Ciba Foundation Symposium 192

THE MOLECULAR BIOLOGY AND PATHOLOGY OF ELASTIC TISSUES

1995

JOHN WILEY & SONS

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Symposium on The molecular biology and pathology of elastic tissues, held at the Windsor Golf and Country Club, Nairobi, Kenya, 1–3 November 1994

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Preface

Although based in London, the Ciba Foundation is truly an international organization whose mission is to foster scientific cooperation globally. For this reason, we have a worldwide panel of scientific advisers drawn from most countries where there is substantive scientific research. We encourage our advisers to visit our headquarters in Portland Place whenever opportunities present and it was during such a visit that our scientific adviser for Kenya, Professor Joseph Mungai, suggested we should consider holding one of our symposia in Nairobi. Professor Mungai is an anatomist with particular interests in the elastic tissues of animals and it seemed a very happy coincidence, therefore, that we received almost simultaneously symposium proposals in this area from him and (entirely independently) from Dr Leslie Robert (France) and Professor Bob Mecham (USA). Given the crucial role of elastin in ageing and pathology, the stimulus of the cloning of the genes for the soluble elastin precursor (tropoelastin) and for several microfibrillar proteins, and the discovery of elastin receptors, the proposal was clearly very timely with research in the area progressing rapidly.

It gives me very great pleasure to acknowledge publicly the enthusiastic encouragement and help which Professor Mungai and his colleagues Professor Kimani and Miss Shah gave the Foundation in the organization of the symposium. I should also like to thank them and Dr Davy Koech, Director of KEMRI (the Kenya Medical Research Institute) for their assistance and advice with the subsequent open meeting, a collaborative venture with KEMRI and the Kenyan Commission for Higher Education (of which Professor Mungai is Secretary). We were delighted to have the opportunity for the Foundation to re-visit the African continent for an occasion which was memorable not only for the excellence and camaraderie of the scientific discourse but equally for the warmth and enthusiasm of our hosts.

Derek J. Chadwick
Director, The Ciba Foundation

Chairman's introduction

Leslie Robert

Laboratoire de Biologie Cellulaire, Université Paris VII, 3 Place Jussieu, F-75005 Paris, France

About 500–600 million years ago, during the great Cambrian explosion, many new variants of life appeared. Among these were the oxygen-producing cyanobacteria. The progressive enrichment with oxygen of what was previously a reducing atmosphere continued during the Precambrian era. Because most of the previously existing species were adapted to an oxygen-free atmosphere, many (if not most) of these disappeared. New mutations allowed some species to adapt to an aerobic way of life, and these were therefore able to develop and diversify further.

Diploblasts, which developed in the aquatic milieu, gave rise to triploblasts. These had the further developmental potential offered by the possession of the mesenchyme, which had already made an appearance in a primitive form in the sponges. Many sponges contain collagen fibres and glycoproteins, some of which are very similar to fibronectin.

Soon after the Cambrian radiation, the chordates appeared, followed by the appearance of the neural tube and notochord. Vertebrates turned out to be very successful in colonizing all possible ecological niches, from the depths of the sea to the skies. Their extraordinary success was to a large extent a consequence of their perfect adaptation to aerobic life. This involved the creation of conditions maximizing accessibility of their cells to oxygen, which was so efficiently used by their mitochondria to generate energy in the form of ATP. Oxygen, gathered first through gills and later through lungs, was propelled by the developing cardiovascular system to the most remote parts of the body. Without these extraordinary inventions, no further increase in size, or the specialization of organ systems, would have been possible.

We can consider it to be an extraordinary coincidence that the elastin gene appeared at the same time. Lampreys (agnatha, notochord throughout life) possess a not-too-dissimilar protein, lamprin (as has been shown by Fred Keeley). As far as we can tell, subsequent development of the elastin gene occurred rapidly, from teleost fishes to terrestrial quadrupeds. This will be discussed by several participants at this meeting who have been pioneers in this field, especially Joel Rosenbloom and Charles Boyd.

Elastin is, in evolutionary terms, much younger than most collagens; the only similarity between these two types of protein is that both possess

high proportions of glycine, a similarity that confused some of the early workers.

The physicochemical properties of elastin are very unusual, and its discovery was apparently the result of its extraordinary resistance to chemical, physical and enzymic attack, at least in aqueous media. Mörner, a 19th-century German chemist, noticed that when tissues were treated with calcium hydroxide (a very aggressive hydrolytic agent), a yellowish fibrous material (elastin) resisted this treatment. Eulenberger, in 1836, isolated elastic tissue from vascular walls using boiling water. Interestingly, the resistant properties of elastin collapse in the presence of organic solvents. As we showed some thirty years ago, in aqueous-organic solvents, mild alkali treatment will completely hydrolyse insoluble elastin fibres in peptides (κ-elastin) which were extensively used for biochemical and pharmacological studies, and which also found their way into industrial applications. This observation opened the way for a physicochemical interpretation of the structure and elasticity of elastin that is largely based on strong hydrophobic interactions between the predominantly aliphatic amino acid side chains. The elucidation of elastin's tertiary and quaternary structure, which will be recalled by Dan Urry, Tony Tamburro and Peter Winlove, also explained its strong affinity for Ca^{2+} and lipids. The progressive deposition of Ca^{2+} and lipids in elastic fibres with ageing is part of the atherosclerotic process and will also be discussed during this meeting.

The isolation of the first proteolytic enzyme that rapidly degrades elastin, pancreatic elastase, by J. Balo and I. Banga in Budapest in the 1950s, began a period of rapid increase in interest in this curious protein. Several other elastases have been identified and characterized. Some, such as polymorphonuclear leukocyte elastase, play important roles in pathological processes, for instance, lung emphysema. The elastin–elastase inhibitor balance hypothesis (increased elastolytic damage to tissues through lack of elastase inhibitor), proposed after the discovery of genetic deficiencies in elastase inhibitors (α_1Pi) by Klas Bertil Laurell, further stimulated elastin research and resulted in an upsurge of synthetic elastase inhibitor patents.

The elucidation of the structure of elastin was initially a difficult task. The first breakthrough was the identification and characterization of the cross-linking amino acids, desmosine and isodesmosine, by Miles Partridge, whose recent death deprived this field of one of its founders. Allen Bailey will recall this crucial step in the elucidation of elastin's structure. The production of a soluble precursor, tropoelastin, from the aorta of pigs raised on a copper-free diet in Bill Carnes' laboratory, enabled Larry Sandberg to produce the first amino acid sequences of tropoelastin. The important observation was the presence of tetra-, penta- and hexapeptide repeating sequences, which further demonstrated the peculiar nature of elastin. Polymers made with such repeating units in Dan Urry's laboratory had many of the interesting properties of elastin.

Because of its extraordinary resistance, elastin was initially considered to be inert. For this reason, it was only recently that the study of elastin cell biology was initiated in the laboratories of Bob Senior and Bob Mecham, and also in ours. These studies have largely focused on the fibroblasts of the bovine ligamentum nuchae and the smooth muscle cells of large blood vessels, but chondrocytes and skin fibroblasts have also been used. These systems have enabled us to study the regulation of elastin biosynthesis and the mechanism of elastin fibre deposition, a field pioneered by Bob Mecham, who will talk about this important aspect of elastin cell biology. In our laboratory, we demonstrated an interaction between cells and elastic fibres that is mediated by a membrane receptor and an inducible adhesive component of cell membranes. This mechanism is important in physiological processes such as the regulation of blood vessel wall tension and also in tumour cell metastasis. The demonstration of the elastin receptor on several cell types enabled us to explore (with M. P. Jacob and T. Fülöp) its biological role. However, most of our recent knowledge of elastin has come from gene sequencing. The demonstration of alternative exon usage in several species means that cells can secrete hundreds of possible tropoelastin isoforms.

The degradation of elastic fibres is a hallmark of vascular ageing. Degradation products, such as elastin peptide fragments, are present in the circulation and may well play an important role in the pathogenesis of some age-dependent diseases. Loss of tissue elasticity, however, is not always accompanied by loss of elastic fibres. Increased elastin fibre deposition in senile or solar elastosis, as well as in some forms of breast cancer, still presents an enigma to elastin biochemistry.

It appears, therefore, that the phylogenetic appearance of elastin was vital in order for vertebrates to develop highly efficient respiratory and circulatory systems. This advantage is offset by the relatively rapid decay of elastic fibres, which lose their elasticity with time and which are also involved in several of the most common diseases of old age. This demonstrates once more the fact that the 'invention' of new genes serves mainly the success of the individual in terms of reproductive success, not for long survival. Thus, elastin biology is an area where pharmacology should come in with original, innovative research to alleviate the very widespread disorders involving elastic tissues and which commonly result in early mortality, as in the case of cardiovascular and pulmonary diseases. Let us hope that the papers presented at this symposium will encourage young researchers to enter this fascinating field of biological research and develop it further.

Molecular biophysics of elastin structure, function and pathology

Dan W. Urry, Chi-Hao Luan and Shao Qing Peng

Laboratory of Molecular Biophysics, School of Medicine, The University of Alabama at Birmingham, VH 300, Birmingham, AL 35294-0019, USA

Abstract. Owing to the presence of the recurring sequence XPGX′ (where X and X′ are hydrophobic residues), the molecular structure of the sequences between cross-links in elastin is viewed primarily as a series of β-turns which become helically ordered by hydrophobic folding into β-spirals, which in turn assemble hydrophobically into twisted filaments. Both hydrophobic folding and assembly occur when the temperature is raised above T_t, the onset of an inverse temperature transition. Using poly[f_v(VPGVG),f_x(VPGXG)] (where f_v and f_x are mole fractions with $f_v + f_x = 1$ and X is now any of the naturally occurring amino acid residues), plots of f_x versus T_t result in a new hydrophobicity scale based directly on the hydrophobic folding and assembly processes of interest. With the reference values chosen at $f_x = 1$, the most hydrophobic residues of elastin, Tyr (Y) and Phe (F), have low values of T_t, -55 and $-30\,°C$, respectively, and the most hydrophilic residues, Glu (E^-), Asp (D^-) and Lys (K^+), have high values of 250, 170 and 120 °C, respectively. Raising the average value of T_t for a chain or chain segment from below to above physiological temperature drives hydrophobic unfolding and disassembly; lowering T_t does the reverse. This ΔT_t mechanism has been used reversibly to interconvert many energy forms and is used here to explain initiating events of elastogenesis, pulmonary emphysema, solar elastosis and the paucity of elastic fibres in scar tissue. In general, oxidation and/or photolysis convert(s) hydrophobic residues into polar residues with the consequences of irreversibly raising T_t to above 37 °C, hydrophobic unfolding and disassembly (fibre swelling), and greater susceptibility to proteolysis.

1995 The molecular biology and pathology of elastic tissues. Wiley, Chichester (Ciba Foundation Symposium 192) p 4–30

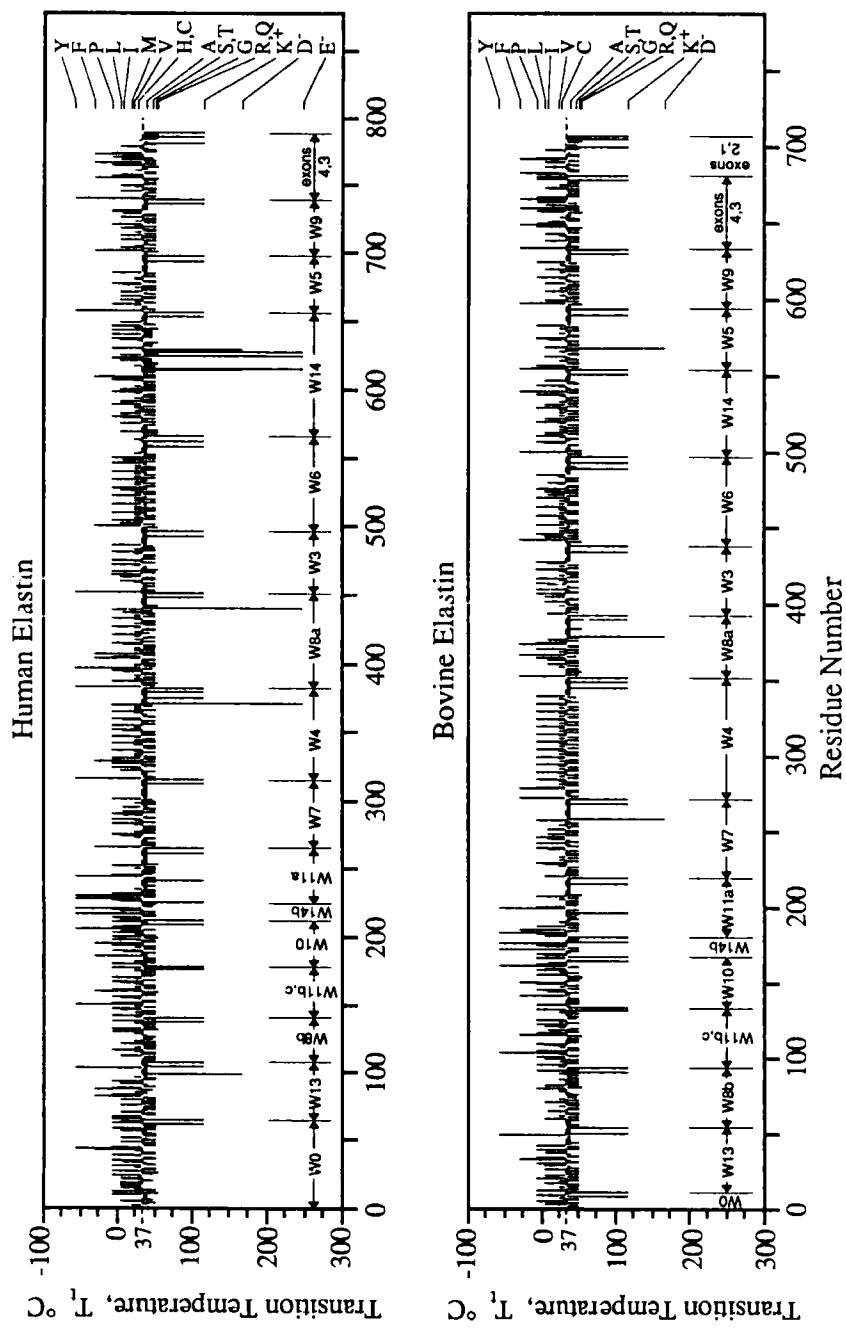
The study of the polypentapeptide of elastin, $(Val^1\text{-}Pro^2\text{-}Gly^3\text{-}Val^4\text{-}Gly^5)_n$ or poly(VPGVG), and many of its analogues and chemical modifications thereof, has led to significant insight into the factors that control hydrophobic folding/unfolding and assembly/disassembly. This has occurred just prior to the growing appreciation that hydrophobic folding can be the primary process in protein structure formation. It has been possible for us to substantiate the understanding developed on the poly(VPGVG) model by designing

compositional variants, fashioning these protein-based polymers (high molecular weight polymers of repeating peptide sequences) into cross-linked elastic bands and driving contraction (hydrophobic folding and assembly) to produce the useful mechanical work of cyclically lifting and lowering a weight. The input energies shown to be capable of driving contraction and relaxation of these elastic bands are thermal, chemical, pressure, electrochemical and light energy. In other words, we have designed elastic protein-based polymers using the basic poly(VPGVG) structure to achieve thermo-, chemo-, baro-, electro- and photomechanical transduction (for a review, see Urry 1993). Also, the elastic protein-based polymers have been designed to convert light energy and electrochemical energy into the chemical energy of increased proton concentration (that is, to perform photo- and electrochemical transduction as occurs in photosynthesis and in the first energy conversion step of oxidative phosphorylation).

Because we have achieved this degree of functional protein engineering by starting with the most striking repeating sequence of elastin, it seems appropriate to ask what insights this knowledge may provide towards our understanding of elastin itself. Such an effort begins with knowledge of primary structure and is followed by the determination of molecular structure and the development of the principles that are fundamental to structure formation, function and dysfunction.

Primary structure and hydrophobicity plots

The first substantial information on primary structure of elastin came from Sandberg, Gray and their co-workers (Gray et al 1973, Sandberg et al 1985), who identified in porcine elastin repeating peptide sequences occurring between potential cross-linking sequences. This occurred some years after Partridge (1969) had identified desmosine and isodesmosine and Franzblau & Lent (1969) had identified lysinonorleucine as the principal cross-links, all of which derived from the side-chains of lysine residues. The primary structures for human (Indik et al 1987), bovine (Yeh et al 1987) and porcine (Sandberg et al 1985) elastins are given in Fig. 1 in terms of single-residue hydrophobicity plots using the T_t-based hydrophobicity scale which we developed using poly(VPGVG) (Urry 1993). This repeating sequence is apparent as the longest and most regular repeating sequence seen in the hydrophobicity plots for bovine and porcine elastin in Fig. 1; following the Sandberg nomenclature, it is indicated as the W4 sequence. Other repeating sequences are apparent in Fig. 1, for example, the W6 repeat, $(Ala^1\text{-}Pro^2\text{-}Gly^3\text{-}Val^4\text{-}Gly^5\text{-}Val^6)_n$. Also apparent are the periodic Lys (K^+) residues seen as negative deflections. Four lysines, two from each of two chains, combine to form desmosine and isodesmosine cross-links, and two lysines combine to form lysinonorleucine cross-links. The development of the T_t-based hydrophobicity scale is a central


Human Elastin
Bovine Elastin
Transition Temperature, T_t °C
Residue Number
-100
0
37
100
200
300
0
100
200
300
400
500
600
700
800
Y
F
P
L
I
M
V
H,C
A
S,T
G
R,Q
K+
D-
E-
W0
W13
W8b
W8c
W11b,c
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exons 4,3
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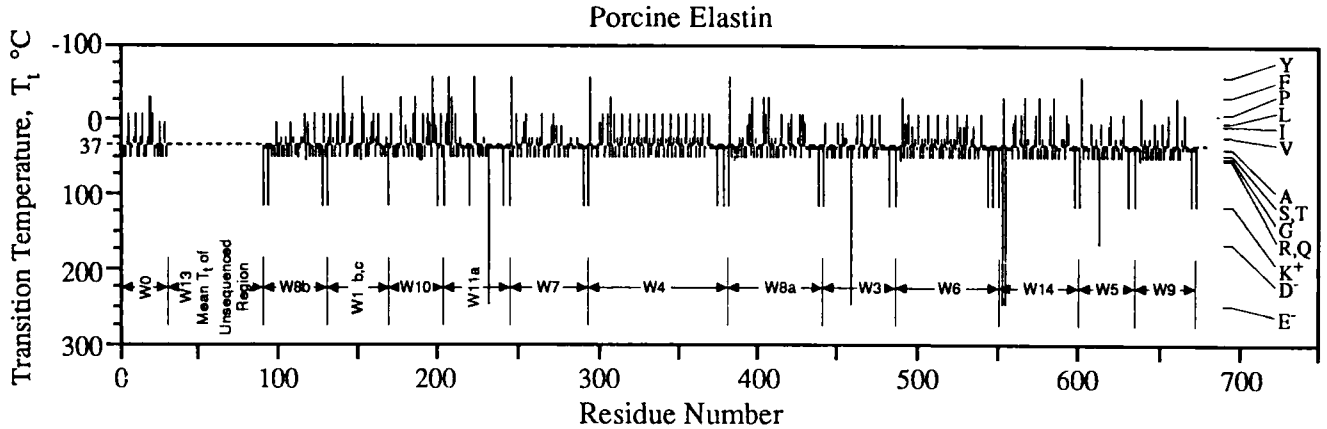


FIG. 1. Single-residue hydrophobicity plots for human, bovine and porcine tropoelastins using the T_t-based hydrophobicity scale listed in Table 1. The plots are given as deflections from the 37 °C line: the larger the upward deflection the more hydrophobic the residue, and the greater the negative deflection the more polar the residue. The single-letter codes for the amino acid residues are given on the right-hand side at the point of maximum deflection. Along the bottom of each plot, in addition to the amino acid residue number, is given the sequence according to the Sandberg nomenclature. Perhaps most striking is the repeating VPGVG sequence so apparent in the W4 sequence of the bovine and porcine proteins. There is also the repeating APGVGV hexapeptide of W6 most apparent in the human and porcine proteins, and throughout the non-cross-link sequences there is a prominent occurrence of PG, which is the most common means of inserting a β-turn. Finally, there are the regularly recurring negative deflections to 120 °C, the position of the charged lysine (K^+) residues which become involved in the cross-links.

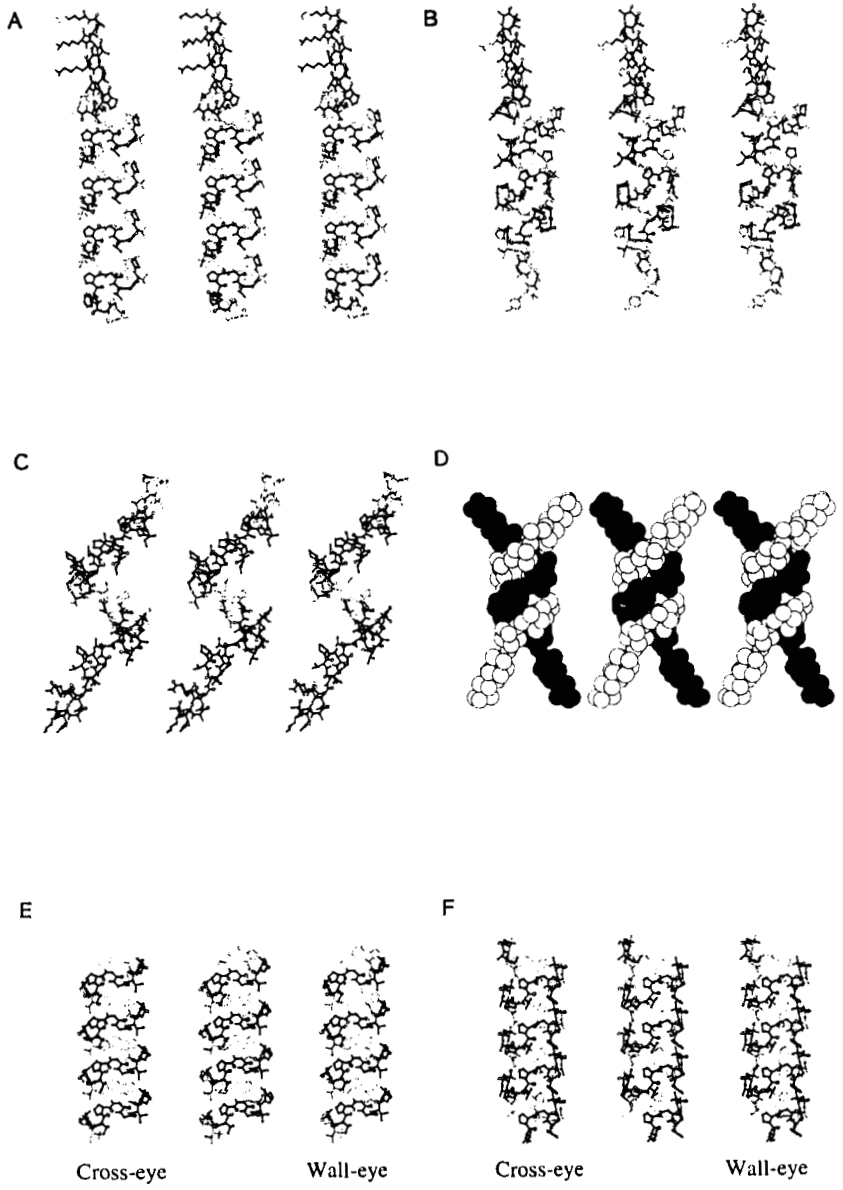


FIG. 2. Proposed molecular structures, shown in stereo perspective, for particular sequences of elastin. (A) Bovine W4; (B) human W4; (C) human W6 (single chain); (D) human W6 (association of a pair of chains shown in space-filling representation); (E) the polytetrapeptide, $(VPGG)_n$; and (F) the polynonapeptide $(VPGFGVGAG)_n$. In each case for the three structures, the left-hand pair are for cross-eye viewing and the right-hand pair are for wall-eye viewing. (C.-H. Luan & D. W. Urry, unpublished results.)

component in our understanding of the factors that control hydrophobic folding and unfolding.

β-spiral structures for repeating peptide sequences of elastin

As a key step in moving towards an understanding of elastin structure, function and pathology, the proposed and working-model β-spiral molecular structures are presented in Fig. 2 for the bovine and human W4 sequences, the human W6 sequence and the repeating consensus sequences—poly(VPGG) and poly(VPGFGVGAG). These structures are based on extensive physical characterizations of the chemically synthesized protein-based polymers and on molecular mechanics and dynamics calculations.

Elastogenesis and inverse transitions

As may be judged from its amino acid composition, elastin is a hydrophobic protein which has many hydrophobic residues (those with upward deflections from the 37 °C line of Fig. 1) and an extreme paucity of charged residues (those with the large negative deflections in Fig. 1, e.g. the Glu [E^-], Asp [D^-] and Lys [K^+] residues). A fibrous protein (e.g. myosin) has quite the inverse distribution. Accordingly, elastogenesis, the process of elastic fibre formation, is reasonably expected to be directed by hydrophobic folding and assembly. In fact, each of the high polymers of the repeat sequences, the precursor protein (tropoelastin) and the fibre fragmentation product (α-elastin) is soluble in water at a sufficiently low temperature but exhibits a phase separation of hydrophobic folding and assembly as the temperature is raised above a critical temperature (designated as T_t). As the polypeptide part of the system is characterized by an increase in order with increase in temperature, this hydrophobic folding and assembly transition is called an inverse temperature transition (Urry 1992).

The ΔT_t mechanism for controlling hydrophobic folding and assembly

Since the polypeptide chains are unfolded and disassembled when the temperature is below T_t (the onset temperature for the transition), but have completed their folding and assembly when the temperature is some 15–20 °C above T_t, the control of T_t becomes the means with which to control whether the fibre forms or whether it dissociates to the extent allowed by the cross-links that may have formed. In short, instead of raising the temperature to drive the hydrophobic folding and assembly transition, in the ΔT_t mechanism the transition temperature, T_t, is lowered from above to below the working temperature to drive the hydrophobic folding and assembly transition isothermally (Urry 1992, 1993).

Consequences of the ΔT_t mechanism of hydrophobic folding and assembly for structure, function and pathology

If the value of T_t for a set of chains is above 37 °C, fibre formation will not occur. This can happen, for example, when there is too much hydroxylation of proline residues. If the fibre is already formed with cross-links in place and an external process such as oxidation occurs to raise the value of T_t above 37 °C, the fibre will swell, with a marked loss of elastic strength (i.e. a decrease in elastic modulus), and extend with loss of function. In addition, it has been our experience that when the synthetic model elastic fibres are hydrophobically folded and assembled they are resistant to proteolytic degradation, but when the T_t is raised above 37 °C, as occurs when a carboxamide breaks down to a carboxylate, proteolytic digestion occurs. Thus, we propose this to be a factor, for example, in the aetiology of pulmonary emphysema. Furthermore, should photo-oxidation occur, the value of T_t would be raised and again there would be swelling followed by proteolytic degradation with the result being solar elastosis.

Molecular structures for sequences between cross-links

The most extensively characterized elastic sequence of elastin is that of the prominent (VPGVG) repeat of the bovine W4 sequence. This molecular structure, given in stereo pairs in Fig. 2A, includes the lysine-containing, alanine-rich, α-helical cross-linking sequence at the C-terminus. The VPGVG repeat is characterized by a Type II Pro^2-Gly^3 β-turn that has a Val^1 C–O···H–N Val^4 10-atom hydrogen-bonded ring. On raising the temperature in water through the transition temperature range, the series of β-turns wrap up into a helical structure, called a β-spiral, by optimizing intramolecular hydrophobic contacts in which the β-turns, with hydrophobic contacts, function as spacers between turns of the spiral. Two or more of these β-spiral structures, again by hydrophobic association, supercoil to give twisted filaments as observed in electron micrographs of negatively stained incipient aggregates (Urry 1992).

The calculated molecular structure of the human W4 sequence, which does not exhibit such a well-defined repeat, is given in Fig. 2B. We have synthesized, polymerized, cross-linked and physically characterized this sequence; we find that it also becomes a predominantly entropic elastomer at temperatures above the inverse temperature transition (Gowda et al 1995).

The human W6 sequence and the component APGVGV consensus sequence have also been synthesized, polymerized and physically characterized, and were found to be inelastic (Rapaka et al 1978a). The conformation of this quite-rigid structure, on the basis of extensive nuclear magnetic resonance and computational characterization, is shown in Fig. 2C as stereo pairs of a single chain. The single chain is seen to form an intertwined structure in

space-filling stereo perspective (Fig. 2D) (C.-H. Luan & D. W. Urry, unpublished results). This rigid intertwining of chains is considered to be an important aligning element of elastogenesis prior to the formation of the natural cross-links.

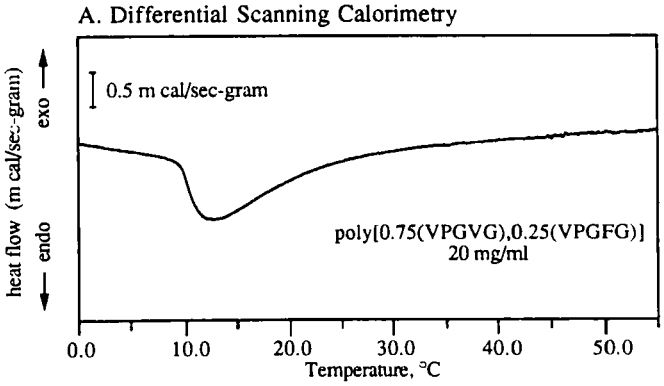
Also included in Fig. 2 are the proposed and working-model structures of elastic poly(VPGG) (Fig. 2E) (Luan et al 1991) and elastic poly(VPGFGVGAG) (Fig. 2D) (C.-H. Luan & D. W. Urry, unpublished results), respectively. These repeats are less common but they have also been synthesized, polymerized, physically and computationally characterized, cross-linked and found to be elastomeric.

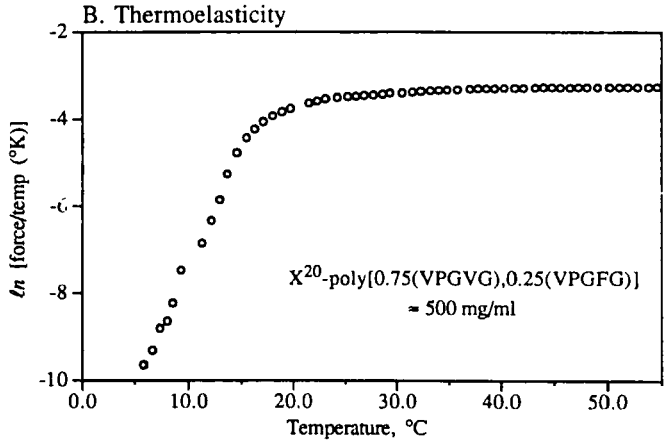
Because of the prominent recurrence of the Pro-Gly sequence apparent in Fig. 1, the repeat sequences of elastin are thought to form β-spiral structures. So far, the repeating sequences that contain VPG... have been found to be elastic, whereas those that contain APG... are inelastic.

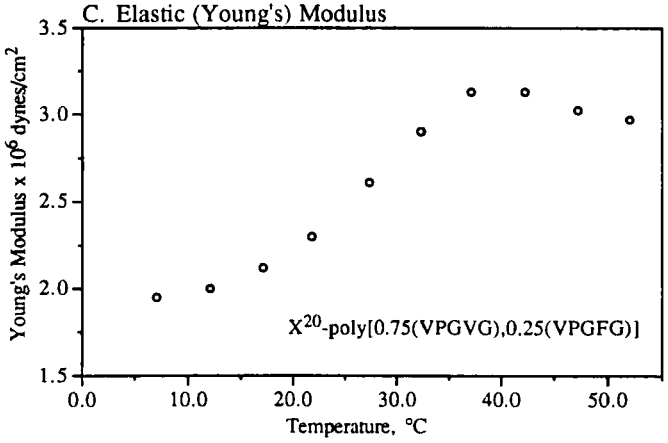
Hydrophobic folding and assembly (an inverse temperature transition)

The elastin repeat peptides have the correct balance of hydrophobic (apolar) and polar (peptide) moieties to increase in order in water as the temperature is raised through a transition temperature range. This is unambiguously demonstrated by the cyclic analogue, cyclo(APGVGV)$_2$, of the repeating (APGVGV) sequence of W6. Just as with poly(APGVGV), cyclo(APGVGV)$_2$ is soluble in water at a sufficiently low temperature, and it aggregates as the temperature is raised through the transition temperature range. Whereas poly(APGVGV) shows twisted filaments in the transmission electron micrographs of negatively stained aggregates (Urry 1982), cyclo(APGVGV)$_2$ reversibly forms crystals (Urry et al 1978). These molecules go from being randomly distributed in solution at low temperature to being precisely positioned in a crystal when the temperature is raised. The polypeptides unambiguously increase in order as the temperature is increased. Quite similarly, poly(VPGVG) is seen in the electron micrographs to form twisted filaments (Urry 1982), and the cyclic structure, cyclo(VPGVG)$_3$, which has a nearly identical conformation, is found in the crystal state to associate by hydrophobic contacts between molecules within the stacks of molecules (in analogy to intramolecular folding to form the β-spiral) and between stacks of molecules (in analogy to intermolecular assembly to form twisted filaments) (Cook et al 1980). Similarly, tropoelastin and α-elastin go from a random distribution in solution to parallel aligned twisted filaments as the temperature is raised through the transition temperature range (Urry 1982).

The general finding of folding and assembly into regular structures as the temperature is raised, which is confirmed by the crystallization of cyclo(APGVGV)$_2$ and cyclo(VPGVG)$_3$, indicates that the dominant inter- and intramolecular interactions of elastin are hydrophobic, a result that can be


A. Differential Scanning Calorimetry
0.5 m cal/sec-gram
heat flow (m cal/sec-gram)
exo
endo
poly[0.75(VPGVG),0.25(VPGFG)]
20 mg/ml
0.0
10.0
20.0
30.0
40.0
50.0
Temperature, °C


B. Thermoelasticity
-2
-4
-6
-8
-10
ln [force/temp (°K)]
X20-poly[0.75(VPGVG),0.25(VPGFG)]
≈ 500 mg/ml
0.0
10.0
20.0
30.0
40.0
50.0
Temperature, °C


C. Elastic (Young's) Modulus
3.5
3.0
2.5
2.0
1.5
Young's Modulus x 10^6 dynes/cm^2
X20-poly[0.75(VPGVG),0.25(VPGFG)]
0.0
10.0
20.0
30.0
40.0
50.0
Temperature, °C

predicted from the amino acid composition so apparent in Fig. 1. This increase in order with temperature arises because there is structured water surrounding the hydrophobic side chains when they are dissolved at low temperature that becomes less-ordered bulk water during hydrophobic association as the temperature is raised through the transition. The entropy (the disorder) of the entire system (polypeptide or protein plus water) increases with increasing temperature in keeping with the second law of thermodynamics; but there is a larger increase in disorder of the water part of the system and a smaller, though very important, increase in order for the polypeptide part of the system. That the folding and assembly occurs as a transition is apparent from Fig. 3A, where the transition is seen to be endothermic. Heat is required to destructure the water of hydrophobic hydration. Because our focus is on the polypeptide folding and function and because there is an increase in order instead of the usual decrease in order with temperature, this is called an inverse temperature transition. Accordingly, the folding and assembly of the structures of Fig. 2 are the result of an inverse temperature transition to increased order with increased temperature (Urry 1992).

The T_t-based hydrophobicity scale

The temperature for the onset of the inverse temperature transition is designated as T_t. An easy measure of T_t is shown in Fig. 4A. The onset of aggregation when the temperature is raised results in an onset of light scattering, and the temperature at which half-amplitude is achieved in a plot of turbidity versus temperature is defined as T_t. As is apparent in Fig. 4A, the value of T_t changes with amino acid composition in a systematic way.

Using the general formula poly[f_v(VPGVG),f_x(VPGXG)], where f_v and f_x are mole fractions, $f_v + f_x = 1$, and X is any amino acid, we see that plots of f_x versus T_t result in straight lines (Fig. 4B) such that it becomes possible to

FIG. 3. (A) Differential scanning calorimetry using a 20 mg/ml sample of poly[0.75(VPGVG),0.25(VPGFG)] showing an endothermic transition beginning just below 10 °C. This shows the inverse temperature transition. (B) Thermoelasticity curve for 20 Mrad γ-irradiation cross-linked poly[0.75(VPGVG),0.25(VPGFG)] where the resulting elastic band, held at fixed length, is seen to contract with the dramatic development of elastic force as the temperature is raised through the range of the inverse temperature transition for hydrophobic folding and assembly. Above the transition, the plot of *ln*(force/temperature) versus temperature is seen to approach a zero slope (particularly above 37 °C), which is classically interpreted to indicate a dominantly entropic elastic force. (C) Elastic (Young's) modulus for the cross-linked elastic strip, X^{20}-[0.75(VPGVG),0.25(VPGFG)], where the elastic modulus of the same strip is seen to increase as the temperature is raised through the range of the inverse temperature transition. The hydrophobic folding and assembly that occur on passing through the inverse temperature transition contribute to the elastic force.

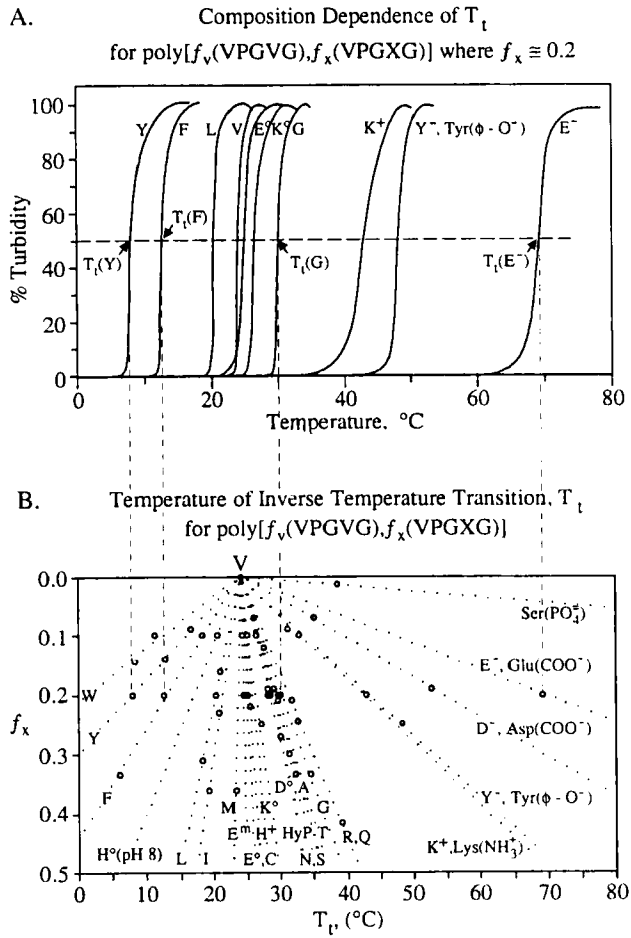


FIG. 4. (A) Measurement of T_t and composition dependence of T_t for poly[f_v(VPGVG),f_x(VPGXG)] where $f_x = 0.2$. T_t is taken as the temperature at which 50% turbidity is obtained. Clearly, as the substituted residue becomes more hydrophobic, the value of T_t lowers and, as the residues become more polar, the value of T_t rises. Note the very large effect of side chain ionization. These values of T_t, along with those for different values of f_x for all of the naturally occurring amino acid residues, are plotted as f_x versus T_t in (B), where the plots for each amino acid residue define a straight line which when extrapolated to $f_x = 1$ gives a measure of the relative hydrophobicities as given in Table 1. (Adapted with permission from Urry 1993.)

extrapolate to $f_x = 1$ and obtain a set of relative T_t values. It is obvious that an increase in hydrophobicity causes a decrease in T_t and a decrease in hydrophobicity causes an increase in T_t such that a hydrophobicity scale is produced which, for the first time, is dependent on the hydrophobic folding and assembly process of interest (Urry et al 1992).

The T_t-based hydrophobicity scale, which we have developed using the most striking repeating sequence of elastin as the host sequential polypeptide, is given in Table 1. This scale was used for the hydrophobicity plots for human, bovine and porcine elastin in Fig. 1.

Hydrophobic folding and assembly in proteins is now taking on greater significance. Dill et al (1993) have developed a theoretical foundation for cooperative hydrophobic folding transitions which, of course, is what we have been describing for the elastin sequences. Dobson et al (1994), in their studies of hen lysozyme, show hydrophobic collapse to be the initial folding step, followed by the sorting out of the best secondary structure that can occur given the hydrophobic domain that forms. Furthermore, it is becoming more apparent that the periodicity at which hydrophobic residues appear in a sequence can dictate the secondary structure that will result. Accordingly, the presence of a hydrophobic residue every third and/or fourth residue dictates an amphiphilic α-helix; the occurrence of hydrophobic side chains at alternating residues dictates β-chains, etc. The hydrophobic side chain periodicity of poly(VPGVG) is as required for a β-turn, residues i and $i+3$, coupled with an extended β-like chain which gives the β-spiral structure.

The ΔT_t hydrophobic paradigm for protein folding and function

As seen in Fig. 4, raising the temperature above T_t (the temperature for the onset of the inverse temperature transition) drives aggregation, which occurs by hydrophobic folding and assembly. In the ΔT_t hydrophobic paradigm the temperature is kept constant and the value of T_t is changed. Lowering T_t from above to below physiological temperature drives hydrophobic unfolding and disassembly.

Importantly, there are many ways by which the value of T_t can be changed: (1) the value of T_t decreases as the concentration and chain length of the protein-based polymer are increased; (2) changing the composition of the protein-based polymer changes the value of T_t; (3) increasing the concentration of salts decreases the value of T_t; (4) introduction of organic solutes such as sodium dodecylsulfate, guanidine hydrochloride, urea, etc. increases the value of T_t; (5) increasing the side chain charge markedly increases T_t; (6) phosphorylation, e.g. of a serine side chain, most dramatically increases the value of T_t; (7) application of pressure, particularly with aromatic residues present, increases the value of T_t; (8) reduction of a bound prosthetic group, such as a nicotinamide, markedly lowers T_t; (9) absorption of light by a bound chromophore, which thereby converts from a *trans* to a *cis* isomer, or which in other ways makes the polymer more polar, raises T_t; and (10) salt neutralization of a charged side chain markedly decreases the value of T_t (Urry 1993). In general, any change that causes chains to become more polar and less hydrophobic will raise the

TABLE 1 T_t-based hydrophobicity scale for proteins

Residue X		*T_t(°C), linearly extrapolated to $f_x = 1$*	*Correlation coefficient*
Lys(NMeN, reduced)[a]		−130	1.000
Trp	(W)	−90	0.993
Tyr	(Y)	−55	0.999
Phe	(F)	−30	0.999
His (pH 8)	(H^o)	−10	1.000
Pro	(P)[b]	(−8)	calculated
Leu	(L)	5	0.999
Ile	(I)	10	0.999
Met	(M)	20	0.996
Val	(V)	24	reference
Glu($COOCH_3$)	(E^m)	25	1.000
Glu(COOH)	(E^o)	30	1.000
Cys	(C)	30	1.000
His (pH 4)	(H^+)	30	1.000
Lys(NH_2)	(K^o)	35	0.936
Pro	(P)[c]	40	0.950
Asp(COOH)	(D^o)	45	0.994
Ala	(A)	45	0.997
HyP		50	0.998
Asn	(N)	50	0.997
Ser	(S)	50	0.997
Thr	(T)	50	0.999
Gly	(G)	55	0.999
Arg	(R)	60	1.000
Gln	(Q)	60	0.999
Lys(NH_3^+)	(K^+)	120	0.999
Tyr(ϕ-O^-)	(Y^-)	120	0.996
Lys(NMeN, oxidized)[a]		120	1.000
Asp(COO^-)	(D^-)	170	0.999
Glu(COO^-)	(E^-)	250	1.000
Ser($PO_4^=$)		1000	1.000

[a]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; the reduced state is *N*-methyl-1,6-dihydronicotinamide.
[b]The calculated T_t value for Pro comes from poly(VPGVG) 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 $Val^1\gamma CH_3$ and $Pro^2\beta CH_2$ moieties.
[c]The experimental value determined from poly $[f_v(VPGVG)_1 f_P(PPGVG)]$.
T_t = temperature of inverse temperature transition for poly$[f_v(VPGVG)_1 f_x(VPGXG)]$.
Adapted from Urry et al (1992).

value of T_t and favour unfolding and disassembly (i.e. will cause swelling when cross-linked).

Consequences of the ΔT_t hydrophobic paradigm for elastin structure formation, function and pathology

All of the ways by which the value of T_t can be changed can either enhance fibre formation (by lowering T_t) or interfere with fibre formation (by raising T_t). As the fibre is formed, lowering T_t can stabilize the fibre with increased elastic modulus and improved function, or raising the value of T_t can lead to fibre swelling with decrease in elastic modulus, with increased incidence of tearing and, in general, with loss of function and enhanced susceptibility to proteolytic degradation.

Elastogenesis and wound repair

Those factors which lower T_t will favour fibre formation, e.g. increasing precursor protein concentration, conversion of lysine to aldehydes and the addition of salts. But the well-known post-translational modification of prolyl hydroxylation, as suggested early on (Urry et al 1979), would interfere with elastin fibre formation (see the relative values of Pro^c of $-8\,^\circ C$ and HyP of $50\,^\circ C$ in Table 1). That prolyl hydroxylation interferes with fibre formation in culture has been shown by Franzblau and colleagues (Barone et al 1985). In wound repair there is a high level of prolyl hydroxylase, which ensures abundant high-quality collagen due to hydroxylation of many of the prolyl residues of collagen (Rapaka et al 1978b). The same enzyme hydroxylates tropoelastin (Bhatnagar et al 1978), raising T_t above physiological temperature and preventing fibre formation. This provides an explanation for the paucity of elastic fibres in scar tissue. Furthermore, in the assembly process there are specific charged residues that need to be neutralized, for instance by side-chain ion pairing, during assembly. In particular, the human W4 sequence has a Glu residue that must be neutralized to bring the chain segment T_t sufficiently below $37\,^\circ C$.

Function: the development of entropic elasticity

The remarkable durability of mammalian elastic fibres, each of which is capable of some 2 000 000 000 demanding stretch/relaxation cycles in the aortic arch during its lifetime, requires that the elastomeric force be dominantly entropic. In Figs 3B and C, force development is seen to occur as the temperature is raised above T_t and through the range of the inverse temperature transition; that is, elastomeric force develops over the temperature range in which fibre entropy must decrease and for which all of the foregoing discussion shows structure formation to occur by hydrophobic folding and assembly. By the classical argument of a near zero slope in the plot