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Hydrophobicity Scale for Proteins Based on Inverse Temperature Transitions

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SYNOPSIS

In general, proteins fold with hydrophobic residues buried, away from water. Reversible protein folding due to hydrophobic interactions results from inverse temperature transitions where folding occurs on raising the temperature. Because homoiothermic animals constitute an infinite heat reservoir, it is the transition temperature, T_t, not the endothermic heat of the transition, that determines the hydrophobically folded state of polypeptides at body temperature. Reported here is a new hydrophobicity scale based on the values of T_t for each amino acid residue as a guest in a natural repeating peptide sequence, the high polymers of which exhibit reversible inverse temperature transitions. Significantly, a number of ways have been demonstrated for changing T_t such that reversibly lowering T_t from above to below physiological temperature becomes a means of isothermally and reversibly driving hydrophobic folding. Accordingly, controlling T_t becomes a mechanism whereby proteins can be induced to carry out isothermal free energy transduction. © 1992 John Wiley & Sons, Inc.

INTRODUCTION

The repeating sequence, $(Val^1\text{-}Pro^2\text{-}Gly^3\text{-}Val^4\text{-}Gly^5)_n$, occurs in all sequenced elastins[1-5]; in particular, it repeats in bovine elastin with $n = 11$ without a single substitution.[2] High polymers of this repeating sequence, poly(VPGVG), are soluble in water below 25°C; but on raising the temperature to 37°C, this polypentapeptide undergoes a folding and assembly transition to a more ordered filamentous state.[6,7] Consistent with this being an inverse temperature transition to increased order on raising the temperature, the temperature of the transition T_t, depends on the hydrophobicity of the constituent amino acid residues[8,9]; in normal saline, T_t is 7°C for poly(VPGLG), 24°C for poly(VPGVG), and 46°C for poly(VPGAG). The key point is that the Leu^4 and Val^4 polypentapeptides are folded at 37°C, whereas poly(VPGAG) is not.

As some substitutions are beyond the accessible temperature range, partial replacements may be used as in poly[f_v(VPGVG), f_x(VPGXG)] where f_v and f_x are mole fractions with $f_v + f_x = 1$ and X stands for the guest residue to be evaluated. The transition temperature for poly(VPGSG) obtained by substitution at different values of f_x gives a straight line in a plot of f_x vs T_t such that the value of T_t at $f_x = 1$ is 52°C, whereas the value for X = Ser-phosphate is a remarkable 1000°C.[10] Thus, the addition of one phosphate in a mean molecular weight of 20 kDa can shift the temperature of the folding transition some 20°C.[10] Accordingly, if the composition were such that T_t = 25°C before phosphorylation with the polypeptide being completely folded at 37°C, phosphorylation of one residue in over 200 residues would cause complete unfolding.

Similarly for $f_x = 0.2$ in phosphate-buffered saline, T_t is different for X = Glu(COOH), i.e., 24°C than for X = Glu(COO^-), i.e., 69°C,[11] such that for poly[0.8(VPGVG), 0.2 (VPGEG)] at 37°C, the polypeptide is folded at low pH ($\sim$ 3) and unfolded at pH 7. Interestingly, on cross-linking, the proton-driven folding is seen as a contraction capable of

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lifting weights a thousand times greater than the dry weight of the cross-linked elastomeric matrix.[12] This very simple assay for chemomechanical transduction or mechanochemical coupling utilizes the principle of changing the temperature of the inverse temperature transition, and the forces developed can be visually seen to be real and substantial, of the same magnitude as developed by frog sartorius muscle. Accordingly, what is reported here is a particularly functional hydrophobicity scale for the naturally occurring amino acid residues and several interesting chemical variations; it is an experimental scale based directly on the protein folding process of interest rather than on more distantly related comparisons[13-16] such as the partitioning of amino acids and analogues between water and organic solvents, effects on surface tension, etc.

MATERIALS AND METHODS

More than 50 polypentapeptide syntheses and their verifications provide the specific data base for the scale presented below and the syntheses of more than 200 polypeptides provide the general informational base required for an adequate substantiation of the presented data. The general approach to the synthesis has been presented previously.[17,18] The specific syntheses and verifications of structure

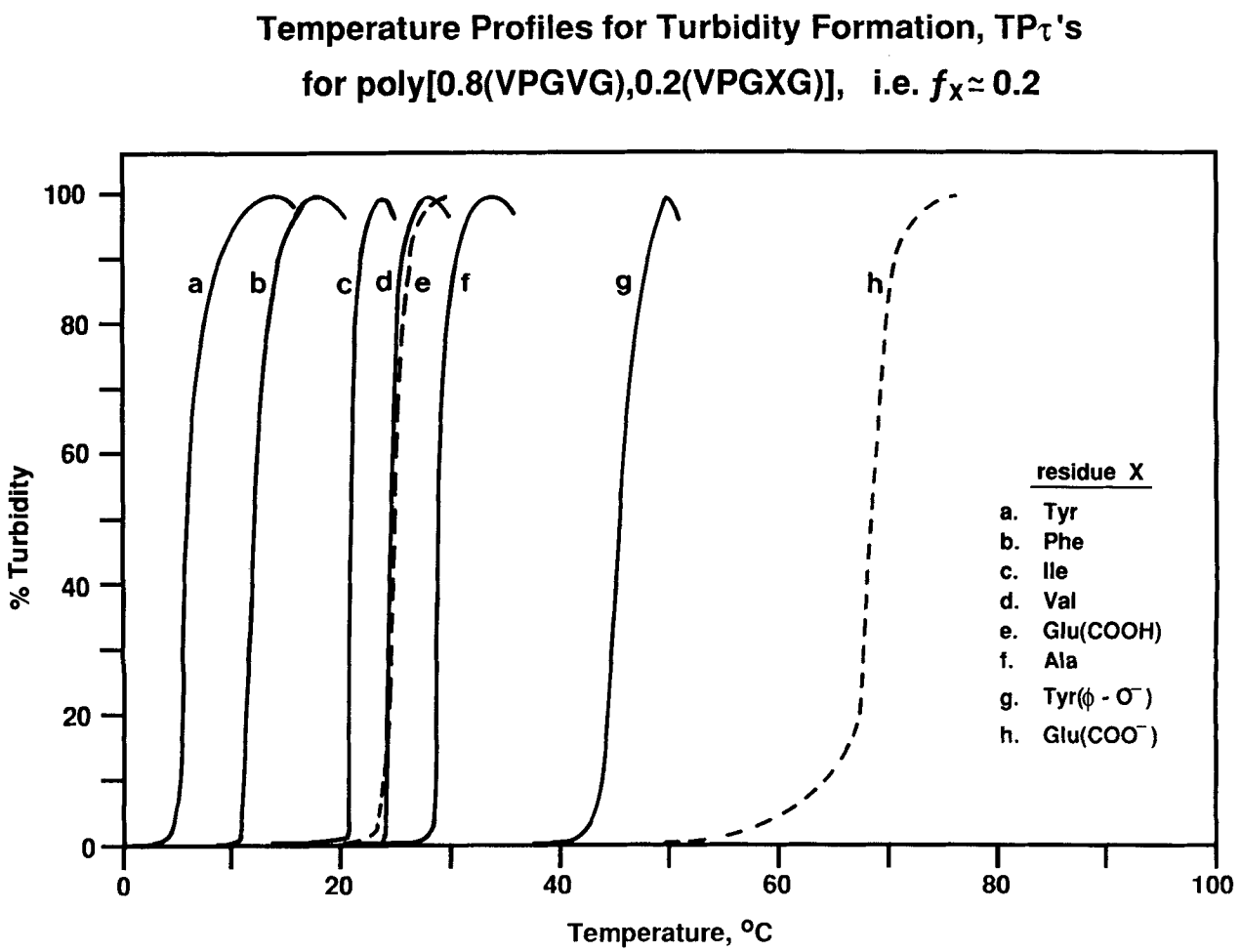

Figure 1. Temperature profiles for turbidity formation, TPτ's. The series of polypentapeptides, poly[0.8(VPGVG),0.2(VPGXG)] where X is the guest residue, is soluble in phosphate-buffered saline at low temperature. Raising the temperature causes the polypentapeptides to undergo an inverse temperature transition of folding and assembly. The onset of the transition is followed by the development of turbidity. T_t, the temperature of the inverse temperature transition, is defined by midpoint temperature for the normalized curves. T_t is found to be inversely proportional to hydrophobicity such that T_t is much larger for the charged state of an amino acid with functional side chains than for the uncharged state. The dashed curves appeared previously[11] and are included for reference.

will be presented elsewhere, mostly in a series of differential scanning calorimetry studies (in preparation). For purposes of discussion of conformation, the permutation chosen for numbering is (VPGVG) due to a Pro^2-Gly^3 Type II β-turn[17]; for purposes of synthesis of high molecular weights, the permutation used is GVGVP or GXGVP.[17,18] The resulting polymers are identical except for the end two or three residues that, with very high degrees of polymerizations as in the present case, are negligible. The composition, that is, the value of f_x, is determined by ^{13}C-nmr and by amino acid analyses, and the purity is verified by thin layer chromatography, amino acid analyses, and nmr.

The determination of the temperature T_t, for the onset of the inverse temperature transition, i.e., for the onset of the folding and assembly transition, was by means of following the onset of turbidity in 40 mg/mL of phosphate-buffered saline (0.15N NaCl, 0.01M phosphate) at 300 nm as the temperature was scanned from near 0 to about 70°C. The concentration of 40 mg/mL was chosen as further increases in concentration of poly(VPGVG) do not lower the value of T_t. The spectrophotometer used was the Cary Model 14 modified by Aviv and equipped with a 300-Hz vibrator to keep the nascent aggregates in suspension. Additional details of the methodology are given elsewhere.[19] A series of representative temperature profiles for turbidity formation (TPτ's) are given in Figure 1 for a set of polypentapeptides with $f_x = 0.2$. The curve is seen to rise abruptly and then reach a maximal value. The temperature for the inverse temperature transition T_t, is defined as the temperature at which turbidity reaches the half-maximal value. These values are then used to determine how T_t changes as a function of the mole fraction of the guest residue in a pentamer.

RESULTS

The temperatures of the inverse temperature transitions T_t for some 50 polypentapeptides are given in Figure 2. In general, the values of T_t are plotted as a function of f_x, and the data points are used to

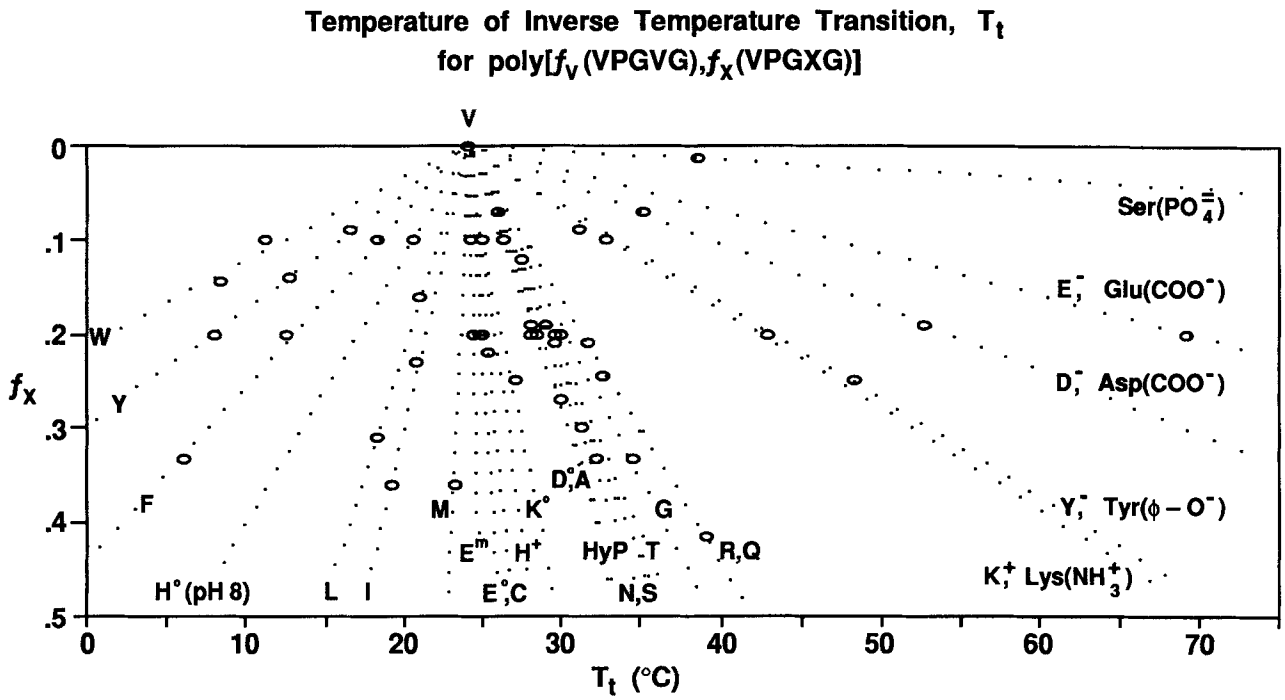

Figure 2. Plots of the temperature of the inverse temperature transition T_t, as a function of the mole fraction f_x, of guest residue X. The zero, plus, and minus superscripts stand for the uncharged, positively, and negatively charged states of the side chains; the "m" superscript on E stands for the methyl ester of the Glu residue, HyP stands for hydroxyproline, and the other letters are the standard single-letter codes for the naturally occurring amino acids. The values of T_t for the residues provide an experimental hydrophobicity scale.

define a least-squares fitted straight line. For the $f_x = 1$ substitutions, the value of T_t is the transition temperature for the particular poly(VPGXG). Where the transition temperature is outside of the accessible range or where the guest residue at higher substitution may cause structural changes, the value of T_t at $f_x = 1$ is the extrapolated value that poly(VPGXG) would exhibit should the temperature be accessible or should the guest residue not disrupt the β-spiral structure of poly(VPGVG) and the supercoiling of β-spirals to form twisted filaments.[20] The values of T_t at $f_x = 1$ for phosphate-buffered saline range from the most hydrophobic residue Trp(W) at −90°C to the most hydrophilic residue Glu(COO^-) at 250°C. The value of T_t at $f_x = 1$ for each amino acid residue is given in Table I, with the values rounded off to the nearest 5°C in the ±55°C range and to the nearest 10°C beyond. Also included is the correlation coefficient, indicating the goodness of fit to the data points. A correlation coefficient of 1.0 occurs when there are only two data points, otherwise there were three or more data points. The linearity of T_t as a function of f_x is best demonstrated for the Trp, Tyr, Phe, Leu, Ile, Met, Ala, and Gly residues.[8]

As Pro has a unique structural role in poly(VPGVG) of inserting a Pro^2-Gly^3, type II β-turn,[18] and as substitution of Pro into position 4 would disrupt the conformation of poly(VPGVG) as it undergoes the folding and assembly process of the inverse temperature transition, the value of T_t for Pro was chosen such that the mean value of T_t for VPGVG would be 30°C, which is the approximate midpoint for the folding and assembly transition for poly(VPGVG) as seen in nmr relaxation studies[21,22] and in the thermally driven contraction of macroscopic cross-linked matrices of poly(VPGVG).[20]

A significant point in this scale is that values are obtained for both the charged and uncharged side chains. Whether or not the residue is charged has a dramatic effect on the value of T_t. Among the amino acid residues, this is most striking for the Glu(E) residue where for $f_x = 1$ in phosphate-buffered saline T_t is 30°C for Glu(COOH) and a remarkable 250°C for Glu(COO^-). A similar shift, but not quite so large, is found for Asp(D) with Asp(COOH) at 45°C and Asp(COO^-) at 150°C. The shifts due to positive charges are not quite as large, i.e., Lys(NH_2) is at 35°C and Lys(NH_3^+) is at 120°C, and even less pronounced for His(Im) at −10°C and His(Im^+) at 30°C. The hydrophobicity of Tyr(Y) is markedly changed on ionization to the tyrosinate from −55 to 120°C. Most remarkable is the chemical perturbation of phosphorylation of a Ser residue where the value of T_t shifts from 50°C to an astounding 1000°C. The actual polymer used in this case was poly[30(IPGVG),(RGYSLG)] in the 2-(N-morpholino) ethane sulfonic acid/magnesium acetate buffer medium.[10]

Table I Temperature of Inverse Temperature Transition T_t for Poly[f_V (VPGVG), f_X (VPGXG)] (A New Hydrophobicity Scale)

Residue X	T_t at $f_X = 1$	Correlation Coefficient
Trp	−90	0.993
Tyr	−55	0.999
Phe	−30	0.999
His (pH 8)	−10	1.000
Pro	(−8)	
Leu	5	0.997
Ile	10	0.996
Met	20	0.997
Val	24	
Glu ($COOCH_3$)	25	1.000
Glu (COOH)	30	1.000
Cys	30	1.000
His (pH 4)	30	1.000
Lys (NH_2)	35	0.919
Asp (COOH)	45	0.999
Ala	45	1.000
HyP	50	0.995
Asn	50	0.997
Ser	50	0.975
Thr	50	1.000
Gly	55	0.999
Arg	60	1.000
Gln	60	0.999
Lys (NH_3^+)	120	0.996
Tyr (ϕ-O^-)	120	0.990
Asp (COO^-)	150	0.996
Glu (COO^-)	250	1.000
Ser ($PO_4^=$)	1000	1.000

DISCUSSION

Inverse temperature transitions as exhibited by polypeptides and proteins are folding and aggregational transitions in which hydrophobic side chains of amino acids go from an aqueous milieu where each hydrophobic moiety is surrounded by waters of hydrophobic hydration to an environment where the hydrophobic side chains are in direct contact

with polypeptide. Inverse temperature transitions are endothermic, characterized by the endothermic heat of the transition and by the temperature at which the transition occurs.[8] For those homoiothermic species, maintenance of a constant temperature provides the heat for the transition. Therefore, the critical issue with respect to whether the polypeptide is hydrophobically folded and/or assembled is whether the temperature of the inverse temperature transition T_t, is above or below the physiological temperature. If the value of T_t is above the physiological temperature, the polypeptide or protein will be unfolded or disassembled; if T_t is below the physiological temperature, the polypeptide or protein will be folded and/or assembled.

The folding and assembly temperature of a protein or polypeptide can be changed by changing the hydrophobicity; increase the hydrophobicity, as in replacing Val by Ile, Leu, Phe, Tyr or Trp, and T_t will be lower; decrease the hydrophobicity, as in replacing Val by Gln, Asn, Ser, Thr, Gly, or Ala, and T_t will be higher. The relative values of T_t for the amino acid residues are listed in Table I. In addition to mutational means of changing T_t, this functional hydrophobicity can be changed by changing the degree of ionization. While previous hydrophobicity scales have not delineated between charged and uncharged states of a side chain, e.g., Glu(COOH) and Glu(COO^-), the value for the effective hydrophobicity of this residue on the transition temperature scale changes by over 200°C. Even more remarkable is the natural biochemical process of phosphorylation of a serine residue in a cyclic AMP dependent protein kinase site, (RGYSLG), where the transition temperature is changed by 1000°C. It now seems quite apparent why phosphorylation/dephosphorylation is such an effective biochemical control mechanism.[23]

Insight into the mechanism whereby these changes in polarity so effectively alter the temperature of inverse temperature transitions is provided by several experimental findings. There is a hydrophobicity-induced pK_a shift: the pK_a for the Glu residue in poly[0.8(VPGVG),0.2(VPGEG)] is near 4.4 whereas for poly[0.8(IPGVG),0.2(IPGEG)] under the same conditions it is a pH unit higher.[24] This is not simply due to a small decrease in water present because there is also observable a large stretch-induced pK_a shift for γ-irradiation cross-linked poly[0.8(VPGVG),0.2(VPGEG)].[25] On stretching of this matrix, hydrophobic side chains become more exposed to water; and even though there is an increase in water in the matrix, the pK_a is raised and there is a corresponding increase in the steepness of the acid–base titration curve.[25] This is interpreted as a competition between hydrophobic moieties and carboxylate moieties for hydration. Even though the stretched matrix is more hydrated, the waters are largely structured as waters of hydrophobic hydration and as such are not suited for hydration of the polar COO^- moieties. Thus there is a delay in the onset of ionization as the pH is raised. But once a few COO^- moieties do form, they begin to pull waters into their hydration shells, destructuring the waters of hydrophobic hydration, and once begun, the COO^- moieties cooperatively destructure the waters of hydrophobic hydration resulting in a steepened and positively cooperative acid–base titration curve.[25]

This perspective is reinforced by the differential scanning calorimetry study showing that the endothermic heat of the transition, which is largely the heat required to destructure the waters of hydrophobic hydration, is reduced to almost one-fourth on forming two COO^- moieties per 100 residues.[26] The interpretation is that the chemical process of forming two COO^- moieties per 100 residues in the process of raising the value of T_t by less than 20°C destroyed three-quarters of the thermodynamically identifiable waters of hydrophobic hydration. Accordingly, the mechanism whereby the temperature of the inverse temperature transition is shifted has been described as an hydration mediated apolar–polar repulsive free energy of interaction.[27]

Now that a hydrophobicity scale based on the temperature for folding and assembly transitions is at hand, it is of interest to see how it might be utilized in what has now become a rather traditional use of hydrophobicity scales, that is, to identify possible transmembrane helical segments in membrane proteins.[28] This is shown in Figure 3 for several well-characterized membrane proteins, bacteriorhodopsin, halorhodopsin, glycophorin A, and human cytochrome b_5.[29–32]

In reading these mean transition temperature plots, $\langle T_t \rangle_{11}$, where the subscript indicates the number of residues over which the transition temperature is averaged, a mean value of less than 37°C would indicate a hydrophobically folded or hydrophobically interacted sequence as would be the case for a hydrophobic α-helix interacting with the lipid layer of a cell membrane. It should also be appreciated that, when a Glu(E), Asp(D), Lys(K), or His(H) residue resides in a sufficiently hydrophobic sequence, the pK_a can be shifted by sequence position alone such that the value for the un-

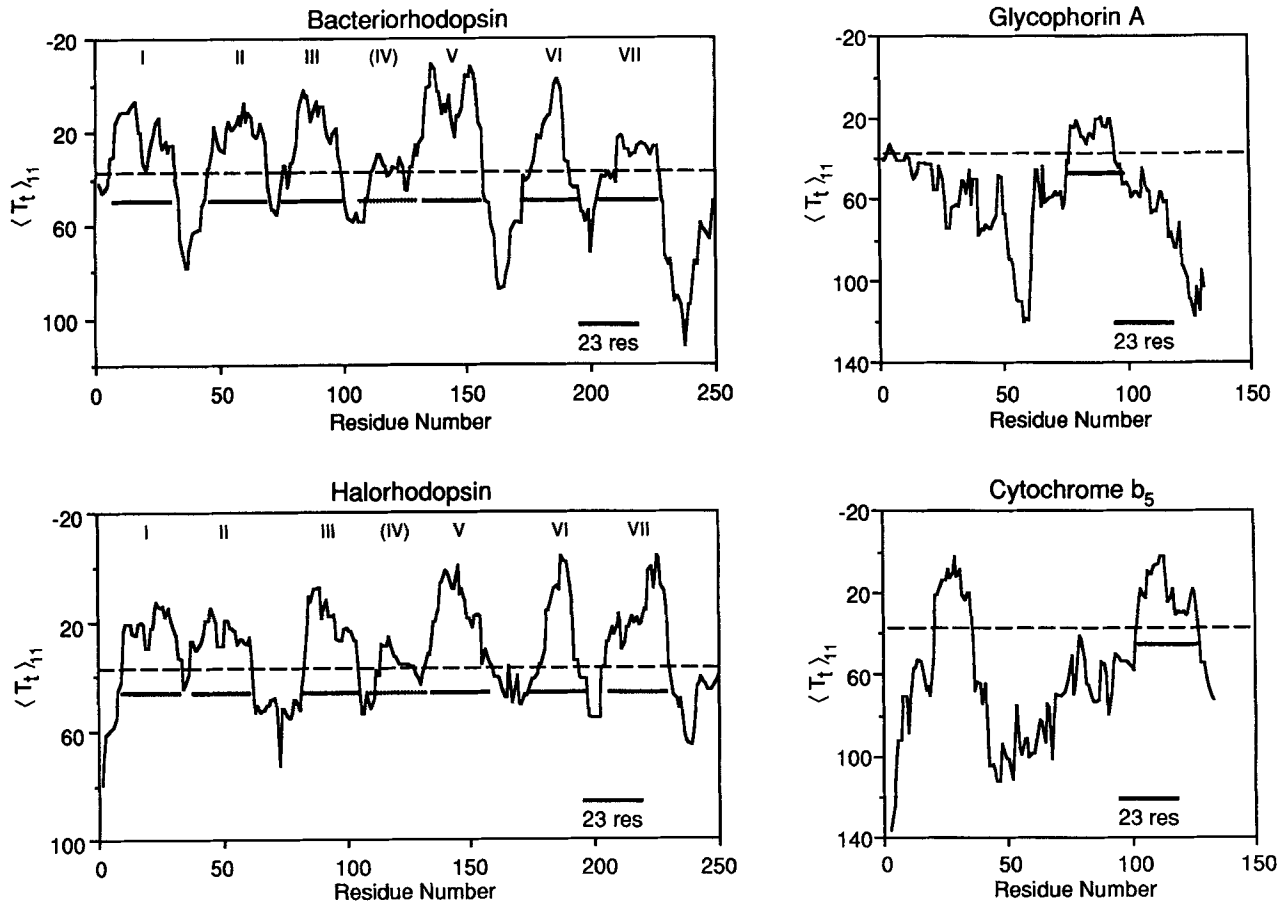

Figure 3. Mean transition temperature, $\langle T_t \rangle_{11}$, plots averaged over an 11-residue sliding window for bacteriorhodopsin (A), halorhodopsin (B), glycophorin A (C), and cytochrome b_5 (D). When the values of $\langle T_t \rangle_{11}$ are greater than 37°C, the sequences would not be hydrophobically folded, whereas when the values are less than 37°C, the sequences would be hydrophobically folded or interacted as would be the case for a transmembrane helix. The bar indicates in each case a length of 23 residues, which is the approximate number of residues whereby an α-helix could span a lipid bilayer. The transmembrane helices are observed for each membrane protein.

charged side chain should be used. Comparing poly[0.8(VPGVG),0.2(VPGEG)] and poly[0.8-(IPGVG),0.2(IPGEG)] with omission of the E residue in the summation, the mean value of T_t are 30 and 27°C, respectively. Yet with this relatively small change due to the more hydrophobic Ile residue having sequence proximities of i to $i - 3$ and i to $i + 2$, and with approximately 50% water in the folded and assembled phase, a pKa shift of one pH unit has been observed.[24] Therefore it can be expected that more hydrophobic and more proximal residues, by virtue of sequence alone, could shift the pK by as much as three pH units. It is not unreasonable, therefore, for sequences with mean T_t values of lower than 20°C to consider the Glu, Asp, Lys and His residues to be uncharged.

In the mean transition temperature plot for bacteriorhodopsin in Figure 3A, six of the seven transmembrane helices are clearly observed. The seventh (Roman numeral IV) occurs in the neutral zone because of three Ala and five Gly residues in the sequence. Nonetheless, this is clearly not a polar sequence and could be considered for a transmembrane helix. For halorhodopsin, the same six helices are well delineated and transmembrane helix IV is slightly better delineated. For the two proteins with well-characterized single transmembrane helices, glycophorin a and cytochrome b_5, these are clearly identified. It is perhaps worth noting that the identification of the transmembrane helices has been achieved directly with the experimental T_t values of Figure 2 and Table I, with no adjustment of values

or rationalizations to bring the results into conformity and with a basis that is more relevant to folding of a fibrous or globular protein in an aqueous milieu than to the insertion of helices into a lipid membrane.

Accordingly, a new experimental basis for a hydrophobicity scale has been developed that uses the temperature for the relevant folding and assembly transitions where hydrophobic interactions are the basis for the folding and assembly. The new scale is most similar to the Nozaki and Tanford scale[13] as completed by Levitt[33]; it is also quite similar to the developing Privalov scale[34] and the Wertz and Scheraga scale,[35] with a significant difference being that the present scale has Tyr as more hydrophobic as do the Nozaki and Tanford[13] and Levitt[33] scales.

It should also be noted that with this understanding of the importance of the temperature for an inverse temperature transition comes the means and the significance of shifting the temperature of the transition. By effecting changes in T_t, the folding and assembly transition can be driven under isothermal conditions. The means whereby T_t can be changed, that is, where folding and unfolding can be controlled isothermally, are numerous: by phosphorylation/dephosphorylation,[10] by changing the degree of ionization of a functional side chain,[12] by esterification and deesterification (see Table I for the methyl ester $COOM_e \rightarrow COO^-$), by chemically modifying the side chain as by hydroxylation,[36] etc., by deamidation (see Table I for $CONH_2 \rightarrow COO^-$), by changes in the concentration of salts[37,38] and other solutes in the medium,[39,40] by a change in the pressure applied to the system,[41] and as been proposed, by a change in the oxidative state of prosthetic groups.

Thus controlling the temperature of inverse temperature transitions becomes a unifying mechanism for the many free energy transductions that are performed by proteins in biological systems. Thermal, mechanical (pressure volume and force length), chemical (polypeptide-based and solvent-based chemical changes), and electrochemical (oxidation and reduction reactions) forms of free energy would be reversibly transduced (interconverted) by changing the temperature of inverse temperature transitions, and the hydrophobicity scale reported herein provides the fundamental information for understanding these free energy transductions. The inverse temperature transition that is so modulable in this molecular system is quite analogous to cold denaturation in proteins[42] and is proposed to be an important mechanism whereby protein modulation results in function.

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