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ELECTROMECHANICAL TRANSDUCTION: REDUCTION-DRIVEN HYDROPHOBIC FOLDING DEMONSTRATED IN A MODEL PROTEIN TO PERFORM MECHANICAL WORK

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SUMMARY: It has long been appreciated that hydrophobic folding is an important element of protein structure formation. Here it is demonstrated for the first time that the electrochemical or chemical reduction of a nicotinamide in a model protein, which increases hydrophobicity, can drive hydrophobic folding and assembly in such a way as to lift a weight or otherwise contract against a constant tensional force.

The model protein, poly[0.73(GVGVP),0.27(GK{NMeN}GVP], can be γ-irradiation cross-linked to form an elastic matrix which contracts on raising the temperature from below to above the transition range for hydrophobic folding and assembly. On reduction of the N-methyl nicotinamide, {NMeN}, the transition temperature range is lowered from above to below 20°C to drive contraction due to hydrophobic folding with the performance of mechanical work.

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The protein-based polymer, poly(GVGVP) where G is Gly, V is Val and P is Pro, is soluble in water below 25°C in all proportions but, on raising the temperature above 25°C, it hydrophobically folds and assembles to form a viscoelastic phase. The temperature for the onset of this inverse temperature transition of hydrophobic folding and assembly is designated as T_t. When the protein-based polymer is made more hydrophobic as on addition of a CH_2 moiety per pentamer by changing the Val of GVG to an Ile(I), the value of T_t is lowered about 15°C and when two CH_2 moieties are removed by changing the Val of GVG to an Ala(A), the temperature for the onset of the hydrophobic folding and assembly transition(T_t) is raised some 30°C. Thus, increasing hydrophobicity lowers T_t and decreasing hydrophobicity raises T_t (1).

Using protein-based polymers of the general composition, 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 naturally occurring amino acid or chemical modification thereof, a T_t-based hydrophobicity scale has been developed for all of the naturally occurring amino acid residues and interesting derivatives. In this scale, Trp is the most hydrophobic residue and Glu with the carboxylate side chain is the most polar, least hydrophobic

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residue (1). Phosphorylation of a serine(S) dramatically increases T_t (2) as to a lesser extent do ionizations of Glu(E) and Asp(D) carboxyl side chains (1). When a chromophore such as azobenzene is attached by amide linkage to a Glu(E) side chain or such as cinnamic acid is attached by amide linkage to a Lys(K) side chain, their presence lowers T_t, but when light of the appropriate wavelength is absorbed, presumably converting from the *trans* to the *cis* geometrical isomer, there is an increase in T_t (3). Under the correct conditions, therefore, the absorption of light can drive hydrophobic unfolding. When aromatic side chains are present, as is the case for the Trp(W), Phe(F) and Tyr(Y) residues, a relatively small increase in pressure can raise the value of T_t and cause hydrophobic unfolding (4). When N-methyl nicotinic acid is attached by amide linkage to a Lys residue to form the nicotinamide, reduction to form the N-methyl-1,6-dihydronicotinamide markedly lowers T_t and drives hydrophobic folding and assembly (5).

Because the basic protein-based polymer is elastic, the polymer can be cross-linked to form elastomeric bands. It, therefore, becomes possible to observe the hydrophobic folding as a contraction with performance of the mechanical work of lifting a weight. When poly(GVGVP) is γ-irradiation cross-linked with a 20 Mrad dose of Cobalt-60 radiation to form the elastomeric band, designated as X^{20}-poly(GVGVP), and a weight is attached to the extended matrix at 20°C, on raising the temperature to 40°C the band contracts due to the hydrophobic folding and assembly and the weight is lifted (1). This visualization of thermo-mechanical transduction provides a particularly graphic demonstration of the force of hydrophobic folding and assembly. When the elastomeric matrix is X^{20}-poly[0.8(GVGVP),0.2(GEGVP)] and a weight is attached to the extended matrix at neutral pH, lowering the pH to below the pKa of the Glu residue causes the matrix to contract and lift the weight (6). This is chemo-mechanical transduction, chemically-driven contraction. Also, when pressurized X^{20}-poly[0.79(GVGVP),0.21(GFGVP) with an attached weight is depressurized, contraction occurs with the lifting of the weight (7); this is baro-mechanical transduction. When X^{20}-poly[0.8(GVGVP),0.2(GK{CA}GVP] where {CA} stands for cinnamic acid attached by amide linkage to the Lys(K) side chain to form the cinnamide, illumination with 300 nm light causes hydrophobic unfolding seen as swelling (Heimbach and Urry, unpublished). This elastomeric band will be illuminated in a chamber with a weight attached to properly demonstrate photo-mechanical transduction. Since lowering T_t, the temperature of the hydrophobic inverse temperature transition, drives hydrophobic folding and the lifting of a weight and since raising T_t results in hydrophobic unfolding and the lowering of the weight, this is called the ΔT_t-mechanism of free energy transduction (1).

In the present report, the elastomeric band, X^{20}-poly[0.73(GVGVP), 0.27(GK{NMeN}GVP)] where {NMeN} stands for N-methyl-nicotinic acid attached by amide linkage to the Lys(K) side chain to result in the N-methyl-nicotinamide, is formed; it is placed in an appropriate chamber with weight attached where rhodium bipyridine and ferro/ferricyamide are the mediators for electron transfer to and from the electrodes, electrochemical reduction results in contraction with the lifting of a weight, and electrochemical oxidation shows relaxation with the lowering of the weight. In a chamber capable of video recording with a somewhat faster chemical reduction using dithionite and oxidation using ferricyanide, the reversible contraction and relaxation, with repeated raising and lowering of the weight, is readily seen. Electro-mechanical transduction is demonstrated for the first time in a protein-based polymer by the direct lifting and

lowering of a weight. Reduction of a nicotinamide prosthetic group in an enzyme in solution or in an electron transport protein in the inner mitochondrial membrane could lower T_t and drive hydrophobic folding, and oxidation could be expected to raise T_t and could be expected to result in hydrophobic unfolding to achieve protein function.

MATERIALS AND METHODS

Peptide Synthesis. Chemical synthesis of the protein-based polymer poly[0.73(GVGVP),0.27(GK{NMeN}GVP] where {NMeN} signifies N-methyl nicotinic acid attached by amide linkage to the Lys(K) side chain to form the nicotinamide was described previously (5). Briefly, the pentamers are synthesized by classical solution methods by synthesizing and purifying the dipeptide VP and the tripeptides, GVG and GK(2-Clz)G where 2-Clz is 2-chlorobenzyloxycarbonyl and coupling using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDCI) and 1-hydroxybenzotriazole (HOBt) to form the two pentapeptides. The pentapeptides were activated at their Pro carboxyls as the para-nitrophenyl ester, mixed in dimethyl sulfoxide in the appropriate ratios and polymerized for two weeks in the presence of N-methyl morpholine. The 2-Clz group was deblocked; nicotinic acid was reacted with the ε-amino of Lys to form nicotinamide which was then N-methylated using methyl iodide. Accurate amino acid compositions were determined by amino acid analysis to determine the coefficients 0.73 and 0.27 and the polymer was further verified by nuclear magnetic resonance.

Cross-linking to Form Elastic Redox Matrix. The model protein, poly[0.73(GVGVP),0.27(GK{NMeN}GVP)] was dissolved in water in an appropriate mold. The temperature was raised some 20°C above T_t and the resulting viscoelastic phase, formed in the shape of band, was γ-irradiation cross-linked with 20 Mrad of Cobalt-60 radiation to form the elastomeric redox matrix with an initial elastic (Young's) modulus of 1.2×10^6 dynes/cm^2 at 37°C.

Stress-strain Apparatus. The instrument used in these studies is a modification of the stress-strain apparatus previously described (8). Force measurements are recorded using a Sensotec Model 31 force transducer conditioned by an Analog Devices 3B16 strain gauge module. The force data is recorded by a personal computer using a Metrabyte Dascon-1 multi-function board. The moving platform of the stress-strain unit is driven by a stepper motor and a Whedco SMD-1150 translator. The translator is interfaced with the computer allowing software control of the drive motor. Position data is recorded from a potentiometer coupled to the platform drive screw. Under software control the force on the sample can be held constant and the platform position recorded.

For the electrochemical reduction and oxidation studies, the sample is enclosed in a sealed chamber which is clamped to a housing enclosing the force transducer. One sample grip is attached to the transducer with a latex diaphragm providing a chamber seal. The other sample grip is attached to the moving platform by a thin stainless steel rod through a Teflon seal in the end of the sample chamber.

Electrochemical Studies. Electrochemical reduction was carried out using a three-electrode potentiostat designed and built in this laboratory. Monitoring of current and recording current-voltage curves utilized a Keithly 427 Current Amplifier, the Fluke 8300A digital voltmeter and a Gould x-y recorder. A graphite electrode was used as the working electrode; platinum and silver-silver chloride electrodes served as the counter and reference electrodes, respectively.

An electron mediator was used to carry electrons from the working electrode into the cross-linked matrix. An effective reducing agent for nicotinamide and its analogs is rhodium(+3) in complex with bipyridine (9). This complex was synthesized in this laboratory. Rhodium bipyridine complex, $Rh(bpy)_3Cl_3$, was dissolved in 0.1 M potassium carbonate buffer containing 0.1 M potassium chloride. The pH was 9.0. This solution was degassed 20 min. with nitrogen gas. Reduction was conducted at – 0.9 V vs. the silver-silver chloride reference electrode. As the mediator adsorbs on the graphite electrode, the current was turned off after 6 hours and the oxidized form of the mediator, $Rh(bpy)_2^{3+}$, was allowed to diffuse into the cross-linked matrix. For electrochemical oxidation potassium ferrocyanide (0.02 M) was used in place of rhodium bipyridine complex. A voltage of + 0.4 V vs. the silver-silver chloride reference electrode was applied. The ferrocyanide ion is converted to ferricyanide at the graphite electrode and then diffuses into the cross-linked matrix where it oxidizes the reduced NMeN moieties.

Chemical Reduction and Oxidation The γ-irradiation cross-linked sample was gripped in the above-described horizontal stress-strain chamber. Contracted dimensions of the sample were 3.3 mm width and 0.4 mm thickness. The solution was 0.1 M K_2CO_3 with 0.1 M KCl at pH 9.0 that had been degassed for 20 minutes with N_2. After 1 hour, a stretching force of 1.0 g was applied. One mL of 0.1 M sodium dithionite in the same degassed buffer was added to the chamber which made the solution 0.01 M in dithionite. Data were collected at 15 min. intervals at 1.0 g constant force as the dithionite diffused into the cross-linked matrix. The sample contracted as the N-methyl nicotinamide moieties were reduced. For oxidation the solution was replaced by 0.02 M potassium ferricyanide in the same carbonate-KCl buffer.

Experimental Description of Video Cell. The elastic redox matrix, X^{20}-poly[0.73(GVGVP),0.27(GK{NMeN}GVP)], was suspended in a vertical flow-through cell composed of plexiglass. A mass of 3 grams (when suspended in solution) was attached to the bottom end of the sample. The cell dimensions were 1 cm x 1 cm x 8 cm. A liquid reservoir 8 cm in height was connected to the cell at the top. An aluminium water jacket covered the cell on 3 sides. Redox solution was delivered by a Masterflex Model 7016 variable speed pump. A system of solenoid valves allowed flushing of delivery tubing. Reduction was carried out with 0.01 M sodium dithionite in 0.1 potassium carbonate buffer at pH 9.2 at a flow rate of 6 mL/min. Oxidation utilized 0.02 M potassium ferricyanide in the same 0.1 M carbonate buffer. Time lapse photography at 20 seconds per frame recorded changes in length of the sample. The time lapse film was converted to video tape at UAB Medical Television. Data was taken from the video tape for length vs. time plots.

RESULTS

The electrochemical reduction and oxidation results for the redox model protein elastomeric band, X^{20}-poly[0.73(GVGVP),0.27(GK{NMeN}GVP] are given in Figure 1A and B, respectively, at 21°C using a graphite working electrode, a platinum counter electrode and a silver/silver chloride reference electrode. Reduction, using a potential of – 0.9 V with respect to Ag/AgCl, is seen to drive contraction against a constant load of 1 gm; oxidation, using a potential of + 0.4 V with respect to the Ag/AgCl reference electrode, drives extension. Using the steepest slopes of Figure 1, the rates of contraction and extension are approximately 1% and 2.5% of total length/hr, respectively, in the horizontal stress/strain apparatus at a constant positive force (tension) of one gram where 10 to 15 hours are required to reach the fully contracted or extended states.

For chemical reduction by dithionite and oxidation by ferricyanide in the horizontal stress/strain apparatus with a constant one-gram load, the rates of contraction and extension

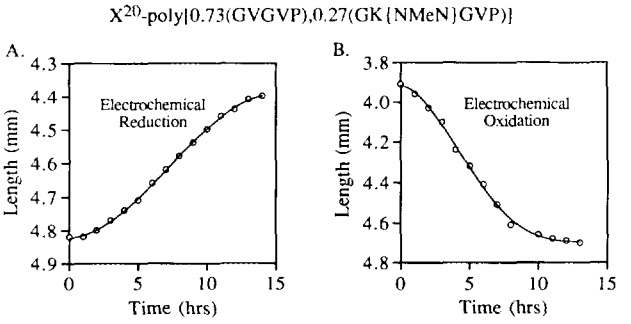

Figure 1. (A) Electrochemical reduction drives contraction and (B) electrochemical oxidation results in extension. This is electro-mechanical transduction. See text for details.

obtained from the steepest slopes of Figure 2A and B are 5.2% and 4.0% of total length/hr, respectively. The elastomeric bands of Figures 1 and 2 were of the same width, 3.3 mm, and of the same thickness, 0.4 mm, such that diffusional issues are similar. Comparison of the data in Figures 1 and 2 suggests that the electrode reactions are rate limiting for the electrochemical reduction and oxidation. Comparison of Figures 2A and B shows that dithionite can drive contraction faster than ferricyanide can drive extension in spite of the extending tensional load. Since reduction of poly[0.73(GVGVP),0.27(GK{NMeN}GVP)] in solution by dithionite goes to 60% completeness in 100 min, it would appear either that diffusion in and out of the matrix is the rate limiting process for the chemical reduction or the rate of hydrophobic folding and assembly within the matrix is rate limiting. Since contraction can be driven by salt at 20°C at a faster rate (1), it appears that the diffusional process is rate limiting.

In the flow-through vertical cell of Figure 3 with a three-gram load suspended from an elastomeric band of X^{20}-poly[0.73(GVGVP),0.27(GK{NMeN}GVP)], the rate of contraction using dithionite as reductant and the rate of extension using ferricyanide as oxidant are quite similar, about 13 to 14% of total length/hr. The band is of the same thickness as for the horizontal cell, 0.4 mm, but it is somewhat wider, 4.5 mm. Accordingly, the three-fold faster rate is likely due to the flow-through cell supplying a steady concentration of reactant at the matrix interface, 0.01 M dithionite or 0.02 M ferricyanide.

DISCUSSION

The unique effects of hydrophobic association in proteins and related molecules in aqueous systems have been appreciated for more than half a century from the work of Edsall (10,11), Frank and Evans (12), Kauzmann (13), Scheraga and coworkers (14-16), Tanford (17) Ben-Naim (18), Privalov (19), etc. More recently, Dill and coworkers (20) have provided a theoretical basis for understanding the cooperativity of hydrophobic folding transitions and Dobson and coworkers

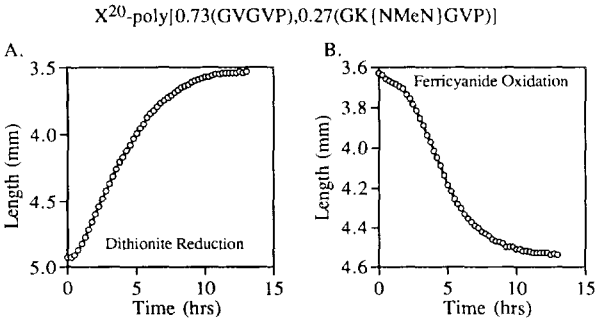

Figure 2. (A) Chemical reduction driven contraction using dithionite which appears to be faster than for the electrochemical reduction seen in Figure 1A. (B) Chemical oxidation-driven extension which also appears to be faster than electrochemical oxidation using the same oxidant as mediator. See text for details.

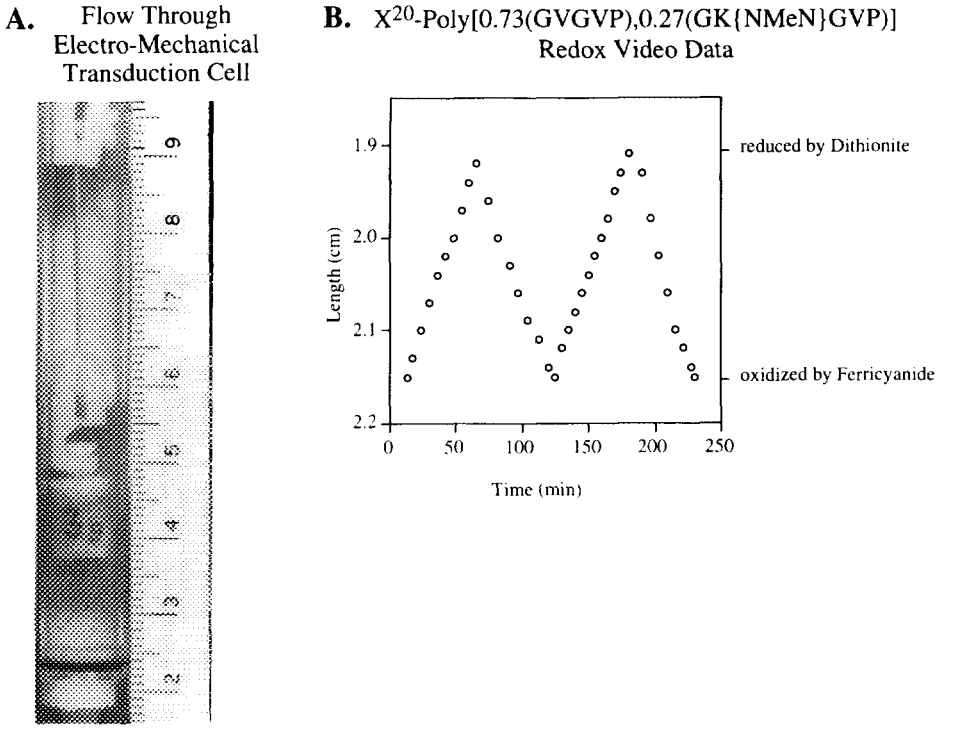

Figure 3. Repeated reduction/oxidation cycles observed in a transparent, thermostatted, flow-through cell (A). Plots of data obtained by time lapse photography using approximately a 55-minute reaction period for each half of the cycle (B). See text for details.

(21) have experimentally shown hydrophobic folding to be the primary event in the folding of hen lysozyme, an event which precedes stable secondary structure formation.

Hydrophobic folding occurs as a transition involving a limited temperature range as perhaps best shown by model proteins like those of this report (1). Thus, raising the temperature from below to above the transition temperature range drives hydrophobic folding. Of greatest significance to warm-bloodied animals, however, are the many ways that the transition temperature range for hydrophobic folding can be moved from above to below physiological temperature to drive folding or from below to above a working temperature to drive unfolding. The onset of the hydrophobic folding and assembly transition as the temperature is raised is designated as T_t, and many of the changes which are central to protein function cause changes in T_t. This is the ΔT_t-mechanism for protein function most easily discussed in terms of energy conversion. Figure 4 summarizes the demonstrated and putative energy conversions using the ΔT_t-mechanism. This report provides the arrow connecting the electrical and mechanical force apices. In particular, electrochemical or chemical reduction of a nicotinamide is seen to result in the very real physical force of lifting a weight in the performance of mechanical work. It is achieved because reduction of nicotinamide makes the matrix more hydrophobic, lowers T_t from above to below the operating temperature of 21°C and thereby drives hydrophobic folding.

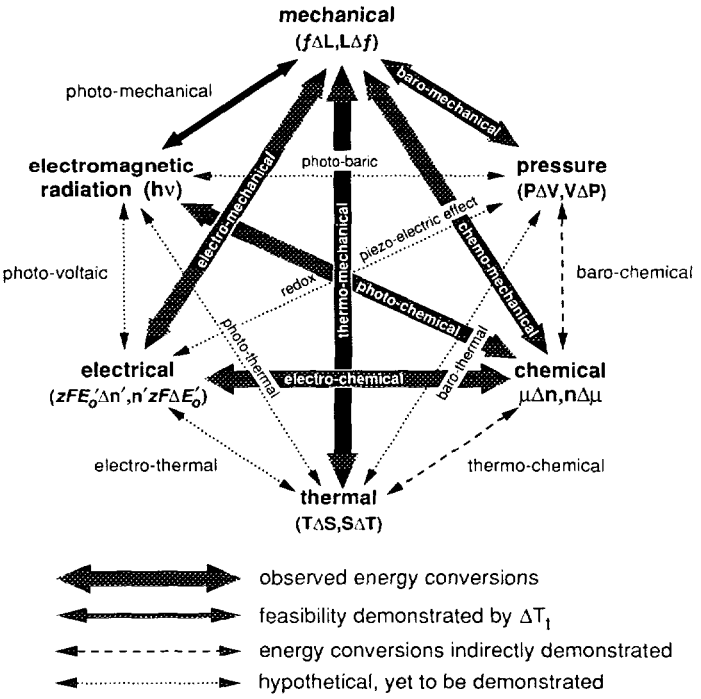

Figure 4. Demonstrated and putative energy conversions using the ΔT_1-mechanism of free energy transduction. This report is the first demonstration of electro-mechanical transduction, that is, it provides the basis for the solid arrow connecting the electrical and mechanical apices.

Recently it has also been shown quite exhaustively (22-26) that increasing hydrophobicity causes changes in the pKa of functional groups such as the carboxyls of Glu and Asp residues and the ε-amino of the Lys residue. For example, raising the hydrophobicity increases the pKa of the Asp or Glu carboxyl such that increasing hydrophobicity can do the chemical work of taking up a proton. It has also been shown that increasing the hydrophobicity by the reduction of the nicotinamide of the present study can increase the pKa of an Asp residue and do the chemical work of picking up a proton. Thus, by increasing hydrophobicity, electrochemical reduction can result in the performance of mechanical work as shown herein, and, with the properly designed model protein, it can result in the performance of chemical work (in preparation).

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