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Non-linear hydrophobic-induced pK_a shifts: Implications for efficiency of conversion to chemical energy

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Abstract

By using one Asp or one Glu per thirty residues in a polytricosapeptide capable of exhibiting a hydrophobic folding and assembly transition and stepwise converting a set of the five Val residues (most proximal to the Asp or Glu residue) to more-hydrophobic Phe residues, a non-linear hydrophobic-induced pK_a shift was observed with a ΔpK_a of 0.4 (Asp) and 0.3 (Glu) on addition of 2 Phe residues per 30mer but with a ΔpK_a of 4.7 (Asp) and 2.7 (Glu) on going from 4 Phe/30mer to 5 Phe/30mer. As a shift in pK_a can be equivalent to the conversion to chemical energy from whatever energy input – mechanical, chemical, electrochemical, pressure or light – which effects a change in hydrophobicity, the non-linear hydrophobic-induced pK_a shift means increased efficiency of energy conversion with increased hydrophobicity of the protein-based polymer.

1. Introduction

Hydrophobic folding of elastic protein-based polymers of the general composition poly[f_v(GVGVP), f_x(GXGVP)] where f_v and f_x are mole fractions with $f_v + f_x = 1$, G is Gly (glycine), V is Val (valine), P is Pro (proline), and X is a particular amino acid guest residue or chemical modification thereof, has been shown on γ-irradiation to form elastic matrices capable of performing mechanical work with appropriate design and input free energies, the intensive variables of which are temperature, pressure, chemical potential, electrochemical potential and electromagnetic radiation [1,2].

The physical basis underlying the function of these designed molecular engines has been described as arising from the competition for hydration between apolar side chains such as those of the valyl, phenylalanyl (Phe, F), isoleucyl (Ile, I), etc. residues and the polar side chains such as those of charged aspartic acid (Asp, D), glutamic acid (Glu, E), lysine (Lys, K), etc. residues. This has been called an apolar–polar repulsive free energy of hydration [1].

One expression of this competition for hydration, that is of the repulsive free energy of hydration, has been seen in the stretch-induced pK_a shifts [3], in the large decrease in the endothermic heat of the hydrophobic folding transition on the occurrence of two carboxylate residues per 100 residues [4], and in the large pK_a shifts observed for poly [f_v(GVGIP), f_x(GXGIP)] as f_x is varied from 1.0 to 0.06 for X = D, E, or K [5–7]. Also using the fixed primary structures of polytricosamers containing one Asp or

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Glu residue and five Phe residues per 30mer, dramatic pK_a shifts have been observed from 3.9 to 10.1 for Asp (a ΔpK_a of 6.2 pH units) and from 4.3 to 8.1 for Glu (a ΔpK_a of 3.8 pH units) [8,9]. Accordingly, changes in hydrophobicity effect remarkable changes in pK_a.

Chemical energy is $\Delta\mu \times \Delta n$ [10] where $\Delta\mu$ is the change in chemical potential (expressible in terms of pK_a shifts as $\Delta\mu = -2.3RT\Delta pK_a$) and Δn is the change in moles of species (e.g. of protonated or deprotonated species) resulting from the ΔpK_a. Changes in pK_a, therefore, become measures of the chemical energy outputs resulting from free energy inputs which change the hydrophobicity of the protein-based polymer. Relevant free energy inputs which cause changes in hydrophobicity of the protein-based polymer are the electrochemical reduction of an attached redox couple and the absorption of light by an attached chromophore that undergoes as suitable photochemical reaction [1,2].

This Letter reports the dependence of the pK_a values of Asp and Glu residues on changes in the number of Phe residues that have replaced Val residues per thirty residues in polytricosapeptides. A dramatic non-linearity is observed in the ΔpK_a values as the numbers of Phe residues per 30mer are increased from two to five. The finding becomes fundamental to the design of protein-based polymers for the efficient conversion to chemical energy of different energy inputs such as light or electrochemical reduction. Such considerations are fundamental to the primary process whereby living organisms utilize light (as in photosynthesis) or reduction of electron carriers on oxidation of foods (as in respiration) to develop transmembrane proton gradients which are then used to phosphorylate ADP (adenosine diphosphate) to produce adenosine triphosphate (ATP), the ubiquitous biological energy currency of living organisms [11].

2. Materials and methods

2.1. Peptide synthesis

The preparation of the following tricosapeptides is not only labor intensive, but requires considerable care in the preparation and purification of the building blocks that are used in making the high molecular weight polymers. Once the high molecular weight polymers are obtained, the side chain protection of the glutamic and aspartic acid residues must be removed. In order to minimize the glutamimide and aspartimide formation, cyclohexyl ester (OCHx) [12] was used in the synthesis and deprotected using hydrogen fluoride with *p*-cresol as a scavenger. The polymers were then verified using amino acid analysis and ^{13}C NMR.

The peptides Boc-Gly-Val-Gly-OH, Boc-Val-Pro-OBzl, Boc-Gly-Val-Gly-Val-Pro-OBzl, Boc-Gly-Val-Gly-Val-Pro-ONp, Boc-Gly-Glu(OCHx)-Gly-OH, Boc-Gly-Glu(OCHx)-Gly-Val-Pro-OBzl, Boc-Gly-Phe-Gly-OH, and poly[0.8(Gly-Val-Gly-Val-Pro), 0.2(Gly-Glu-Gly-Val-Pro)], referred to below as polymer **VI**, were all prepared as previously described [13–15].

Synthesis:

Boc-Phe-Pro-OBzl (**I**). Boc-Phe-OH (Boc: tert-butyloxycarbonyl; 90.00 g, 0.339 mol) was dissolved in 500 ml of dimethylformamide (DMF) and cooled to $-15°$ C. 1-hydroxybenzotriazole (HOBt; 50.43 g, 0.373 mol) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDCI; 71.54 g, 0.373 mol) were added. After 20 min, a pre-cooled solution of HCl-H · Pro-OBzl (OBzl; benzyl ester; 81.99 g, 0.339 mol) and N-methylmorpholine (NMM; 37.30 ml, 0.339 mol) was added. The reaction mixture was stirred overnight at room temperature. The DMF was removed under reduced pressure and the residue was extracted into $CHCl_3$. The $CHCl_3$ extract was washed with water, 10% citric acid, 5% $NaHCO_3$, water and dried over Na_2SO_4. The solvent was removed under reduced pressure and dried to obtain 125.12 g (yield 81.51%) of **I**.

Boc-Asp(OCHx)Gly-OBzl (**II**). Boc-Asp(OCHx)-OH (OCHx; cyclohexyl ester; 75.00 g, 0.238 mol) was coupled to Tos · H · Gly-OBzl (Tos; *p*-toluenesulfonyl; 80.22 g, 0.238 mol) using EDCI with HOBt. The reaction was worked up by acid and base extractions to obtain 100.64 g (yield 91.52%) of **II**.

Boc-Gly-Asp(OCHx)-Gly-OBzl (**III**). Compound **II** (100.00 g, 0.216 mol) was deblocked with 4.0 N HCl/dioxane for 1.5 h. Excess HCl and dioxane were removed under reduced pressure, triturated with ether, filtered, washed with ether and petroleum ether and dried (yield 97.80%). This was coupled to Boc-Gly-OH (37.05 g, 0.211 mol) using EDCI with

HOBt. The reaction was worked up by acid and base extractions to obtain 99.11 g (yield 90.21%) of **III**.

Boc-Gly-Asp(OCHx)G-OH (**IV**). Compound **III** (99.00 g, 0.191 mol) in glacial acetic acid (1000 ml) was hydrogenated in presence of 10% palladized charcoal catalyst at 40 psi. The catalyst was filtered off and the solvent was removed under reduced pressure. The residue was triturated with ether, filtered, washed with ether and petroleum ether and dried to obtain 71.18 g (yield 86.99%) of **IV**.

Boc-Gly-Asp(OCHx)-Gly-Val-Pro-OBzl (**V**). Boc-Val-Pro-OBzl (23.64 g, 0.058 mol) was deblocked with HCl/dioxane and coupled to compound **IV** (25.1 g, 0.058 mol) using EDCI with HOBt. The reaction was worked up by acid and base extractions to obtain 41.10 g (yield 94.03%) of **V**.

Boc-Gly-Asp(OCHx)-Gly-Phe-Pro-OBzl (**VI**). Compound **I** (26.45 g, 0.058 mol) was deblocked with HCl/dioxane and coupled to compound **IV** (25.10 g, 0.058 mol) using EDCI with HOBt. The reaction was worked up by acid and base extractions to obtain 40.21 g (yield 90.06%) of **VI**.

Boc-Gly-Phe-Gly-Phe-Pro-OBzl (**VII**). Compound **I** (26.45 g, 0.058 mol) was deblocked with HCl/dioxane and coupled to Boc-Gly-Phe-Gly-OH (23.23 g, 0.058 mol) using EDCI with HOBt. The reaction was worked up by acid and base extractions to obtain 35.79 g (yield 85.78%) of **VII**.

Boc-Gly-Val-Gly-Phe-Pro-OBzl (**VIII**). Compound **I** (26.45 g, 0.058 mol) was deblocked with HCl/dioxane and coupled to Boc-Gly-Val-Gly-OH (19.37 g, 0.058 mol) using EDCI with HOBt. The reaction was worked up by acid and base extractions to obtain 35.12 g (yield 90.23%) of **VIII**.

Boc-Gly-Asp(OCHx)-Gly-Val-Pro-ONp (**IX**). Compound **V** (8.75 g, 0.012 mol) was hydrogenated and reacted with bis(4-nitrophenyl carbonate) (bis-PNPC; 1.5 equiv.) in pyridine (90 ml). When the reaction was complete, as determined by TLC, the solvent was removed under reduced pressure. The residue was worked up by acid and base extractions. The solvent was removed under reduced pressure, triturated with ether, filtered, washed with ether and petroleum ether, and dried to obtain 7.35 g (yield 78.64%) of **IX**.

Polymer **I**: Poly[0.83(Gly-Val-Gly-Val–Pro), 0.17(Gly-Asp-Gly-Val-Pro)] (**X**). Boc-Gly-Val-Gly-Val-Pro-ONp (4.11 g, 0.006 mol) and compound **IX** (1.01 g, 0.001 mol) were deblocked together using trifluoroacetic acid (TFA) and a one molar solution of the TFA salt in DMSO was polymerized for 18 days using 1.6 equiv. of NMM as base. The polymer was dissolved in water, dialyzed using 3500 mol. wt. cutoff tubing and lyophilized. The material was then deblocked using HF: *p*-cresol (90:10, v/v) at 0° C for 1 h. It was triturated with ether and then dissolved in water, dialyzed using 50000 mol. wt. cutoff tubing and lyophilized to obtain 1.03 g (yield 39.42%) of **X**.

The pentamers above were then used to construct a series of polytricosapeptides, namely:

polymer **II**: poly(GDGFP GVGVP GVGVP GVGVP GVGVP, GVGFP);

polymer **III**: poly(GDGVP GVGVP GVGFP GFGFP GVGVP GVGVP);

polymer **IV**: poly(GDGFP GVGVP GVGFP GVGFP GVGVP GVGFP);

polymer **V**: poly(GDGFP GVGVP GVGFP GFGFP GVGVP GVGFP);

polymer **VII**: poly(GEGFP GVGVP GVGVP GVGVP GVGVP GVGFP);

polymer **VIII**: poly(GEGVP GVGVP GVGFP GFGFP GVGVP GVGVP);

polymer **IX**: poly(GEGFP GVGVP GVGFP GVGFP GVGVP GVGFP);

polymer **X**: poly(GEGFP GVGVP GVGFP GFGFP GVGVP GVGFP);

polymer **XI**: poly(GDGVP GFGFP GFGVP GVGVP GFGFP GVCVP);

polymer **XII**: poly(GEGVP GFGFP GFGVP GVGVP GFGFP GVGVP);

polymer **XIII**: poly(GDGFP GVGVP GVGVP GVGVP GFGFP GFGFP). These tricosapeptides were synthesized by the [(5 + 5 + 5) + (5 + 5 + 5)] fragment coupling strategy in solution phase. In the synthesis, the Boc groups' removal was effected by TFA. All coupling reactions and polymerizations were carried out by the EDCI/HOBt method. The final deprotection and purification by dialysis were carried out as for polymer **I** above. Key structures are shown schematically in Fig. 1.

2.2. The experimental procedure of acid–base titration

The acid–base titrations were carried out on the Titralab (Radiometer American, Inc.) and the Mettler

DL21 Titrator, each connected to a dedicated PC controller whereby the computer software controls the experiment and provides necessary flexibility in intervals ensuring that equilibrium has been reached after each addition of titrant.

Before performing pH measurements, the system is calibrated with standard pH solutions at pH 4, 7 and 10. The VIT90 performs 1- and 2-point calibrations for four different electrode pairs. After the pH calibration, a quantity in the range of 30 to 100 mg of the sample is dissolved in 1.5 ml type I water (low conductivity with a resistance greater than 12 MΩ) within a 5 ml vessel in the SAM90 or the Mettler DL21 sample stations. Using 1 N HCl or NaOH solution to adjust pH to the desired low or high value, the PC controller program is used to start the titration. The time between each drop of NaOH or HCl with a concentration of 0.1 N was set to 45 min. Particular care must be taken in the titrations of aspartic acid containing polytricosapeptides. The pH adjustments must be carried out at low temperature (around 2 to 5° C) to prevent the aspartic acid residue in the peptide chain from undergoing α–β exchange. To prevent CO_2 dissolution, the acid–base titrations were carried out under an argon atmosphere.

All titrations began more than 2 pH units below the approximate pK_a value and were stopped more than 2 pH units above the estimated pK_a value. It should be noted for all polymers that the COO^- state was soluble and the COOH state was hydrophobically folded and assembled at the 20° C temperature of the titration.

3. Results and discussion

3.1. Acid–base titration results

A series of acid–base titration curves, for polymers **I** to **IV** containing the Asp amino acid residue are shown in Fig. 2A and for polymers **VI** to **IX** containing the Glu amino acid residue, are given in Fig. 2B. In each case, the progression to higher pK_a values occurs systematically with the increase in number of Phe residues per 30mer. The same five positions within the 30mer are used for replacement of Val by Phe as the number of substitutions progresses from 2 to 3 to 4 and finally to 5. The acid–base titration results for the proximal five Phe residues/30mer have appeared previously [8,9]. It is

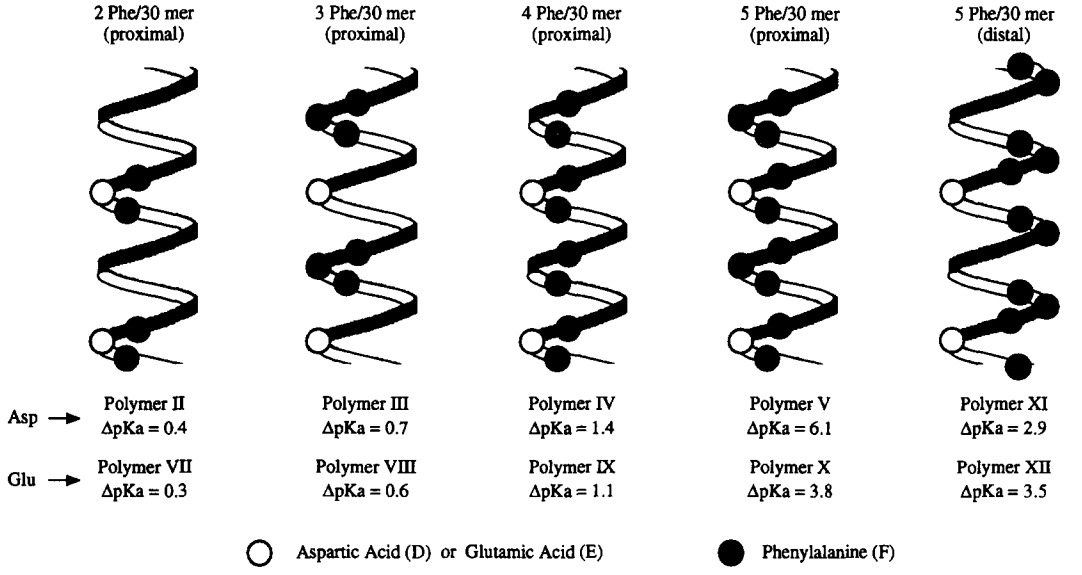

Fig. 1. Schematic representations of polymers **II** to **V** and polymers **VII** to **X** showing the relative orientation of Asp or Glu, (○), residues to the varying number of Phe (●) residues for the β-spiral structure and of polymers **XI** and **XII** with five distal Phe (●) residues/30mer [1].

essential for the present comparative study to use the same set of positions because with the Asp residue, for example, five Phe residues can be placed as proximal as possible to the Asp given retention of the β-spiral structure to give a ΔpK_a of just greater than 6 or the five Phe residues can be placed as distal as possible from the Asp with retention of the β-spiral structure to give a ΔpK_a of only 2.8 [9]. In the present study, the five proximal positions were used. The data of Fig. 2 are plotted in Fig. 3 along with the previously published data for the five Phe residues/30mer [8,9] as the ΔpK_a versus the number of hydrophobic Phe residues per tricosamer for both the Glu- and Asp-containing polytricosapeptides. A very non-linear curve is obtained. This is the result of a regular structure, because, when the composite pentamers of the polytricosapeptides for each of the five Phe residues (distal and proximal) were

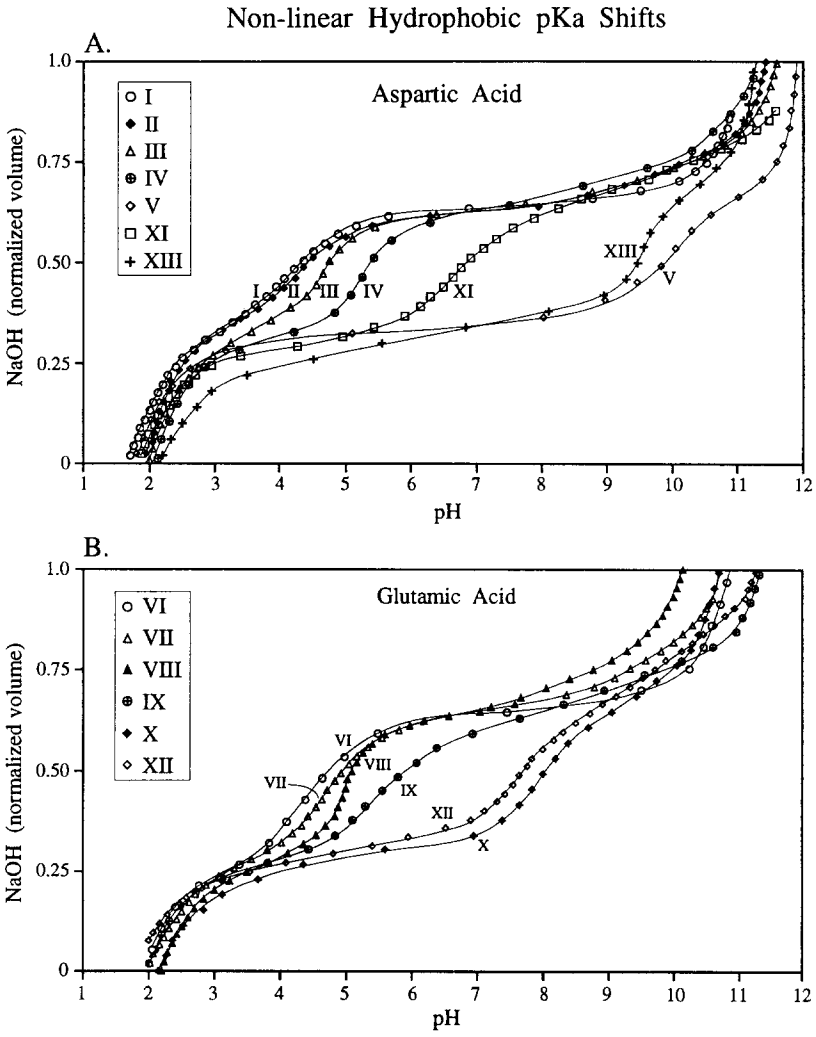

Fig. 2. Acid–base titration curves. (A) For the Asp-containing polymers **I**, **II**, **III**, **IV**, **V**, **XI**, and **XIII**. (B) For the Glu-containing polymers **VI**, **VII**, **VIII**, **IX**, **X**, and **XII**. The titration curves for polymers **V**, **X**, **XI**, and **XII** were previously reported [8,9] and are included here to show the continuity of the pK_a shifts. Each titration began at a somewhat different pH value. Because each titration began at a somewhat different pH value and there was some variation in the concentrations used, for easier visual comparison the vertical scale for each titration was shifted and expanded or contracted as necessary in order that the sigmoid curves begin and end at similar y axis locations.

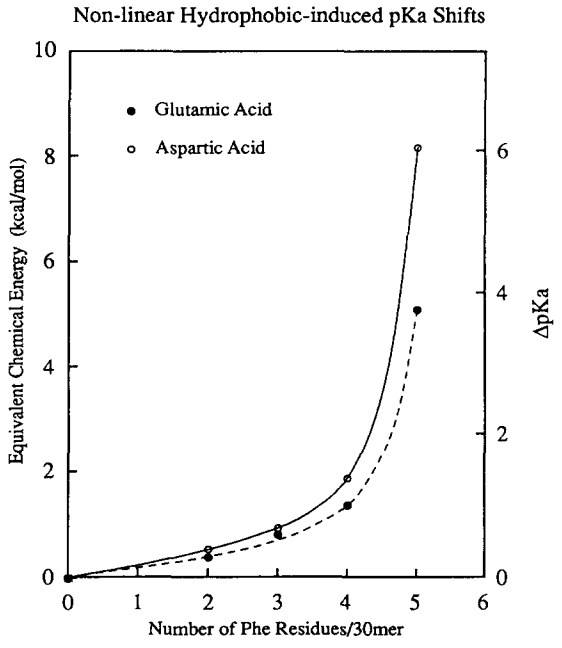

Fig. 3. Plots of the ΔpK_a values of the polytricosapeptides of Fig. 1 (with the data for polymer I as reference for Asp-containing polymers and the data for polymer VI as reference for the Glu-containing polymers) as a function of the number of Phe residues per tricosapeptide in the five structurally most proximal positions. As the ΔpK_a, given as the right ordinate, has a chemical energy equivalence, given as the left ordinate, this non-linearity becomes the basis for the efficiency of energy conversion. Please see text for discussion.

mixed in the correct ratios and randomly polymerized, the highest ΔpK_a was 1.3 rather than 2.8 and 6.1 for the fixed/primary sequences [16].

3.2. Non-linearity in pK_a versus hydrophobicity plots

Non-linearity in the pK_a versus increasing hydrophobicity has been previously seen in the family of protein-based polymers, poly[f_v(GVGIP), f_x(GXGIP)] where f_v and f_x are mole fractions with $f_v + f_x = 1$, where f_x was varied from 1.0 to 0.06 and where X = Glu [6], Asp [5] and Lys [7]. In considering the physical basis responsible for the ΔpK_a values, charge–charge repulsion [10,17–19], electrostatic (ion) self-energy [20] in which macroscopic dielectric constant data and in which a microscopic dielectric analysis based on Langevin (water) dipoles and peptide bond dipoles of the local environment of the chargeable species were considered [21–23] and a competition between apolar and polar species for hydration [1], i.e. an apolar–polar repulsive free energy of hydration [1,24], were each discussed with the latter being concluded as the physical basis.

In the previous studies [5–7], the pentamers GVGIP and GXGIP were not ordered in the polymer and simultaneously the chargeable residue X was decreasing as the more-hydrophobic V (valyl) residue was increasing. In the present studies, X is kept constant, one per 30mer, and the hydrophobicity is increased in a systematic manner with 2, 3 and 4 and finally 5 Phe residues replacing a specific set of Val residues, that is, using the set of five Val positions most proximal to the X residue.

3.3. Relevance of ΔpK_a to chemical energy

Chemical energy can be defined as the product of the change in chemical potential, $\Delta\mu$, and the change in moles of chemical species, Δn, i.e. $\Delta\mu\Delta n$. Chemical potential is defined as $\mu = RT \ln a$ where R is the gas constant (1.987 cal/mol deg), T is the temperature in K, and a is the activity, but at the low proton concentrations, $[H^+]$, of concern here $\mu = RT \ln [H^+]$. This may be rewritten as $\mu = 2.3RT \log[H^+]$, and since $pH = -\log[H^+]$, as $\mu = -2.3\,RT\,pH$. Using the pK_a as the point of reference, the change in chemical potential becomes $\Delta\mu = -2.3\,RT\Delta pK_a$. Of course a ΔpK_a can contribute no more to the change in chemical energy than that change necessary to cause a functional moiety at a given pH to go from being completely protonated to completely deprotonated. This depends on the number of protons involved per functional moiety and the steepness of the acid–base titration curve which are elements of the Δn term. For our purposes here, the ΔpK_a will be thought of as setting a limit to the chemical energy change.

3.4. Dependence of the temperature, T_t, of an inverse temperature transition on hydrophobicity

What is called hydrophobicity arises out of the thermodynamic properties of the waters that surround a hydrophobic (an apolar) moiety dissolved in water. The formation of water of hydrophobic hydration is exothermic, i.e. the enthalpy, ΔH, is negative,

but the water of hydrophobic hydration is more-ordered than bulk water such that its formation is associated with a decrease in entropy, i.e. a negative ΔS. Accordingly, the change in Gibbs free energy ($\Delta G = \Delta H - T\Delta S$) governing solubility is comprised of two terms of significant and similar magnitudes but of opposite sign. The result of this for polymers containing both polar and apolar components is that the polymers can be soluble and unfolded at a sufficiently low temperature, but, as the temperature is raised above a critical temperature, T_t, they fold and assemble as water of hydrophobic hydration becomes less-ordered bulk water [1,24,25]. This is called an inverse temperature transition as the polymer part of the system becomes more-ordered with increase in temperature, and there are examples with analogues of the present polypeptides where crystallization occurs on raising the temperature and dissolution of the crystal occurs on lowering the temperature below T_t, the temperature of the inverse temperature transition [1,26].

Using the protein-based polymer, poly[f_v(GVGVP), f_x(GXGVP)], it has been found that increasing hydrophobicity as on adding a CH_2 moiety as occurs when Val is replaced by Ile lowers the temperature, T_t, of the inverse temperature transition by about 15° C for $f_I = 1$ and that decreasing the hydrophobicity as by removing two CH_2 moieties per pentamer as occurs when Val is replaced by Ala increases the value of T_t for $f_A = 1$ by about 30° C. Thus, the value of T_t can be a functional measure of hydrophobicity and a T_t-based hydrophobicity scale has been developed for all of the naturally occurring amino acid residues and a number of chemical modifications thereof [1,27]. By this scale, Ile is more hydrophobic than Val, that is, $\Delta T_t(\text{Val} \rightarrow \text{Ile}) \approx -15°$ C, and, since $\Delta T_t(\text{Val} \rightarrow \text{Ile}) \approx -50°$ C by this scale, Phe is some three times more hydrophobic relative to Val than is Ile. It should be recognized that T_t is approximately the enthalpy change divided by the entropy change for the transition, i.e. $T_t \approx \Delta H_t / \Delta S_t$.

3.5. *Different energy inputs that effect a change in hydrophobicity, i.e. a change in T_t*

There are many energy inputs that change the functional hydrophobicity of a protein-based polymer: mechanical, chemical, pressure, electrochemical and light. Doing the mechanical work of stretching a hydrophobically folded polymer causes partial unfolding and exposure of hydrophobic side chains and can effect a hydrophobic-induced pK_a shift [3]. Doing the chemical work of adding salts in general lowers T_t, increasing functional hydrophobicity [1]. Doing the chemical work of charge neutralization by change in protonation, by charge screening or by ion binding dramatically lowers T_t [28]. Increasing pressure, particularly with polymers containing aromatic residues, raises T_t because the volume occupied per molecule for water of hydrophobic hydration is less than that for bulk water [29]. The work of electrochemical or chemical reduction of a redox moiety attached to the polymer increases hydrophobicity and lowers T_t [30]. Finally, the absorption of light, effecting a trans to cis geometrical isomerization of an attached azobenzene, decreases hydrophobicity and raises T_t [2].

3.6. *Relevance of non-linear hydrophobic-induced pK_a shifts to efficiency of energy conversion*

Consider a change in hydrophobicity, a ΔHPB, due to any of the above energy inputs equivalent to the replacement of two Val by Phe residues. This would be represented by a bar the length of two divisions on the x axis of Fig. 3. If the energy input and polymer composition were such that the hydrophobicity change were equivalent to going from zero to two Phe residues/30mer, then there would be little change in pK_a and little conversion to chemical energy. If, on the other hand, the polymer were more hydrophobic and the same change were carried out, i.e. the same energy input performed, equivalent to going from three to five Phe/30mer, then there would be a large change in pK_a and a much more efficient conversion to chemical energy. These effects have now been observed for both photo-chemical, transduction, light-driven proton release, and electro-chemical transduction with reduction-driven proton uptake [1]. Thus, the significance of the non-linear hydrophobic-induced pK_a shifts is

[1] In preparation.

in the efficiency of conversion of a given free-energy to chemical energy of increasing or decreasing proton concentration. Thus, non-linearity is an experimental observation which becomes important in understanding efficiency of energy conversion. The non-linearity presumably arises out of the nature of the competition between apolar and polar groups for hydration which has been called an apolar–polar repulsive free energy of hydration [1].

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