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THE HYDROPHOBICITY OF VERTEBRATE ELASTINS

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Summary

An evolutionary trend towards increasing hydrophobicity of vertebrate arterial elastins suggests that there is an adaptive advantage to higher hydrophobicity. The swelling and dynamic mechanical properties of elastins from several species were measured to test whether hydrophobicity is associated with mechanical performance. Hydrophobicity was quantified according to amino acid composition (*HI*), and two behaviour-based indices: the Flory–Huggins solvent interaction parameter (χ_1), and a swelling index relating tissue volumes at 60 and 1 °C. Swelling index values correlated with χ_1 and, for most species studied, with *HI*, suggesting that the different approaches used to quantify hydrophobicity are equally valid. Dynamic mechanical properties were measured both in a closed system, to control the effects of water content, and in an open system, to determine whether the increased swelling of hydrophobic materials at low temperatures offsets the direct stiffening effect of cold. There were no biologically significant differences in mechanical behaviour in either open or closed systems that could be attributed to hydrophobicity. Therefore, although the original function of hydrophobicity in an ancestral elastin may have been to produce molecular mobility, mechanical performance did not drive a subsequent increase in hydrophobicity. Higher hydrophobicities may have arisen to facilitate the manufacture of the elastic fibre.

Key words: hydrophobicity, vertebrate, elastin, biomechanics, swelling.

Introduction

The aortic wall of all vertebrates except agnathans contains a rubbery protein called elastin that allows the vessel to expand under the high pressures associated with cardiac contraction. In expanding, energy from the blood is temporarily stored in the elastin as elastic energy, but is promptly returned to the blood when the elastin recoils in diastole. This recoil acts as a second pump, forcing the blood on downstream and smoothing out pressure fluctuations. The total work of the heart is reduced as long as the transfer of energy into and out of the elastin is efficient. Elastin achieves both efficiency and long-range deformation with a high molecular mobility, although it is not clear how this mobility is achieved. Covalent crosslinks that unite individual molecules in an insoluble extracellular network ultimately limit this mobility and so allow the network to return to its original dimensions without permanent strain. Both high molecular mobility and insolubility are unusual for a protein, but they are understood to be necessary for elastomeric performance.

Elastin is also unusually hydrophobic and has become increasingly so with evolution (Sage and Gray, 1979, 1980, 1981). This trend suggests that there is an adaptative advantage to higher hydrophobicity, but it is not known whether the advantage is associated with elastomeric function. In at least mammalian and avian elastins, the precursor tropoelastin contains two distinct domain types, one hydrophilic and one hydrophobic. The hydrophilic regions are rich in alanine and lysine and are involved in crosslinking; the hydrophobic regions are elastomeric. The association of elastic behaviour with the hydrophobic regions suggests that hydrophobicity does contribute to the mechanical functioning of the protein, and the correlation between an increase in elastin hydrophobicity and the development of a closed, high-pressure circulatory system (Sage, 1982) suggests that mechanical performance has driven this aspect of the evolution of elastin. Alternatively, hydrophobicity may be advantageous for some other reason, such as facilitating crosslinking, a process associated with the manufacture of elastin, not with its function. In this paper, we address the question of why elastins are hydrophobic and why some elastins are more hydrophobic than others. We begin by advancing three hypotheses, two based on mechanical performance and one based on a manufacturing issue.

Hypothesis 1: hydrophobicity provides elasticity

The behaviour of elastin can be described by the kinetic theory of rubber elasticity which requires substantial mobility of the protein chains. To produce this mobility, there must be a number of available conformations separated by low-energy barriers that permit a fast equilibration between the different conformations. Selection may therefore have favoured the

inclusion of small amino acids such as glycine and valine, to minimize steric hindrance, and of proline, for its tendency to form β-turns. These amino acids tend to be hydrophobic. Evolution may also have selected against charged or polar amino acids that promote interactions between neighbouring elastin chains, possibly stabilizing the secondary structure. Such amino acids are hydrophilic. High proportions of small amino acids are also found in other elastomers such as the invertebrate rubbers abductin (Sage and Gray, 1976) and resilin (Anderson, 1971) and some spider silks (Guerette et al., 1996). These characteristics are not necessary for elastomeric behaviour: octopus arterial elastomer contains little glycine and many polar and charged amino acids (Shadwick and Gosline, 1985a,b), and the elastomeric sequences of spider silks are rich not only in glycine but also in glutamine, which makes them hydrophilic (Lewis, 1992; Guerette et al., 1996). However, more than one strategy may produce molecular mobility.

Hypothesis 2: hydrophobicity yields a variable water content that results in thermally independent behaviour

The mechanical properties of a sample of elastin reflect a combination of thermal and hydrational processes; increasing either temperature or water content reduces the friction between elastin chains, increasing molecular mobility and reducing the stiffness of the elastin network. The water content of a protein-based elastic network represents, in part, the thermodynamic drive of the water to mix with and thereby to swell the network. Since a protein has hydrophilic and hydrophobic regions, its interaction with water varies throughout the network. Upon exposure to water, hydrophobic regions show a positive free energy change indicating that their association with water is unfavourable and that water is a poor solvent for that region of the protein (Tanford, 1980). However, the magnitude of the free energy change is smaller at lower than at high temperatures, indicating that water is not as bad a solvent at lower temperatures and, consequently, the hydrophobic regions swell more at lower than at high temperatures. Hydrophilic regions, including the hydrophilic side chains and the polar regions of the peptide bonds, typically show the opposite response to water: a negative free energy change (which indicates a favourable association with water) and a swelling profile in which water content is either constant or increases with increasing temperature.

The interaction between water and elastin is heavily influenced by its hydrophobic regions. Thermal swelling profiles, at least for the more hydrophobic elastins, are characterized by moderate water contents at approximately 40 °C and progressively higher water contents as the tissue is cooled. All else being equal, the more hydrophobic the protein, the greater the change in water content. Therefore, if the elastin is allowed to absorb water from its surroundings as its temperature is reduced, the stiffening effect caused by a drop in temperature can be offset by the extra plasticization from the increased water content. Gosline and French (1979) observed that these opposing thermal and hydrational actions largely cancel each other out in the highly hydrophobic bovine ligament elastin, leaving the tissue with temperature-independent mechanical properties. Since constant mechanical properties might be of value to an animal that experiences a range of body temperatures, they proposed that the hydrophobic character of elastin arose during the evolution of the protein to provide an ancestral vertebrate ectotherm with a material whose mechanical properties were independent of temperature.

Hypothesis 3: the segregation of hydrophobic and hydrophilic residues facilitates self-aggregation

The soluble precursor tropoelastin molecules are exported to the extracellular matrix where they are incorporated into the growing elastin network. Large hydrophobic patches may promote self-aggregation, and segregation of hydrophobic and hydrophilic amino acids may present the crosslinking sites for easy enzymatic access (Robson et al., 1993).

We have tested the first two hypotheses, those based on mechanical function. If mechanical performance has driven an increase in hydrophobicity, we would expect to see differences in the mechanical properties of elastins of different hydrophobicity. The first hypothesis focuses on the elastin itself, and so we tested elastins from different species in a closed system where the effects of differing water contents could be controlled. In the second hypothesis, the change in water content with temperature is integral to the hypothesis, and so the elastins were tested in an open system where water content was free to change.

Hydrophobic indices based on swelling behaviour and on amino acid composition

The amount of water associated with a sample of elastin depends on its hydrophobicity, and so the water content of a sample or some other measure of its swelling behaviour can be used as an index of hydrophobicity. But, by associating with a water molecule, an elastin chain is forced into a more open and possibly less energetically favourable conformation that would resist further hydration. Therefore, the water content at equilibrium represents the balance between the entropic and enthalpic drives of the water and elastin to mix and the elastic resistance to deformation of any structures in the elastin (its secondary structure, crosslinks and any structure that results in physical interaction between fibres). The effects of these constraints must be accounted for if hydrophobicity is determined from swelling behaviour. An alternative approach to quantifying hydrophobicity is to calculate a value from an assessment of the hydrophobicity of each amino acid weighted according to the amino acid composition of the elastin. This approach assumes that primary structure and local folding have no impact on the hydrophobic nature of the chain. If structural effects are negligible, both behavioural and compositional approaches will yield valid measures of hydrophobicity. If structural effects are not negligible but are equal in all samples, the approaches will still yield relative values that are appropriate for a comparative study. We have therefore

assessed the hydrophobicity of elastin using both approaches, using two methods based on swelling behaviour and four composition-based methods using literature values of hydrophobicity for each amino acid. By comparing the values generated by the two different approaches, we can infer the influence of structural factors on the behaviour of elastin.

The first of the two methods based on swelling behaviour quantifies the energetic cost of an interaction between a water molecule and elastin: the more hydrophobic the elastin, the greater the cost. The Flory–Huggins solvent interaction parameter, χ_1, provides a measure of the free energy of interaction per $\boldsymbol{RT}$ (where T is absolute temperature and $\boldsymbol{R}$ is the gas constant), having both enthalpic and entropic contributions. According to the Flory–Rehner theory for the swelling of a crosslinked polymer network, the total free energy for dissolving water in an elastin network is given by the sum of the changes in the conformational free energy and the mixing free energy:

$$-[\ln(1-v_2)+v_2+\chi_1(v_2)^2] = V\rho/M_c[(v_2^0)^{2/3}(v_2)^{1/3}-v_2/2], \quad (1)$$

where v_2 is the volume fraction of polymer, v_2^0 is the volume fraction at which crosslinks were introduced, ρ is the density of dry polymer, V is the molar volume of solvent, and M_c is the molecular mass between crosslinks (Flory, 1953). The right-hand term of equation 1 represents the conformational free energy, which can be equated with entropy changes associated with the swelling of the polymer network and takes into account mean crosslink density and assumes random crosslink distribution. The left-hand side represents the mixing free energy: the first two terms represent the ideal entropy of dilution of the elastin by the water, and the χ_1 term considers the chemical interaction between them. Although M_c has not been determined for elastins other than bovine ligament elastin, the sensitivity of χ_1 to v_2 by far overshadows any effects due to variation in V, ρ, M_c or v_2^0. Therefore, comparative values for χ_1 at 37 °C, designated $\chi_{1,37}$, can be calculated from equation 1 and v_2, which can be calculated from water contents.

Our second approach to measuring hydrophobicity is to quantify the temperature-dependence of the interaction between elastin and water. The interaction between water and a non-polar solute becomes progressively more favourable with decreasing temperature, which would cause a hydrophobic network to swell. We have used the increase in volume between 60 and 1 °C as an index of hydrophobicity, which we call the swelling index; the greater the volume change, the greater the hydrophobicity. From an experimental perspective, the swelling index is completely independent of the $\chi_{1,37}$ index, which is based on water contents measured at 37 °C.

One advantage of basing hydrophobicity on swelling behaviour is that water contributes directly to the mechanical performance of elastin. However, for mechanical behaviour to drive an evolution towards increased hydrophobicity, the water molecules that enter the network must be distributed to network regions that can benefit from them. The second hypothesis links hydrophobicity and mechanics through the two assumptions that hydrophobicity determines swelling and that swelling provides plasticization. The first assumption is well founded in thermodynamics; the second assumption is reasonable but untested. Equation 1 applies to the network in swelling equilibrium with water, and the equilibrium or static elastic resistance to swelling is not necessarily influenced in the same way or by the same pool of water molecules as are the biologically important dynamic mechanical properties. Fibre architecture within the vessel wall or the distribution of crosslinks within the network may affect the static swelling behaviour without similar effects on dynamic behaviour. Lillie and Gosline (1996) found a tight connection between swelling and the viscoelastic properties of pig elastin that is maintained over the full viscoelastic spectrum from rubbery behaviour at low frequencies to glassy behaviour at high frequencies. Therefore, in pig elastin, water appears to plasticize all viscoelastic behaviour equally, static as well as dynamic. Whether this holds in other species has not been established.

Materials and methods

Pig (*Sus scrofa*) aortic arches, turkey (*Meleagris* sp.) aortic arches and bovine (*Bos* sp.) ligamentum nuchae were obtained from local abattoirs. Salmon (*Oncorhynchus keta*) bulbus arteriosae and dogfish shark (*Squalus acanthias*) ventral aortae were obtained from local fish plants. White shark (*Carcharodon carcharias*) ventral aortae were obtained from the Natal Sharks Board, Umhlanga Rocks, Republic of South Africa. Frog (*Rana catesbeiana*) aortic arches, turtle (*Chrysemys picta*) aortic arches and alligator (*Alligator mississippiensis*) aortic arches were obtained from healthy animals from university laboratories. All tissues were obtained fresh except the white shark tissue, which was freshly frozen and stored at −20 °C. For each type of experiment described below, each sample came from a different individual except for white shark, for which the tissue came from two animals.

Purification

The selection of a purification protocol is complicated by the lack of sequence data for most elastins, making it impossible to know whether purification is complete. Contaminants must be avoided in tissues used for composition analysis, necessitating an aggressive protocol. However, tissues for mechanical testing cannot tolerate harsh treatment since scission of a single elastin chain preserves the chemical composition but degrades the sample mechanically (Lillie et al., 1998). This rules out the use of sodium hydroxide, which cleaves peptide bonds (Starcher and Gallione, 1976; Lillie et al., 1998), and cyanogen bromide, which requires the absence of methionine, present in many non-mammalian elastins (Sage and Gray, 1979). Fortunately, the primary contaminant significant to mechanical behaviour is collagen, which is easily heat-denatured to gelatin (Lillie et al., 1994).

We have therefore used conservative techniques to avoid hydrolysis of tissues for mechanical testing. Treatment duration was set by the requirements of pig tissue which, because of its

size, offered the largest barriers to diffusion and so should require the longest treatment (Lillie et al., 1994). The tissues were autoclaved at 0.1 MPa for six periods of 45 min each and then extracted for 24 h with 6 mol l^{-1} guanidine hydrochloride. Samples were rinsed thoroughly in distilled water, autoclaved twice more, and then stored in sterile water until used. Samples used in the determination of amino acid composition were further purified by immersion in 0.1 mol l^{-1} NaOH at 70 °C for 25 min.

Amino acid composition and hydrophobic index

Elastin samples from turtle, dogfish shark, white shark and alligator were hydrolysed following a standard protocol (Sage and Gray, 1979) and shipped to the University of Victoria, Canada, for composition analysis.

The primary hydrophobic index (*HI*) used to quantify the differences in hydrophobicity based on amino acid composition is taken from Sage and Gray (1981). The index is calculated as:

$$HI = H_{avg}/FC, \quad (2)$$

where H_{avg} is the average hydrophobicity (Bigelow, 1967).

$$H_{avg} = \sum \Delta F_{t,i} n_i, \quad (3)$$

where $\Delta F_{t,i}$ as given by Tanford (1962) is the free energy of transfer of an amino acid from ethanol to water and n_i is the mole fraction of that amino acid. *FC* is the fractional charge, which is the sum of the mole fraction of charged amino acids: glutamic acid, aspartic acid, histidine, lysine and arginine (Welscher, 1969). Since the amino acid composition data do not differentiate between aspartic acid and asparagine or between glutamic acid and glutamine, the calculated fractional charges are maximal values.

Since there are several physical criteria that could be used to quantify the hydrophobicity of an amino acid, we have selected three other composition-based scales from the literature: the first is based on the coacervation or the precipitous coming out of solution of soluble elastin-like peptides (Urry et al., 1992); the second is a consensus scale based on the energy of transfer from vapour to water and the energy of transfer from the interior of a protein to its surface (Kyte and Doolittle, 1982), and the third is a consensus scale based on the energies of transfer from ethanol to water, from vapour to water, and from the interior to the surface, plus the effects of burial of polar and non-polar groups (Eisenberg et al., 1982). We have determined the hydrophobicity of each elastin according to each scale.

Water content

Water contents at 37 °C were obtained by equilibrating wet tissue at a relative humidity (RH) between 95 and 99.5 % at 37±0.5 °C for 10 days (Bull, 1944). The following numbers of samples were tested: dogfish shark 12, white shark 5, salmon 10, frog 10, turtle 10, alligator 6, turkey 12, pig 12 and cow ligament 12. Since it is not possible to obtain 100 % RH using this method (Parsegian et al., 1986), we have used 99.5 % RH as the saturation value, but in doing so we underestimate the true water contents in all species by approximately 5 %. The samples were dried at 105±1 °C for 2 days to measure dry mass. The water content at 37 °C gives the ratio of water mass to elastin mass (×100 %), which can be converted to a ratio of water volume to elastin volume using 1.23 g cm^{-3} as the density of elastin (Scandola and Pezzin, 1980). If the temperature of a sample of elastin immersed in water is changed from 37 °C, its total volume will change owing to a change in water volume, assuming that the volume of the elastin is constant. Therefore, volume ratios and water contents at other temperatures can be calculated from the water content at 37 °C and the relative volumes from the swelling studies.

Swelling

Thermal swelling tests were run on the following numbers of samples: dogfish shark 9, white shark 8, salmon 7, frog 6, turtle 11, alligator 8, turkey 11, pig 12 and cow ligament 9. Elastin samples approximately 15 mm×5 mm×1 mm were lightly air-dried and then glued using epoxy resin into stainless-steel mounts for swelling tests. Rehydrated samples were impaled with two short parallel segments of 30 gauge hypodermic needle and then suspended in distilled water in a 70 ml plastic culture flask with a glass face. A thermistor approximately 2 mm from the test sample measured temperature to within 0.5 °C. A video camera connected to a video dimension analyzer (VDA, model 303, Instruments for Physiology and Medicine, San Diego, CA, USA) continuously measured the distance between the two hypodermic needles. Measurements were made at 2° decrements as temperature was reduced from above 60 to 1 °C. The test took approximately 2 h, which ensured equilibrium.

Distances were converted to relative lengths, expressed as a proportion of the length at 37 °C, and were then converted into relative volumes, $v_{rel,T}$, by assuming that elastin swells equally in all dimensions:

$$v_{rel,T} = v_{total,T}/v_{total,37}, \quad (4)$$

where $v_{total,T}$ is the total volume at any temperature and $v_{total,37}$ is the total volume at 37 °C. Bovine ligament elastin has been shown to swell isotropically in water (Aaron and Gosline, 1980, 1981) and in dimethylsulphoxide (Mistrali et al., 1971). The volume of a sample is the sum of the water volume and the elastin volume, so that:

$$1 = v_1 + v_2, \quad (5)$$

where v_1 is the volume fraction of water and v_2 is the volume fraction of elastin. The volume fraction of elastin at 37 °C can be calculated from:

$$v_{2,37} = v_{elastin}/v_{total,37}, \quad (6)$$

where $v_{elastin}$ is the volume of elastin.

Using the assumptions of additive volumes and constant elastin volume, the volume fraction of elastin at any temperature, $v_{2,T}$, can be calculated from $v_{rel,T}$, the relative volume at that temperature:

$$v_{2,T} = v_{2,37}/v_{rel,T}. \quad (7)$$

The volume fraction of water at any temperature, $v_{1,T}$, can be calculated from equation 5 and can be converted to water content using appropriate values for water and elastin density.

Finally, to compare the overall swelling ability of different elastins, we define a swelling index, *SI*, as the ratio of the relative volumes at 60 °C and 1 °C:

$$SI = v_{rel,60}/v_{rel,1}\,. \quad (8)$$

Swelling thermodynamics

To establish the hydrophobicity of the elastins, values for χ_1 were calculated from water contents and swelling data using equation 1. The following constants were used for all elastins: V was assumed to be 0.018 l mol^{-1} (Gosline, 1978), M_c to be 6.0 kg mol^{-1} (Aaron and Gosline, 1981), ρ to be 1.23 g ml^{-1}, v_2^0 to be 0.6 (Gosline, 1978) and the lattice coordinate number zx (see below) to be 4.2 (Gosline, 1978).

The free energy, ΔG, associated with the transfer of 1 mole of water from the bulk phase into the swollen elastic network was calculated between 1 and 60 °C for each elastin:

$$\Delta G = \chi_1 \boldsymbol{R} T/zx\,, \quad (9)$$

where $\boldsymbol{R}$ is the gas constant, T is the absolute temperature, and zx is the lattice coordination number. The free energy *versus* temperature data were fitted to a third-order polynomial by least-squares regression. The worst-case fit had a correlation coefficient of 0.998. The polynomial had the form:

$$\Delta G = A + BT + CT^2 + DT^3\,. \quad (10)$$

The coefficients derived from equation 10 were used to calculate values for enthalpy (ΔH), entropy (ΔS) and heat capacity (ΔC_p) (Edelhoch and Osborne, 1976):

$$\Delta S = -\,\mathrm{d}(\Delta G)/\mathrm{d}T = -\,B - 2CT - 3DT^2\,, \quad (11)$$

$$\Delta H = \Delta G + T\Delta S = \mathrm{A} - CT^2 - 2DT^3\,, \quad (12)$$

$$\Delta C_p = \mathrm{d}(\Delta H)/\mathrm{d}T = -\,2CT - 6DT^2\,. \quad (13)$$

Mechanical tests

Mechanical properties were measured on samples of turkey (N=3), pig (N=6), alligator (N=2), white shark (N=4) and salmon (N=4) elastin immersed in distilled water as an open (variable water content) system. Mechanical properties were also measured on turkey (N=3) and white shark (N=2) elastin in a closed (fixed water content) system. The network chains in fully hydrated elastin are highly mobile, and further increasing mobility by raising the temperature above approximately 40 °C produces little mechanical effect. Accordingly, to demonstrate the thermal effects at high temperatures in the closed system better, we reduced the total mobility of the molecules by selecting a moderately reduced water content (approximately 38 %). The closed systems were achieved using two methods. Samples of turkey elastin were equilibrated at 97 % RH at 37 °C for 10 days. Along with the elastin sample to be tested mechanically, three companion pieces of tissue were placed in the same RH chamber for the determination of water content. Samples were tested mechanically while immersed in mineral oil to prevent dehydration. Samples of white shark elastin were equilibrated and tested in 2 mol l^{-1} sucrose solution, which dehydrates the elastin to approximately the same level as achieved at 97 % RH (Lillie et al., 1996; Lillie and Gosline, 1996) and holds the tissue volume constant to within 3 % between 1 and 40 °C. For all mechanical tests in both closed and open systems, data were collected over a temperature range of 1 °C to approximately 70 °C in increments of approximately 5 °C. The samples were held at each temperature for approximately 20 min which, as preliminary tests showed, ensured equilibrium.

The protocols for measuring the dynamic mechanical properties of samples of elastin have been described previously (Lillie et al., 1996). The tissue was deformed in tension driven by a noise function generator. Fourier transforms of the force and displacement signals were collected using a Wavetek model 5820A cross-channel spectrum analyzer, producing spectra with 200 data points at frequencies, f, between 0.1 and 100 Hz. At each frequency, the dynamic stress, $\sigma(f)$, was calculated as the applied force divided by the original cross-sectional area of the tissue, and the dynamic strain, $\varepsilon(f)$, was calculated as the dynamic change in tissue length divided by the original length of the tissue. Original dimensions refer to the undeformed tissue immersed in distilled water at room temperature. The data were converted to storage moduli, $E'(f)=[\sigma(f)/\varepsilon(f)]\cos\delta(f)$, and loss moduli, $E''(f)=[\sigma(f)/\varepsilon(f)]\sin\delta(f)$, where $\delta(f)$ is the phase shift between stress and strain. The storage modulus is proportional to the energy that is stored elastically in the elastin as it is stretched, and the loss modulus is proportional to the energy that is dissipated, primarily as heat. Therefore, the tangent of phase shift is the ratio of energy lost to energy stored, i.e. $\tan\delta(f)=E''(f)/E'(f)$, and is an inverse measure of the efficiency of the material.

Statistics

Correlations were identified using the Pearson product-moment correlation coefficient calculated using SigmaStat software (Jandel Scientific Inc., San Rafael, CA, USA). Differences were considered significant at the 5 % level.

Results

Amino acid composition and hydrophobic index

Amino acid compositions for turtle, alligator, white shark and dogfish shark are presented in Table 1 together with amino acid compositions for pig, salmon, frog and turkey elastin taken from Sage and Gray (1979, 1981) and for bovine ligament taken from Steven et al. (1974). The non-polar amino acid content ranges from 40 to 60 %; the polar amino acid content is relatively more variable, ranging from 3.5 to 15 % (Table 1).

The calculated values for H_{avg}, *FC* and *HI* are also given in Table 1. H_{avg} does not correlate with any evolutionary trend of the vertebrates studied and is approximately equal in all elastins except those from salmon and dogfish shark. With the

Table 1. *Amino acid compositions of selected vertebrate elastins*

	Chondrichthyes				Reptiles			Mammals	
	Dogfish shark	White shark	Teleosts Salmon[a]	Amphibians Frog[a]	Turtle	Alligator	Birds Turkey[a]	Pig[a]	Cow ligament[b]
Lys	13.5	12	8	7	9	14.5	3	5	2
His	3.5	2	6	2	2	–	0.5	1	–
Arg	17	23.5	26	9	7	19	7	8	4
Asx	23	22.5	17	12	11	26	6	6.5	5
Thr	17	23.5	29	23	20	101.5	13	15	7
Ser	19	23.5	30	15	13	17.5	7	12	8
Glx	53.5	66	36	29	29	41	15	19	16
Pro	129	107	86	104	124	100	128	113	124
Gly	284	289	421	402	353	306	353	313	337
Ala	153	143	151	154	189	161.5	187	244	245
Val	136	120	46	83	124	105	154	128	116
Ile	24	41	10	33	15	39	24	18	21
Leu	43	59.5	63	60	64	82	56	54	64
Tyr	51	45	49	42	35	35	14	19	8
Phe	27	16.5	11	15	11	19	22	33	29
Cys	–	–	<1	3	–	–	<1	<1	–
Met	7	8	6	4	2	5.5	1.2	<1	–
Nonpolar (%)	52	59	40	46	52	59	59	57	60
Polar (%)	13	15	12	7	7	12	4	5	3.5
H_{avg}	1.12	1.08	0.82	1.06	1.02	1.04	1.05	1.04	1.03
FC	111	126	94	59	58	101	32	40	28
HI	10	8.5	9	16	17	10.3	33	26	37
HI[a]	8.7		10.6	15.7	23.7	ND	33.5	23.2	
S.D. (*N*)[a]	1.2 (2)		1.4 (9)	0.9 (2)	(1)	ND	2.1 (3)	1.9 (5)	
Des/Ide[a]	3		0.9	1.9	2.7	ND	2.5	2.8	

Values are not corrected for hydrolytic losses and are listed as residues per 1000 residues.

[a]Data from Sage and Gray (1979, 1981).

[b]Data from Steven et al. (1974).

Asx=Asp+Asn; Glx=Glu+Gln.

H_{avg}, average hydrophobicity; *FC*, fractional charge; *HI*, hydrophobic index; S.D., standard deviation for *HI*; *N*, number of species in a class; ND, not determined; Des/Ide, desmosine/isodesmosine.

exception of the alligator, the *FC* decreases from lower vertebrates to higher vertebrates, causing the *HI* to increase following the same sequence. Our values for *HI* are in good agreement with those obtained by Sage and Gray (1979, 1981) for other species from each vertebrate group listed in Table 1. The values of *HI* show that all the elastins are hydrophobic. For comparison, non-hydrophobic proteins such as the octopus arterial elastomer or lysozyme, a typical globular protein, have *HI* values of approximately 3.

Our values of *HI* were used to rank the hydrophobicity of the various elastins (Table 2). Also included in Table 2 are the rankings obtained using other composition-based scales based on different physical criteria. The order depends little on the scale used.

Swelling and hydrophobicity

The swelling behaviour of all the elastins studied was temperature-dependent. The relative volume, $v_{rel,T}$, of each

Table 2. *Rank of elastins according to hydrophobicity based on amino acid composition*

	Sage[a] (HI)	Urry[b]	Kyte[c]	Eisenberg[d]
White shark	1	1	3	2
Salmon	2	2	1	1
Dogfish shark	3	4	2	3
Alligator	4	3	5	4
Frog	5	5	4	5
Turtle	6	6	6	6
Pig	7	7	7	7
Turkey	8	9	8	8
Cow Ligament	9	8	9	9

The method used to calculate hydrophobicity is described in the reference indicated at the top of the column. A higher number indicates a higher hydrophobicity.

[a]Sage and Gray (1981); [b]Urry et al. (1992); [c]Kyte and Doolittle (1982); [d]Eisenberg et al. (1982).

sample in swelling equilibrium in water decreased with increasing temperature. From the relative volume, we have calculated the volume fraction of water, v_1 (Fig. 1A), and from the relative volume and the water content data at 37 °C we have calculated the water content over the full range of temperatures (Fig. 1B). In general, water contents increased from values of approximately 40–50 % (i.e. g water 100 g^{-1} dry protein) at 40 °C to approximately 70–90 % at 1 °C. Salmon elastin showed the smallest change in water content, indicative of a low hydrophobicity. For all species, the swelling index correlated well with both water content at 37 °C (P=0.013, r= –0.779; Fig. 2) and $\chi_{1,37}$ (P=0.017, r=0.760; Fig. 2), but not with the *HI* (P=0.06, r=0.645; Fig. 3). The correlation between swelling index and *HI* is lost only at low values for *HI*, for alligator and possibly dogfish shark elastins, which the behaviour-based indices (Tables 1, 2) rank as more hydrophobic.

The energetic cost of association between water and elastin, given by χ_1 in Table 3, was high for all elastins and increased with temperature. For a good solvent, χ_1 has a value below 0.5; for a poor solvent, χ_1 has a value a value above 0.5 (Flory, 1953). Therefore, water is a poor solvent for elastin at low temperatures and a very poor solvent at high temperatures. However, swelling is governed by the tendency of elastin and water molecules to mix (the left-hand side of equation 1) and the resistance of the network to the deformation (right-hand side of equation 1). Restrictions provided by crosslinks are accounted for in equation 1 if certain assumptions are met, but if other structural features hinder swelling, χ_1 will be overestimated. We can estimate the severity of these possible structural effects by comparing the values of thermodynamic parameters for elastin with those obtained for individual amino acids.

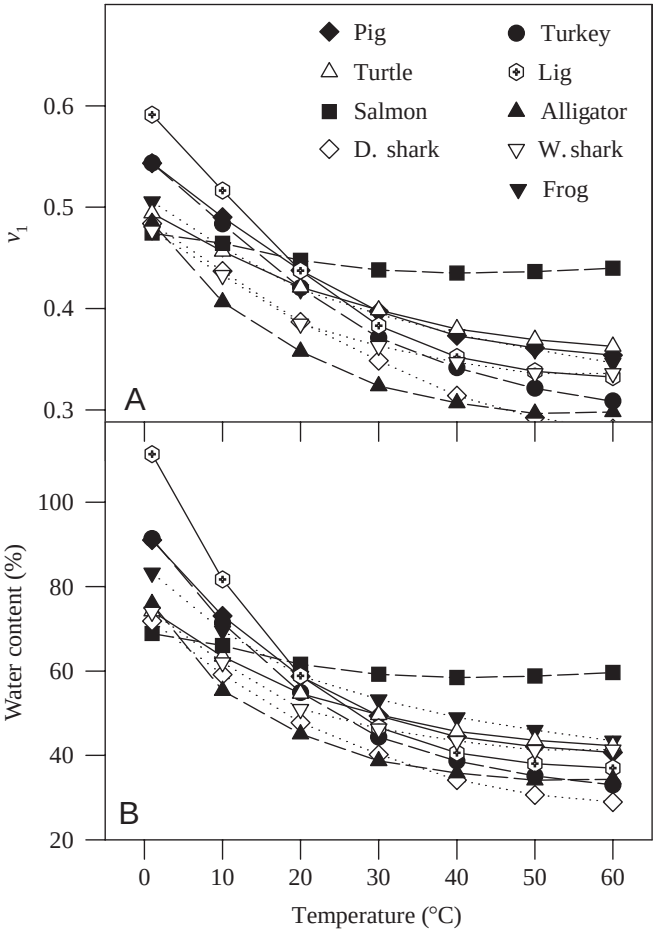

Fig. 1. Thermal dependence of mean water volume fraction v_1 (A) and mean water content (B) for each species. Standard errors are not shown, but values averaged ±0.01 for volume fraction and ±2 % for water content. D. shark, dogfish shark; W. shark, white shark; Lig, bovine ligament.

Both the magnitudes and the thermal dependencies of the thermodynamic parameters for elastin listed in Table 3 are indicative of processes driven by hydrophobic interactions. Both ΔG and ΔC_p are positive, and both ΔH and ΔS are negative. Over the full temperature range, the values for ΔG are close to the values for the transfer of 1 mole of water from the bulk phase to the first layer around an aliphatic amino acid: 377 J mol^{-1} at 1 °C, 490 J mol^{-1} at 20 °C, 582 J mol^{-1} at 40 °C and 645 J mol^{-1} at 60 °C (Nemethy and Scheraga, 1962). The values of ΔG in Table 3 are a few per cent too high because we used water contents at 99.5 % RH for saturation values, but this difference is approximately equal to the uncertainty in the use of the value of 4.2 assigned to zx (see equation 9). The difference in ΔG between 1 and 60 °C is smaller than expected in salmon and white shark, indicating a lower hydrophobicity. The experimental values for ΔC_p are more varied, which is to be expected since they involve the second derivative of the free energy, but most are similar to the values derived from the ΔG data of Nemethy and Scheraga (1962) using the process given in our Materials and methods section: 18.8 J mol^{-1} K^{-1} at 1 °C, 20.1 J mol^{-1} K^{-1} at 20 °C, 21.8 J mol^{-1} K^{-1} at 40 °C and 23.0 J mol^{-1} K^{-1} at 60 °C. Again, salmon and dogfish shark elastin appear to be less hydrophobic than the standard aliphatic amino acid, and alligator and cow ligament more hydrophobic. The ΔC_p values at 37 °C correlate with the swelling index (r=0.716, P=0.03) but not with $\chi_{1,37}$.

The general match between these experimental values and predicted values means that most or all of the swelling behaviour can be accounted for in terms of the solubility of nonpolar compounds in water; the hydration of the peptide and other polar groups remains essentially unchanged, and the water gained or lost in the swelling process is associated primarily with the nonpolar parts of the protein. This observation supports two conclusions: (1) that the swelling index is largely unaffected by possible hydrophilic contaminants, and (2) that constraints on elastin swelling imposed by local secondary structures or by fibre architecture must be minimal. The exceptional response of salmon elastin suggests these conclusions may not be valid for this elastin: either there is a contribution from hydrophilic parts of the elastin or some aspect of its structure is not accounted for by the crosslinking feature of equation 1.

Table 3. *Values for thermodynamic parameters*

	Temperature (°C)	χ_1	ΔG (J mol^{-1})	ΔH (J mol^{-1})	ΔS (J mol^{-1} K^{-1})	ΔC_p (J mol^{-1} K^{-1})
Dogfish shark	1	0.78	427	−1009	−5.2	1.3
	20	0.89	523	−954	−5.1	4.2
	40	1	620	−837	−4.6	8
	60	1.06	707	−640	−4.1	11.7
White shark	1	0.79	427	−950	−5	11.7
	20	0.89	515	−699	−4.1	15.1
	40	0.94	586	−360	−3	18.8
	60	0.96	632	63	−1.7	23.4
Salmon	1	0.79	431	−268	−2.6	6.3
	20	0.82	477	−155	−2.2	5.4
	40	0.82	515	−54	−1.8	4.6
	60	0.83	553	25	−1.6	3.3
Alligator	1	0.78	423	−1494	−7	24.7
	20	0.93	540	−1005	−5.3	26.8
	40	1.01	628	−444	−3.4	28.9
	60	1.03	678	159	−1.5	31.4
Turkey	1	0.73	393	−1235	−5.9	11.3
	20	0.85	498	−1009	−5.1	12.6
	40	0.96	594	−745	−4.3	13.8
	60	1.01	670	−452	−3.4	15.5
Pig	1	0.73	398	−1017	−5.1	10.5
	20	0.83	490	−795	−4.4	13
	40	0.91	565	−507	−3.4	15.9
	60	0.94	628	−159	−2.5	18.8
Cow ligament	1	0.69	373	−1515	−6.9	19.7
	20	0.83	490	−1118	−5.5	22.2
	40	0.94	582	−640	−3.9	25.1
	60	0.97	645	−105	−2.3	28.5

Values are for the transfer of 1 mole of water into the elastin network.

χ_1, energetic cost of association between water and elastin; ΔG, free energy associated with the transfer of 1 mole of water from the bulk phase into the swollen elastic network; ΔH, enthalpy change; ΔS, entropy change; ΔC_p, heat capacity change.

Mechanical properties

In assessing the effects of hydrophobicity on the mechanical performance of elastin, we have looked at five species with extreme values of hydrophobicity assessed on the basis of both the swelling index and the *HI*. We selected turkey and pig, rated highly hydrophobic by both the swelling index and *HI*, white shark and salmon, rated the least hydrophobic by both indices, and alligator, ranked highly hydrophobic according to its swelling behaviour and of relatively low hydrophobicity according to its *HI*.

Hypothesis 1: closed system

Fig. 4 shows the behaviour of a sample of turkey elastin with the water content held constant at 38 %. The storage modulus curves in Fig. 4A represent a small portion of the original data collected at 5, 17 and 40 °C. At 5 °C, the tissue is glassy, with storage modulus values of approximately 10^7 Pa. Raising the temperature to 40 °C reduces the moduli by approximately two orders of magnitude, producing rubbery behaviour at the lower frequencies, but the storage modulus increases (as does the loss modulus; see Fig. 4B) showing that the tissue approaches the start of the glass transition at the higher frequencies.

Reducing the temperature increases the stiffness by increasing the friction between the elastin chains, requiring more time for them to achieve a certain deformation. Increasing the frequency of deformation has an equivalent effect on behaviour because it reduces the time available for deformation; in both cases, it becomes progressively more difficult to achieve the required movement in the allotted time. As long as reducing the temperature slows all molecular motion throughout the network by the same factor, called the shift factor, a_T, time (frequency) and temperature can be considered to be superimposable, and either variable can be adjusted to achieve a certain behaviour. If reducing the temperature from T_1 to T_2 slows the response time by a factor of 100, the equivalent behaviour can be seen at T_1 but at 100 times higher on the frequency scale. Thus, frequency is multiplied by a_T (100 in this case), which is equivalent to shifting the spectra by the factor $\log a_T$ (two decades of frequency in this case), when plotted on a logarithmic scale. The use of time–temperature superposition is a conventional

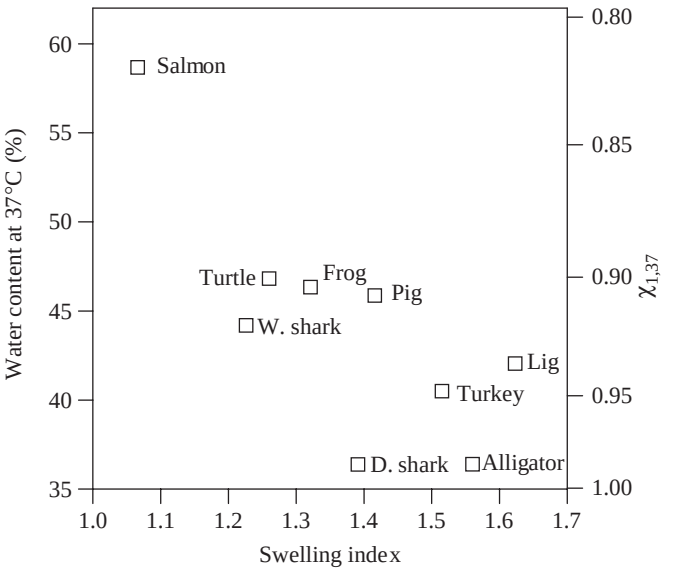

Fig. 2. Correlation between mean water content and mean swelling index, with values for $\chi_{1,37}$ (the value of the Flory–Huggins solvent interaction parameter χ_1 measured at 37 °C) shown on the right-hand vertical axis. Note that the $\chi_{1,37}$ scale is inversely and non-linearly related to water content. Values for standard error averaged ±0.03 for the swelling index (maximum ±0.1 for alligator). P=0.013 for the correlation coefficient (r=–0.779) between mean water content and mean swelling index, and P=0.017 for the correlation coefficient (r=0.760) between $\chi_{1,37}$ and mean swelling index. D. shark, dogfish shark; W. shark, white shark; Lig, bovine ligament.

way to analyze polymers, and we use it here to quantify the change in behaviour induced by changing the temperature (Lillie and Gosline, 1990).

Using the behaviour at 40 °C as the unshifted reference point, individual storage and loss modulus curves for every other temperature were shifted a distance a_T on the frequency axis as necessary to make them overlap and form a continuous, smooth master curve (Ferry, 1970). The solid lines in Fig. 4B show the master curves, each representing 2200 data points collected as individual data sets of 200 points at 11 temperatures between 0.5 and 64 °C. The upper curve is the storage modulus, and the middle curve is the loss modulus. At the bottom of Fig. 4B, tanδ (where δ is the ratio between the loss and storage moduli) reaches a maximum midway through the transition, and the lowest values of tanδ are found at the left-hand end of the curve. Therefore, efficient transfer of energy into and out of this sample of elastin occurred only at the higher temperatures and lower frequencies used. The higher values of tanδ at all other points indicate excessive dissipation of energy which, in an aorta, would increase the demand for cardiac work. The curves for white shark (data not shown) were similar to the one shown in Fig. 4 for turkey.

The turkey spectra collected at 5 °C were shifted by five decades (loga_T=5) to align them with the rest of the master curve. Reducing the temperature from 40 to 5 °C slowed molecular motion by 100 000 times, producing the same change in behaviour as would be observed by holding the temperature constant and increasing the frequency of deformation by five decades, for example from 10^1 to 10^6 Hz. This five-decade shift between 40 and 5 °C can be seen in Fig. 5, in which the shifts used to make the master curves for turkey in a closed system are displayed as a function of temperature. Each point represents the shift from the behaviour at 40 °C. Shifts of this magnitude were also obtained for the less hydrophobic white shark elastin (4.5 and 5.2 decades; Fig. 5) and are comparable with those found previously for pig arterial elastin (Lillie and Gosline, 1990) and bovine ligament elastin (Gosline and French, 1979) in closed systems.

Spectral segments from different parts of the master curve respond to the behaviour of different parts of the network (Ferry, 1970). Behaviour in the rubbery region is dominated by large-scale, long-range motions that encompass several crosslinking segments. As the network moves into the glass transition, its behaviour represents progressively smaller-scale, shorter-range motions that are restricted to shorter sequences between crosslinking domains. Therefore, master curves can reveal substantial (but not subtle) differences in structural features such as crosslink density, entanglements of long chain segments and interactions between spatially close atomic groups. The similarity of the master curves between turkey (Fig. 4) and white shark (not shown) elastin and the overlap of the shift data in Fig. 5 indicate that there are no differences in behaviour that can be attributed to differences in hydrophobicity. Further, if there are structural differences between the two elastins, they do not contribute measurably to the mechanical behaviour of the network.

Hypothesis 2: open system

When water content is held constant in a closed system,

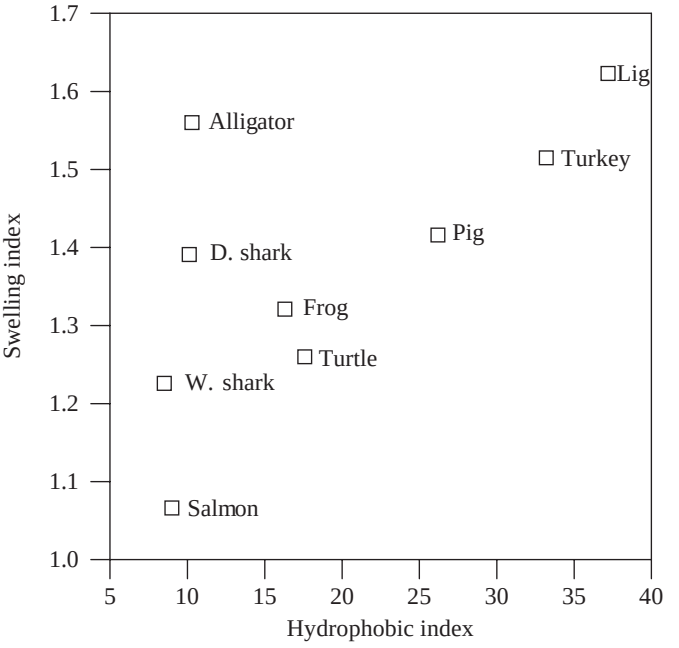

Fig. 3. The correlation between mean swelling index and hydrophobic index. P=0.06 for the correlation coefficient (r=0.645).

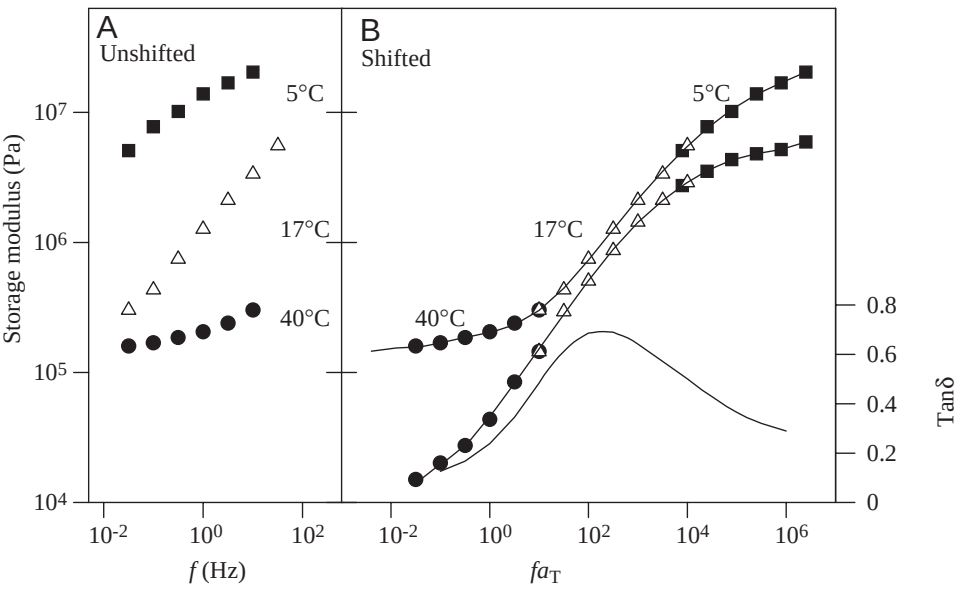

Fig. 4. Mechanical behaviour of one sample of turkey elastin dehydrated to a water content of 38 %. (A) Original unshifted data for storage modulus collected at 5, 17 and 40 °C plotted against frequency (*f* in Hz). Only six or seven of the 200 data points collected per temperature are shown. (B) Master curves created from data collected at 11 temperatures between 0.5 °C and 64 °C showing the storage modulus (upper solid line; left-hand axis), the loss modulus (middle line; left-hand axis) and tanδ (where δ is the ratio between the loss modulus and the storage modulus; see text for explanation; lower line; right-hand axis). Symbols show data from A after the application of a frequency shift (a_T). Data collected at 5 °C were shifted by five decades ($\log a_T$=5), and data collected at 17 °C were shifted by 1.8 decades ($\log a_T$=1.8). The reference temperature is 40 °C so that data collected at 40 °C remain unshifted ($\log a_T$=0). Raising the temperature above 40 °C gave only a modest extension of the storage modulus and no usable data to extend the loss modulus master curve.

cooled elastin shows large changes in behaviour that would be completely incompatible with its function in the arterial wall. When free swelling is permitted in an open system, the deterioration in behaviour largely disappears: turkey elastin in an open system is only slightly stiffer at 5 °C than it is at 40 °C (Fig. 6A). At 5 °C in the closed system (water content 38 %), the sample was glassy with a storage modulus near 10^7 Pa; at the same temperature in the open system (water content 83 %), the sample was still rubbery with a modulus near 10^5 Pa (compare 5 °C curves in Figs 4 and 6A). Although some differences in behaviour are to be expected from the dehydration process in the closed system (Lillie et al., 1996), they appear minimal given the similarity between the curves in the open and closed systems at 40 °C where the water contents are virtually identical: 39 % in the open system, 38 % in the closed system (compare 40 °C curves in Figs 4 and 6A). The difference in hydration accounts for the large reduction in the shift along the frequency axis in the open system compared with the closed system: the 5 °C curve of the open system shown in Fig. 6A has been shifted by only 0.4 decades to achieve suitable overlap with the 40 °C curve, compared with the five-decade shift required in the closed system (Fig. 5). Thus, the swelling of turkey elastin almost completely countered the thermal effect, reducing it by a factor of $10^{4.6}$, i.e. $10^{(5.0-0.4)}$. The high swelling index of turkey elastin gives it a large potential to mitigate the direct thermal effects, falling short by only 0.4 decades. According to the second hypothesis, the less hydrophobic elastins, i.e. those with a lower swelling index, will be unable to maintain constant mechanical properties and will demonstrate a significant shift towards the glass transition when cooled.

Surprisingly, the mechanical properties of the less hydrophobic salmon elastin tested in swelling equilibrium at 5 °C and 40 °C (Fig. 6B) are virtually identical to those observed in turkey elastin. Even though the calculated water content of salmon elastin increased by only 10 % from 58 % at 40 °C to 68 % at 5 °C, the behaviour of the tissue shifted by only 0.6 decades. There is no indication that the much reduced ability of salmon elastin to load water compromises its performance at low temperatures. Over the entire temperature range, the

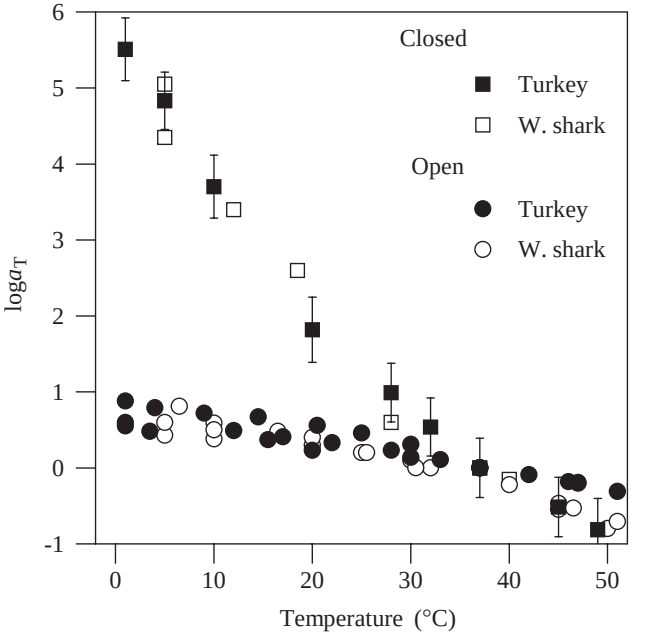

Fig. 5. Shifts ($\log a_T$) used to create the master curves in the closed system in Fig. 4 and in the open system in Fig. 6. Values for turkey elastin in the closed system are means ± S.E.M. (*N*=3). Values for turkey elastin in an open system (*N*=3) and for white shark (W. shark) in a closed (*N*=2) or open system (*N*=4) are individual values.

magnitude of the mechanical shifts for salmon elastin in an open system (not shown) was close to those of turkey and white shark (Fig. 5) and pig (data not shown) elastin. All elastins tested displayed shifts of approximately one decade or less over the temperature range 1–40 °C, with values for tanδ of below 0.15 or a resilience of 62 %. They showed no major differences in their mechanical behaviour despite the measurable differences in their *HI* and swelling indices.

Imperfect reduction in an open system

In creating the master curves for some of the species, we tolerated some imperfect reduction of the spectra at temperatures below approximately 25 °C. Strictly speaking, the overlap of spectra collected at adjacent temperatures must be perfect, and the shape of the adjacent curves must be identical where they overlap. However, as temperature was reduced, the curves for turkey (Fig. 6A), pig and alligator (data not shown) changed shape slightly, with the loss modulus at the higher frequencies decreasing more than expected.

A reduction in the loss modulus that is not accompanied by a similar reduction in the storage modulus produces a reduction in their ratio, tanδ. We can therefore document the changes in spectral shape by plotting tanδ at low (1 Hz) and high (100 Hz) frequencies as a function of temperature (Fig. 7). At 1 Hz, samples from all species tested showed values for tanδ of 0.07 or less that increased only slightly and monotonically with cooling. At 100 Hz, the values for tanδ were somewhat higher, some exceeding 0.1, but the values increased monotonically with cooling in the tissues that showed perfect reduction (white shark and salmon) and increased and then decreased in the tissues that showed imperfect reduction (turkey, pig and alligator). Similar imperfect behaviour has also been observed

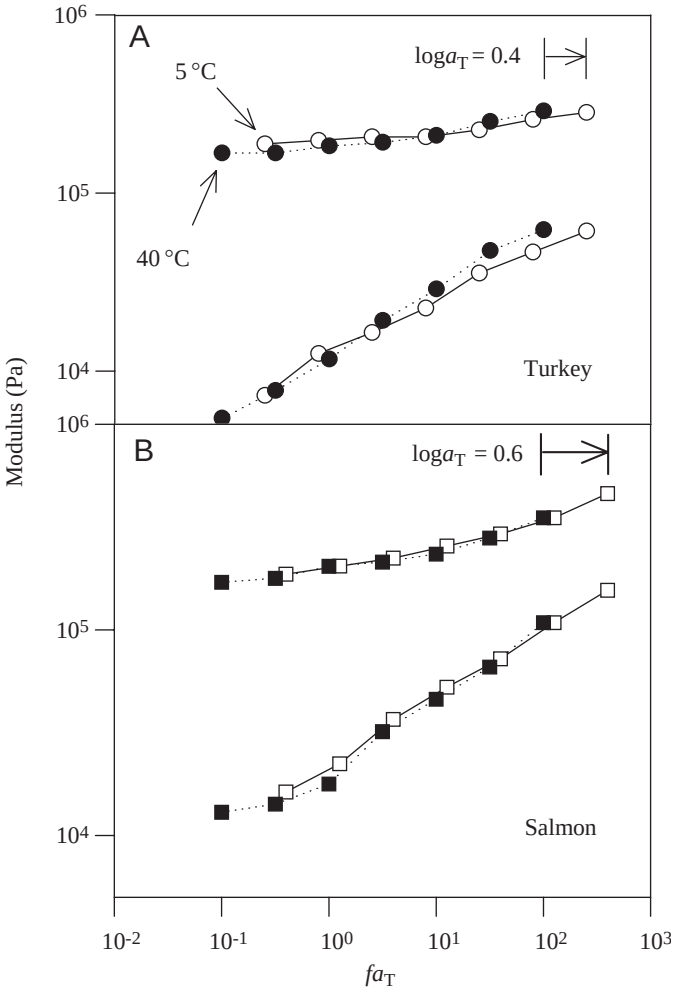

Fig. 6. Storage (upper curves of each panel) and loss moduli (lower curves of each panel) collected in an open system for turkey (A) and salmon (B) elastin. Filled symbols show data collected at 40 °C and open symbols show data collected at 5 °C. The reference temperature is 40 °C. The 5 °C turkey data were shifted by 0.4 decades (loga_T=0.4) to optimize overlap with the 40 °C data. The 5 °C salmon data were shifted by 0.6 decades (loga_T=0.6). Imperfect reduction of the turkey data is evident at higher frequencies (*f*) as a relative reduction in the loss modulus at 5 °C compared with 40 °C. For salmon elastin, the loss moduli at 5 °C and 40 °C overlap, indicating perfect reduction. Water contents for the turkey elastin were 83 % at 5 °C and 39 % at 40 °C. Water contents for the salmon elastin were 68 % at 5 °C and 58 % at 40 °C.

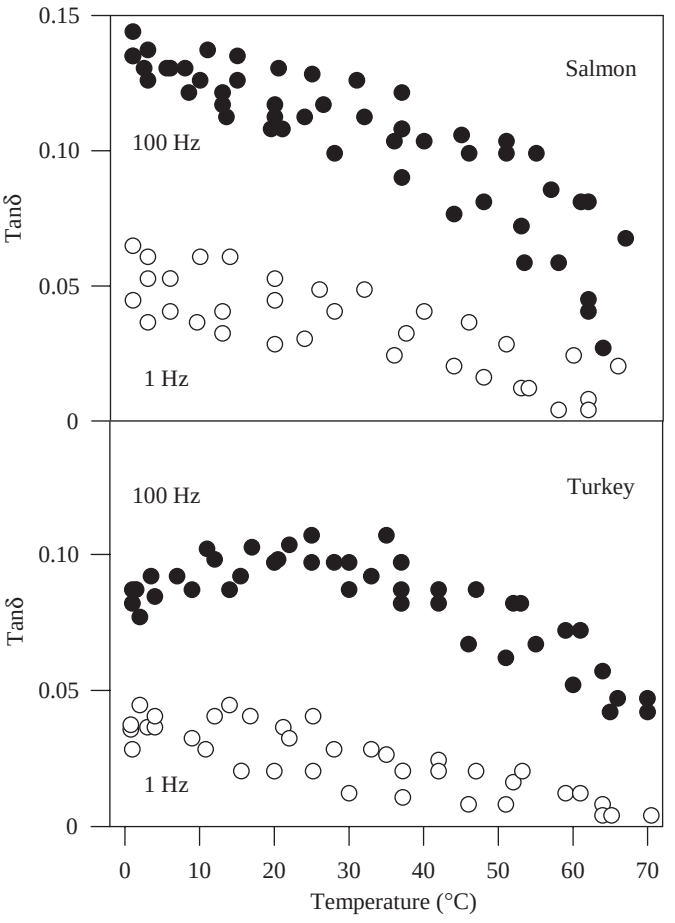

Fig. 7. Values of tanδ at 1 Hz (open symbols) and 100 Hz (filled symbols) for salmon (*N*=4) and turkey (*N*=3), documenting the decrease in turkey tanδ at 100 Hz at lower temperatures. Values are individual measurements collected in an open system. Tanδ is the ratio of loss modulus to storage modulus.

in bovine ligament (Gosline and French, 1979). It is the decrease in tanδ at 100 Hz below approximately 25 °C that causes the difficulty in reducing the data to create master curves.

In reducing such data to form a master curve, we ignored the high-frequency response and calculated the shift from data collected below 10 Hz. The presence of imperfect reduction does not affect our conclusion that the behaviour of elastin in an open system is orders of magnitude less sensitive to a change in temperature than elastin in a closed system, but these slight changes in the spectral shape do indicate that some of the small-scale aspects of elastin behaviour are indeed sensitive to swelling.

Discussion

Hydrophobicity scale

We examined the mechanical properties of elastins from five species, differing in hydrophobicity, to test two hypotheses that explain the high hydrophobicity of elastin in terms of its mechanical performance: either that hydrophobicity provides elasticity or that it yields thermally independent behaviour. We quantified hydrophobicity according to amino acid composition (primarily *HI*) and according to the energetics of the elastin's interaction with water (the $\chi_{1,37}$ and swelling indices), obtaining good overall agreement amongst the indices. This agreement suggests that structural differences at either the molecular or fibrillar level, including differences in fibre disposition (Sage and Gray, 1980), have little impact on swelling behaviour. We drew the same conclusion from the match between our calculated ΔG values and the literature values, which supports the assumption that the v_2 data used to calculate χ_1 and ΔG were dominated by hydrophobic interactions and were not unduly influenced by features of elastin architecture that were not accounted for in equation 1.

Exceptions to the pattern were found in alligator and possibly dogfish shark elastins, for which the swelling indices did not match their low composition-based values of *HI* (Fig. 3). It is possible that their nonconforming behaviour is due to some structural uniqueness. However, since the mismatches are associated with low values of *HI*, it is also possible that the conservative purification protocols were inadequate and resulted in the inclusion of hydrophilic contaminants. No *HI* values for alligator elastin were provided by Sage and Gray (1979), but substituting values from its nearest evolutionary neighbours (33.5 for birds or even 23.7 for a turtle) removes much or all of the mismatch between the swelling index and the *HI* visible in Fig. 3. Further, the presence of imperfect reduction in the alligator mechanical data (see below) suggests that the high value for the swelling index is indeed correct, which makes us suspect that contaminants contributed to the low value of *HI*.

Mechanical properties

There were few differences in the mechanical behaviour of elastins of different species that could be attributed to differences in hydrophobicity. The one difference in mechanical properties that correlated with swelling probably represents fine-scale aspects of molecular dynamics and is probably of no physiological importance. In the more hydrophobic elastins, high levels of swelling below 25 °C decreased the loss modulus and hence tanδ at the high-frequency end of the spectrum where the network approaches its glass transition, resulting in imperfect reduction of the data. This response indicates that, at high levels of swelling, the effects of hydration are not equally distributed through the network, so that the shorter-range molecular motions, which are manifest in the transition, are plasticized more than the longer-range motions, which dominate the rubber plateau. Since our thermodynamic data indicate that the swelling changes are associated primarily with the hydrophobic parts of the protein, high levels of swelling apparently plasticize the elastomeric segments more than the crosslinking segments. Thus, we attribute the imperfect reduction to the uneven distribution of the hydrophobic and non-hydrophobic residues in mammal and bird elastins. Although the sequence for alligator elastin is not known, we predict that it will contain similar domain types to those in mammal and bird elastins. In the less hydrophobic elastins, the partitioning of the network into hydrophobic and hydrophilic domains is less well developed, and the numerous polar amino acids are thought to be evenly distributed throughout the network (Sage and Gray, 1979). The distribution of water molecules throughout the network should be more uniform, which could explain the observed reducibility of their spectra.

There were no measurable differences between the master curves of the highly hydrophobic turkey elastin and the less hydrophobic white shark elastin when tested in a closed system, suggesting that the mobility of their protein chains is fundamentally similar. The similarity in behaviour between the turkey and the less hydrophobic salmon or white shark elastins when tested in an open system shows that there is no advantage to being hydrophobic in producing temperature-independent behaviour. Although the elastins with the lower hydrophobicities do not increase their water contents as much at low temperature, their water contents are apparently adequate to keep them plasticized even at low temperatures. The shift factors were slightly larger for the two elastins with lower hydrophobicities (salmon and white shark) than for the two elastins with higher hydrophobicities (turkey and pig), but any difference in performance over the temperature range actually experienced by an ectotherm would probably be insignificant. Although a mammal that hibernates or a bird that enters a period of torpor will experience a range of body temperatures and so may well benefit from the constancy of behaviour that the swelling would give its elastin, it appears unlikely that a drive towards higher hydrophobicity came from a need to compensate for thermal changes in elastin performance. We therefore conclude that an improvement in mechanical performance did not drive an increase in hydrophobicity and reject the second hypothesis, that elastins are hydrophobic to gain thermally independent mechanical properties.

This conclusion, however, does not necessarily lead to a rejection of the first hypothesis. Although we have identified different levels of hydrophobicity in a range of elastins, all the elastins tested were in fact hydrophobic, with the possible exception of salmon elastin. Sage and Gray (1981) have reconstructed the amino acid composition of an ancestral elastin, predicting it to have been less hydrophobic than present-day mammalian elastin, with higher levels of glutamic acid and glutamine. Although this prediction may need to be modified slightly in the light of our current ranking of teleost elastins as less hydrophobic than shark elastins, the predicted ancestral form appears to be much like that of present-day lower vertebrate elastins, which behave hydrophobically. Thus, the elastomeric part of the ancestral elastin may have been hydrophobic because its particular combination of amino acids made a good, flexible elastomer, as predicted by the first hypothesis. Low hydrophobicity in any extant elastin, such as those of the teleosts, may be related to non-elastomeric domains or may be a more recent evolutionary development. Given the functional constraints on the cardiovascular system to maintain elasticity in any adaptation, all extant elastins may be equally and adequately elastic.

Thus, the original function of hydrophobicity may have been to produce molecular mobility. The observation remains, however, that elastins display different hydrophobicities and, despite our higher than expected ranking of the shark elastins, there still appears to have been an evolutionary trend towards higher values. We therefore return to the question of selection pressures and, having ruled out the first two hypotheses, we consider the third. Beyond its elastomeric function, avian and mammalian tropoelastin has chemotactic and cell- and protein-binding properties. Although some of these functions may have been late evolutionary developments, hydrophobicity may promote the association of elastin with other proteins, particularly with the components of the elastic fibre including itself.

Our findings suggest that hydrophobicity may have arisen to facilitate fibrillogenesis by lowering the solubility of the precursor tropoelastin. Soluble tropoelastin molecules are exported to the extracellular matrix, where they come out of solution and form fibres in all vertebrates. Fibrillogenesis therefore requires that tropoelastin be first soluble and then insoluble. If a tropoelastin molecule is naturally soluble, fibre formation could be initiated by aggregation of large hydrophobic patches, but it may be difficult to collapse the entire molecule. The opposite strategy would be to make tropoelastin itself insoluble, making fibre formation easy, and use to chaperone proteins (Hinek, 1995) to keep it from coming out of solution, or coacervating, prematurely. Since all elastins are hydrophobic, all should have the potential to coacervate, but the coacervation temperature in some is probably too high for it to occur naturally. The coacervation temperature can be lowered by increasing the hydrophobicity. Therefore, hydrophobicity may have increased during evolution to reduce the solubility of tropoelastin, requiring the co-evolution of chaperone proteins and possibly an elevation of body temperature. Although the tendency of elastin to coacervate would remain latent in an ectotherm, whose body temperature during fibrillogenesis is below the coacervation point of its elastin, it might benefit mammals, birds and alligators, which are kept warm during the main period of fibrillogenesis. In these animals, a fine tuning of fibre formation might have driven an increase in hydrophobicity.

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