROACH: To design protein-based polymers capable of energy conversions involving mechanical, pressure, thermal, chemical, electrical, and electromagnetic energies, due to their capacity to exhibit inverse temperature transitions of hydrophobic folding and assembly as the temperature is raised over a characteristic temperature interval, the onset of which is designated as T_t

The protein-based polymers are prepared by means of recombinant DNA technology. The products are purified utilizing the inverse temperature transitional properties of the polymers and verified by the standard physical and chemical means and by biological testing.

The primary purpose of the designed and prepared protein-based polymers was to demonstrate, ultimately, all fifteen pair-wise free energy transductions involving the above six energies. Polymers also can be prepared to demonstrate a very important sixteenth, chemo-chemical, transduction.

MAJOR ACCOMPLISHMENTS: (8 and 1/3 years)

General Perspective: The hydrophobic effect, or hydrophobic folding, has long been appreciated as relevant to protein structure and function. Until this work, there has not been a systematic development of what controls hydrophobic folding nor has there been a reasonable assessment of just how important hydrophobic folding is in protein structure and function. An elastomeric model protein system was used where the many factors controlling hydrophobic folding could be isolated, demonstrated and evaluated and where those factors could be used in de novo designs to achieve function.

It has also long been appreciated that raising the temperature to arrive at a functional protein structure is indicative of hydrophobic folding, as occurs on raising the temperature of a cold denatured protein. Using the special family of elastomeric model proteins that exhibit a phase transition of hydrophobic folding and assembly, we have carefully assessed the hydrophobic folding and assembly process in terms of the temperature for the onset of folding, T_t. The result has been the experimental development of a set of five axioms for protein function and engineering.

Remarkably, using specific designs of our model protein system, virtually every pair-wise energy conversion of metabolism can be demonstrated. This is achieved by means of controlling the temperature of the hydrophobic folding and assembly transition. It involves interconversion of the free energies, the intensive variables of which are – mechanical force, pressure, temperature, chemical potential, electrical potential and electromagnetic radiation. More explicit demonstrations utilize designed model proteins and corresponding energy inputs of heat, chemical energy, pressure, electrochemical energy, and light to drive contraction in visual demonstration of the mechanical energy output of lifting a weight. This is documented in part by a video of designed elastomeric model proteins "pumping iron."

Having demonstrated the phenomenology of changing protein structure and attaining function by means of controlling hydrophobic folding and assembly, it became possible to determine the underlying physical process. It is one of competition for hydration between apolar

(hydrophobic) and polar (e.g., charged) groups constrained to coexist along a protein chain sequence, rather than being free to separate as oil from vinegar. The technical details are developed below.

Stepwise Experimental Development of the Phenomenology: The initial experimental observation was that T_t decreases for more hydrophobic residues and increases for more polar residues. This is the basis for a T_t-based hydrophobicity scale. This is **Axiom 1**. By independent measurement, using microwave dielectric relaxation, the value of T_t is found to be a measure of the amount of hydrophobic hydration. Since hydrophobic association can be observed as a phase transition, $T_t \approx \Delta H_t/\Delta S_t$. The ratio for hydrophobic hydration has been experimentally observed to be smaller (corresponding to a lower value of T_t) for more hydrophobic hydration and larger (corresponding to a higher value of T_t) for less hydrophobic hydration.

Raising the temperature from below to above T_t drives hydrophobic folding and assembly. When the model elastic proteins are cross-linked into an elastomeric band and the temperature is raised from below to above T_t, the band contracts and lifts a weight. This somewhat obvious demonstration of thermo-mechanical transduction represents **Axiom 2**.

Controlling the value of T_t controls the hydrophobically folded and assembled state of the protein. Essentially every variable (energy input) imposed upon an aqueous system containing an appropriately designed model protein changes the value of T_t. Lowering the temperature from above to below an operating temperature drives hydrophobic folding and assembly at constant temperature, and can be used to do mechanical work of lifting a weight when the polymer is cross-linked as an elastic matrix. This is **Axiom 3**. A video has been prepared showing different energy inputs, that lower T_t from above to below an operating temperature, to drive contraction and to perform the mechanical work of lifting a weight.

Any two different energy inputs, which with the properly designed elastic model protein can individually drive hydrophobic folding and assembly by lowering T_t, can be converted one into the other by being part of the same hydrophobic folding domain. This is **Axiom 4**. An example is the conversion of the electrical energy of the reduction of a redox couple into the chemical energy of picking up a proton due to the shift in pKa of the chemical couple caused by formation of the more hydrophobic reduced state. This is an example of electro-chemical transduction as occurs in the development of a proton gradient during electron transport in the inner mitochondrial membrane.

Axiom 5 is the experimental demonstration that energy conversion by this mechanism is more efficient for more hydrophobic domains, hence one of the advantages of oxidative phosphorylation occurring within a lipid bilayer membrane. An experimental basis for this statement resides in the observation of supra-linear hydrophobic-induced pKa shifts discussed below.

Stepwise Experimental Development of the Physical Basis: *Implication of Dependence of T_t on Hydrophobicity and Charge*: On the one hand, adding a CH_2 moiety to a repeating peptide whether a repeating pentapeptide, e.g., $(GVGVP)_n$ going to $(GVGIP)_n$ or a repeating tetrapeptide, e.g., $(GGVP)_n$ going to $(GGIP)_n$, lowers the value of T_t. On the other hand, removing two CH_2 moieties, as in going from $(GGVP)_n$ to $(GGAP)_n$ or in going from $(GVGVP)_n$ going to $(GAGVP)_n$, increases the value of T_t. It is straightforward to deduce that the larger aliphatic moieties would have more water of hydrophobic hydration and that the smaller aliphatic moiety would have less hydrophobic hydration. Furthermore, the formation of a few carboxylates per 100 residues of poly[0.8(GVGVP),0.2(GEGVP)] dramatically increases the value of T_t One proposes from this initial finding that lower values of T_t indicate greater hydrophobicity with more hydrophobic hydration and higher values of T_t indicate lesser hydrophobicity with less hydrophobic hydration and that the formation of anionic carboxylates destroys hydrophobic hydration. This putative relationship is established below by a series of experimental results.

Differential Scanning Calorimetry (DSC): Effect of charge on the heat of the hydrophobic folding and assembly transition: Since the work of Frank and Evans in 1945, one appreciates that dissolution of a hydrophobic group in water is an exothermic reaction and that solubility is limited due to the formation of low entropy, water structure surrounding the hydrophobic group. Accordingly, ΔH is negative and ΔS is positive, resulting in a small value of the Gibbs free energy, $\Delta G = \Delta H - T\Delta S$. To the extent that hydrophobic folding in a model protein can be represented as a reversal of hydrophobic dissolution, the endothermic heat required to drive hydrophobic folding represents the heat required to destructure hydrophobic hydration. Using poly[0.8(GVGVP),0.2(GEGVP)], the heat of the endothermic transition of hydrophobic folding and assembly decreases to one-fourth as two carboxylates form per 100 residues. The smaller ΔH suggests less hydrophobic hydration and hydration of the carboxylate anion occurs at the expense of hydrophobic hydration. This is consistent with a mechanism of competition for hydration between hydrophobic and charged moieties constrained to coexist along a polymer chain.

Stretch-induced pKa shifts: The first compelling insight, that a mechanism other than electrostatic charge-charge repulsion was operative for mechano-chemical transduction exhibited by these elastic protein-based polymers, came on determining the glutamic acid pKa resulting from stretching a hydrophobically folded elastic band of γ-irradiation cross-linked poly[0.8(GVGVP),0.2(GEGVP)]. Stretching increased the pKa of the Glu residue carboxyl from about 4 to above 9 and resulted in a supra-linear increase in pKa with increase in mechanical force. In terms of change in chemical potential, $\Delta\mu$, and change in applied force, Δf, the experimentally determined $\partial\mu/\partial f < 0$, whereas for charge-charge repulsion the sign is reversed, $\partial\mu/\partial f > 0$. This means that in spite of an increase in water in the elastic model protein band on stretching, the carboxylate increases in free energy, i.e., becomes energetically less favored. It is clear that the dominant change in hydration on stretching a hydrophobically folded elastic band would be the formation of water of hydrophobic hydration. The conclusion becomes that hydrophobic hydration is unsuited for carboxylate hydration and that in order to achieve its own hydration the carboxylate must pay for the energy required to destructure hydrophobic hydration. If this is correct, then step-wise increasing hydrophobicity of an elastic model protein should systematically increase carboxyl pKa.

Hydrophobic-induced pKa shifts: Systematically replacing an ionizable residue in poly[f_V(GVGIP),f_X(GXGIP)] where $f_V + f_X = 1$ and X is either Glu (E), Asp (D) or Lys (K) by a hydrophobic Val residue causes the carboxyl pKa values to remarkably increase as f_E and f_D approach zero and the amino pKa values of Lys to decrease as f_K approaches zero. Furthermore, in the family of model proteins, (GVGVP GVGβP GXGβP GVGVP GVGβP GβGβP)$_n$, where X may be either E or D and where β may be either V or F (Phe) and where the number of Phe residues are 0, 2, 3, 4, or 5, a supra linear increase in pKa occurs as F goes from 0 to 5. Indeed, two different types of model protein systems involving three different functional groups demonstrated supra-linear increases in pKa shifts as hydrophobicity is stepwise increased. Hydrophobic-induced pKa shifts are more effective than electrostatic-induced pKa shifts when utilized in performance of chemo-mechanical transduction. *More hydrophobic hydration present in an unfolded polymer causes greater pKa shifts to be exhibited by the ionizable functions of the polymer!*

Proposal of an apolar-polar repulsive free energy of hydration, ΔG_{ap}: Progressive increases in hydrophobicity progressively raise the free energy of charged side chains as reflected in the above noted progressive increases in pKa shifts. As long as T_t is above 0C, the pKa shifts are seen without any change in state. Hydrophobic-induced pKa shifts can be observed while the model protein remains in the fully hydrated unfolded and unassembled state. Thus, the pKa shift can not be ascribed to the change in dielectric constant on going from solution into a folded state. This means that, when a charged species is

tethered to a hydrophobic species, the two species will be positioned in order to achieve the most hydration unaltered by the other species. In order that each species will have the maximal hydration, the lowest free energy at values below T_t for the model protein, they will occupy the most distant positions allowed by their tether. In other words, there exists an apolar polar repulsive free energy of hydration.

One measure of this apolar polar repulsive free energy is the magnitude of the pKa shifts. Since the chemical potential, μ, is given by $\mu = RT\ln a$ where a is the activity, and since at normal values of pH, $a = [H^+]$, $\mu = 2.3RT\log[H^+] = -2.3\ RT\ pH$. The change in chemical potential, $\Delta\mu$, resulting from a shift in pKa, becomes $\Delta\mu = -2.3RT\Delta pKa$. As chemical potential is Gibbs free energy per mole, $\Delta G/\Delta n$, the increase in free energy due to the apolar-polar repulsive free energy of hydration, ΔG_{ap}, is $2.3RT\Delta pKa$. Since hydrophobic-induced pKa shifts as large as 6 pH units have been observed in designed model proteins, a repulsive free energy of greater than 8 kcal/mole has been experimentally observed. This is the magnitude of free energy change whereby much of protein function occurs.

Direct observation of the water of hydrophobic hydration: Using microwave dielectric relaxation to examine the behavior of water in model proteins capable of hydrophobic folding and assembly transitions, a relaxation due to water is observed near 5 GHz, some 10 GHz below that of bulk water. This 5 GHz water results from interacting with the model protein; it increases on making the model protein more hydrophobic, and it disappears as the model protein hydrophobically folds and assembles. Therefore, it is water of hydrophobic hydration!

The key question now becomes, what happens to this hydrophobic hydration on ionization of carboxylates within the model protein. The answer, two-thirds of the water disappears as less than two carboxylates form per 100 residues of model protein. This confirms interpretation of the above-described DSC results where three-fourths of the heat of the endothermic hydrophobic folding and assembly transition disappeared on formation of less than two carboxylates per 100 residues. *Thus, the microwave dielectric relaxation data constitute direct observation of the competition for hydration between apolar and polar moieties!*

CONCLUSIONS: By means of this support we have: 1) experimentally developed five axioms for protein function and engineering by means of hydrophobic folding and assembly transitions, 2) demonstrated the physical basis underlying the "hydrophobic effect" to be an apolar-polar repulsive free energy of hydration, which can be estimated in specific cases by means of hydrophobic-induced pKa shifts, 3) *a priori* designed model proteins capable of performing diverse energy conversions (free energy transductions) of biology, 4) provided data, which argues that protein function by controlling hydrophobic folding and assembly represents a far more efficient process than the commonly considered charge-charge interaction mechanism, 5) noted parallel phenomena with a number of biological systems, implying relevance to protein function in biology, and 6) used the above knowledge to develop medical and non-medical applications with feasibility demonstrated for both areas and with some 30 US patents issued and/or filed.

SIGNIFICANCE: The five axioms for protein function and engineering and the underlying physical process of competition for hydration between hydrophobic and polar moieties represent the nuts and bolts of protein function by means of hydrophobic folding and assembly. A systematic and comprehensive understanding of the role of hydrophobic folding and assembly in protein structure and function has been developed. Controlling hydrophobic folding and assembly can be argued to be a more efficient mechanism for free energy transduction than charge-charge interaction. The axioms for hydrophobic folding and assembly in protein function and the underlying physical process represent fundamental developments in understanding protein function and engineering.

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PATENT INFORMATION:

*f. Patents Issued and Pending since January 1995 (time of last competitive renewal)

D. W. Urry Polymers Responsive to Electrical Energy	European Application PCT/US96/09776 U. S. Application CIP (to #19-A)	**0830509** **Pending**	Issued 3/25/98 Filed 06/07/96 Filed 06/07/95
D. W. Urry, David A. Tirrell, and Catherine Jean Heimbach Photoresponsive Polymers	U.S. Application CIP Serial 08/481,179 (Serial 08/482,179 filed 06/07/95, abandoned 04/06/98 in favor of 09/032/373)	**Pending**	Filed 06/07/95
D. W. Urry, David A. Tirrell, and Catherine Jean Heimbach Photoresponsive Polymers	U.S. Application CIP Serial 08/485,426	**Pending**	Filed 06/07/95
D. W. Urry, et. al. Elastomeric Polytetrapeptide Matrices Suitable for Preventing Adhesion of Biologic Materials	U.S. Application CIP Serial 08/485,495	**5519004**	Issued 05/21/96 Filed 06/07/95
D. W. Urry U. S. Application A Simple Method for the Purification of a Bioelastic Polymer	U.S. Application Serial 08/543,020 (Serial 08/423,516 filed 4/14/95, abandoned 6/14/96 in favor of 08/543,020) International Application PCT/US96/05186 (originally filed 04/15/96 as WO, abandoned) Elected Japan & filed JP application 8-531261	**5954387**	Issued 12/29/98 Filed 10/13/95 Filed 04/14/97
D. W. Urry, et. al. Elastomeric Polypeptide Matrices for Preventing Adhesion of Biologic Materials	U.S. Application Serial 08/246,874 Japanese Application 1-5050221	**5,527,610** **2115962**	Issued 06/18/96 Filed 05/20/94 Issued 12/06/96 Filed 05/20/95
D.W. Urry Superabsorbent Materials	U.S. Application CIP Serial 08.384,566	**5520672**	Issued 05/28/96 Filed 02/06/95
D.W. Urry, et. Al Photoresponsive Polymers	U.S. Application Serial 08/187,441	**Pending**	Filed 01/24/94
D.W. Urry Bioelastomeric Drug Delivery System	U. S. Application Serial #08/316,802 European Application 91302648.0 Japanese Application	**Pending** **EP 0449592** **1994740**	Filed 10/03/94 Issued 11/30/94 Filed 10/03/94 Issued 11/22/95

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D.W. Urry Superabsorbent Materials	U.S. Application Serial 07/688,185	**5393602**	Issued 02/28/95 Filed 04/19/91
	European Application 92.917457.1	**Pending**	Filed 03/10/93
	Japanese Application 4-510189	**Pending**	Filed 03/10/92
D.W. Urry Bioelastomer Containing Tetra/ Pentapeptide Units	U.S. Application PCT/US 88/10949	**4,898,926**	Issued 02/06/90 Filed 06/15/87
	Japanese Application 63-50213	**Pending**	Filed 12/15/89
D. W. Urry and K.U.M. Prasad Segmented Polypeptide Bioelastomers to Modulate Elastic Modulus	Japanese Application 503100/87	**2085090**	Issued 08/23/96 Filed 04/17/87
	U.S. Application (originally filed 4/17/86)	**4870055**	Issued 09/26/89 Filed 04/08/88
	European Application 87.903490.8	**EP 0302892**	Issued 07/13/94 Filed 4/08/88

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