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Temperature-favoured assembly of collagen is driven by hydrophilic not hydrophobic interactions

S. Leikin, D. C. Rau and V. A. Parsegian

It has become almost axiomatic that protein folding and assembly are dominated by the hydrophobic effect. The contributions from this, and other, hydrophilic interactions can now be better distinguished by direct measurement of forces between proteins. Here we report the measurement of forces between triple helices of type I collagen at different temperatures, pH and solute concentrations. We separate repulsive and attractive components of the net force and analyze the origin of the attraction responsible for the collagen self-assembly. In this case the role of the hydrophobic effect appears to be negligible. Instead, water-mediated hydrogen bonding between polar residues is the most consistent explanation.

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Traditionally the relative importance of different energetic contributions to protein folding and assembly has been evaluated from the calorimetric measurement of free energies of transfer of different model compounds and amino acids into water[1,2], from the measurement of stability of protein mutants[3] and from the calculation of the energies of electrostatic interaction between charged residues[4,5]. For a number of years it has been assumed that hydrophobic interactions play the dominant role[6–8]. Evidence for an important contribution from hydrophilic interactions, however, is now accumulating[1–3].

We address this issue through direct measurement of forces between protein molecules by the 'osmotic stress' method[9]. This method allows the measurement of forces as a function of molecular separation within the last 10 Å as surfaces approach contact (for a review see ref. 10). The technique has previously been used with condensed arrays of lipid bilayers[11], DNA molecules[12] and polysaccharides[13].

Here we study interactions involved in the temperature-favoured self-assembly of collagen triple helices. It has long been known that collagen solubility, assembly kinetics and fiber structure depend on pH[14,15], temperature[14] and the presence of salt[14–16] and other solutes such as glycerol[17] and sugars[18,19]. Hydrophobic, electrostatic and hydrogen-bonding mechanisms of assembly have been proposed. Molecular modelling, based on low resolution X-ray data, electron microscopy, the amino-acid sequence and the triple helical structure, showed alignment of both hydrophobic regions and charged/hydrophilic regions on apposing helices[20–22]. By measuring interhelical forces as a function of solution conditions, we determine the separate contributions from repulsive and attractive interactions. Direct measurement of the forces allows us to discriminate between different mechanisms of assembly.

Force-distance curves

The net temperature-dependent force-distance curves measured in fibers reconstituted from collagen triple helices[23] are shown in Fig. 1. These force curves show an exponential repulsion at small separations and a sharp downward turn to an equilibrium spacing in the absence of applied stress. This equilibrium separation represents the balance point between attractive and repulsive interhelical forces.

The downward turn in force curves disappears under several different conditions (Fig. 2). The force becomes purely repulsive, and the exponential now extends over four orders of magnitude from the strongest to the weakest forces measured. Under certain conditions collagen fibers would completely dissolve in the absence of external osmotic stress. In others, some weak residual attraction prevents the complete dissolution of greatly swollen assemblies. Within experimental error, the same, purely exponential force curve is observed by lowering the pH from 7.5 to 6; by adding glycerol or glucose to the bathing solution; for collagen reduced by sodium borohydride; or for collagen fibers stored over a year in PEG solution (Fig. 2). At high osmotic stress and small spacings, this curve coincides with the exponential shown in Fig. 1. Within experimental accuracy, this force is temperature-independent.

Under the experimental conditions examined here,

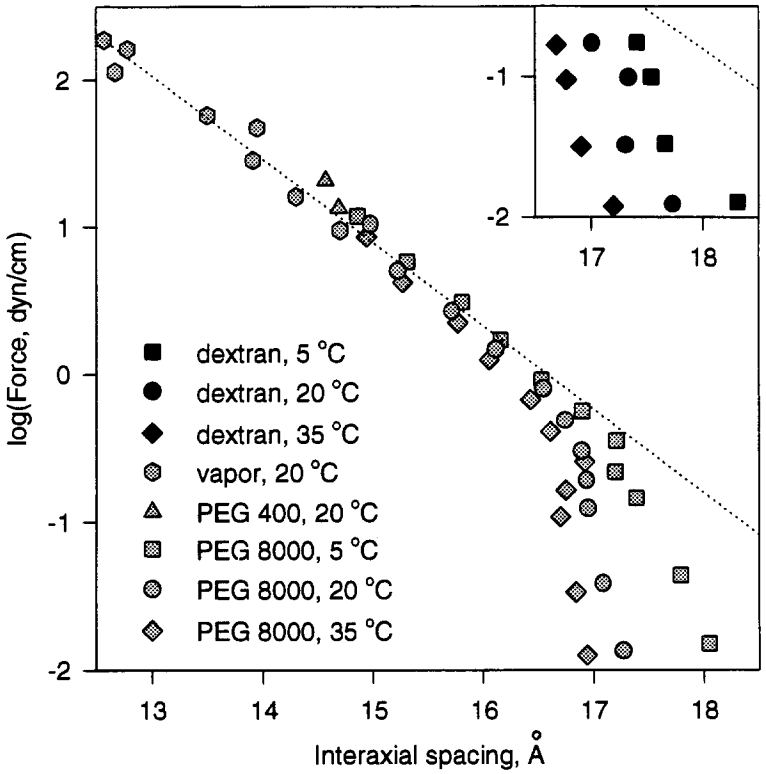

Fig. 1 Temperature-dependent force-distance curves measured between collagen triple helices in 10 mM Tris/2 mM EDTA, pH 7.5. At small separations (high osmotic stress) the force is not sensitive to pH so that the forces above 10 dyn cm^{-1} could be measured by exposure to vapours of different relative humidities without pH buffer. The vapour pressure and osmotic stress measurements overlap demonstrating that both techniques give the same result. The net force is a superposition of a purely exponential repulsive interaction (Figs 2, 3 and outlined here by the dotted line) and a strongly temperature-dependent attraction (Fig. 4). In the insert we show the results of force measurements with dextran as the stressing polymer. The result is the same within the observed sample to sample variation (Fig. 5). The accuracy of determining the osmotic pressure is approximately 10 % (or 0.05 on the $\log_{10}$ scale). The accuracy and reproducibility of interaxial spacings measured on different samples decreases from ±0.1–0.2 Å at d_{int} < 16 Å to ±0.3 Å (d_{int} ~ 17–18 Å) and to about ±0.5 Å at d_{int} ~ 19–20 Å.

this four-decade exponential is a limiting force curve independent of the preparation used to achieve it (Fig. 3). The most straightforward explanation of these observations is that any of these solution conditions or collagen modifications specifically affect the attractive component of the net force, with little or no effect on the repulsive component. The net forces shown in Fig. 1 then result from a combination of the purely exponential repulsion (Figs 2, 3) and a temperature-favoured attraction sensitive to the solution conditions or collagen modifications. Alternatively, but improbably, the very different treatments in each of the cases shown in Figs 2 and 3 create an additional repulsion that has the exact magnitude, distance- and temperature-dependence such that the sum of this new repulsion and the net force gives the observed four-decade exponential.

Repulsive interaction

The rapidly decaying, four decade exponential repulsion (Figs 2, 3) is not consistent with power-law interactions due to motional entropy loss, often invoked[24,25] in the theory of rod-like polymer liquid-crystals. Instead it appears to be qualitatively similar to hydration forces observed between many types of macromolecular surfaces[23]. Several models have been developed to account for these forces, particularly for the interactions between lipid bilayers[10,26,27]. No decisive evidence, however, has brought agreement among different authors who still favour different theories. We have argued that hydration forces are caused by rearrangement of the water hydrogen-bonding network near polar surfaces so that an additional energy (positive or negative) is associated with the removal of this water on mutual approach of the surfaces[10]. For the case of collagen the recent high resolution X-ray crystal structure of a hydrated collagen-like peptide[28] supports this involvement of water. There are no direct contacts between the peptide triple helices which have no flexible side chains. The structure appears to be stabilized by an intricate hydrogen-bonded network of water surrounding the molecules[28].

Within the mean-field theory of hydration forces the expected decay length of the repulsive force, λ, is dependent on both surface structure and water properties[10]:

$$\lambda = \frac{1}{2\ ((1/\lambda_w)^2 + (2\pi/a)^2)^{1/2}} \qquad (1)$$

where λ_w=4–5 Å is a correlation length of the perturbation created by polar groups in water[10]. For collagen, we take a=9.6 Å, the triple helical pitch[29], as the surface periodicity of the perturbation. The agreement between theoretical and measured forces is excellent (Figs 2, 3), but we must note that the calculated decay length is determined almost exclusively by the periodicity of the triple helix and is only weakly sensitive to the value of λ_w so that other interpretations are not excluded. In particular, electrostatic, double–layer repulsion between net neutral surfaces with periodically alternating positive and negative charges has the same exponential form and a similar decay length but with the Debye length replacing λ_w (ref. 10). Collagen is indeed practically net-neutral but charged amino acids are not distributed with the 9 to 10 Å period required to fit the observed decay length. In contrast, it is only necessary that residues along the triple helix be hydrated to apply the 9.6 Å periodicity to the hydration force decay length. Indeed, almost thirty years ago it had been postulated that hydration of the polar peptide backbone results in stable, helical spines of water running parallel with the triple helix[30].

Further experimental and theoretical arguments in favour or against different models of the observed repulsive interaction will be given elsewhere. Here we only suggest the hydration force as one of the possibilities and concentrate on the properties of the attractive interaction responsible for fiber assembly.

Attractive interaction

We estimate the attractive forces by subtracting the exponential repulsion from the net force (Fig. 4). While the accuracy of this subtraction may not be high, it does give a good qualitative estimate of the attraction. In particular, the strong temperature dependence of the at-

tractive force is much larger than experimental error.

The maximum energy of the attraction between pairs of 3,000 Å long collagen triple helices is estimated by integrating attractive force curves as ~ 10 kT, the energy of one or two hydrogen bonds. Many attractive and repulsive interactions can contribute to give this magnitude of net attraction in the 16–20 Å interhelical spacing range. These include: 'hydrophilic' interactions such as coulombic interactions between charged side chains, or image-charge repulsion of the charged side chains from protein backbone, or direct and water-mediated hydrogen bonding; non-specific interactions such as van der Waals' attraction and 'steric repulsion' due to motional entropy loss; and hydrophobic interactions between non-polar residues. Can we single out the force (or forces) responsible for the specificity and strong temperature-sensitivity of collagen self-assembly?

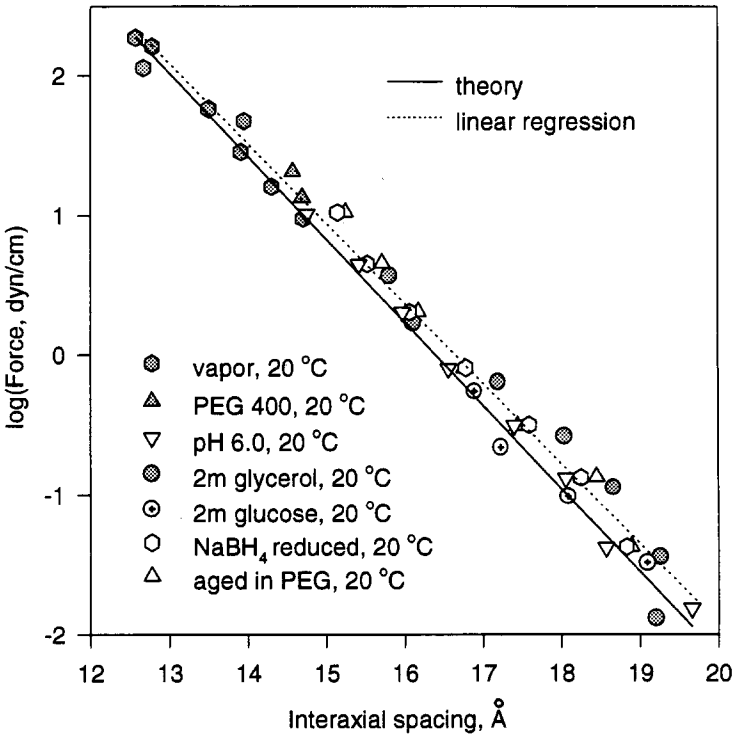

Fig. 2 Repulsive force between collagen triple helices. Several different conditions 'turn off' attraction between the helices resulting in a purely exponential repulsive force. This happens when glycerol or glucose are added to the bathing solution to 2 molal concentration. The same force is seen at pH 6.0 without any extra solutes, with samples reassembled from collagen reduced by sodium borohydride, and with samples stored in 40 % PEG 8000 solution for over a year. The solid line is plotted with the decay length theoretically predicted for hydration repulsion by Eq. 1 with the constraint that the line goes through the point measured at the smallest separation.

The substantial weakening of attraction by lowering the pH from 7.5 to 6.0 is most likely associated with the titration of specific hydrophilic residues. We argue that these hydrophilic residues directly contribute to the observed attraction. If, by fortuitous compensation, protonation created some extra repulsion it must have exactly the same dependence on the separation and temperature as the attraction so that the net force appears purely repulsive. To test such an attraction compensation, we measure the force at pH 6.0 with added 2 m glycerol (Fig. 3). At all temperatures, this is the same limiting force as is observed for collagen either with added 2 m glycerol at pH 7.5 or without glycerol at pH 6.0. Any extra repulsion due to additive glycerol and pH effects, expected for attraction compensation, would have been seen in force curves at low applied stress. Glycerol is acting independently of pH and is not simply shifting the pK of some amino acid. The forces in glycerol do not depend on pH over the extended range from 6 to 8.5. No significant change in the pK of the side chains of free glutamic acid, aspartic acid, histidine, and hydroxylysine is observed in 2 m glycerol.

The very specific effects of several solutes are also consistent with a hydrophilic mechanism of attraction. At a 2 molal concentration, ethanol, methanol and ethylene glycol have no significant effect on forces (Fig. 5), glycerol and glucose cancel the attraction (Fig. 2) while sorbitol pushes the helices closer together (Fig. 5). Sorbitol appears to be partly excluded from the spaces between the helices and may be applying an extra osmotic stress compressing the fibers, in much the same way as the previously reported effect of salt[23].

No correlation with the effect of these solutes on hydrophobic, van der Waals', or electrostatic interactions is evident. For example, it has been argued that glycerol and glucose strengthen the attraction between hydrophobic surfaces[31–33] while methanol and ethanol weaken it[31–34]; the effect of ethylene glycol and glycerol on van der Waals' attractions would be similar, while here it is vastly different; lowering of the dielectric constant with the addition of glycerol or glucose would make electrostatic attraction stronger rather than remove them. The sensitivity of the interaction to specific carbohydrates suggests that attraction could be mediated by a hydrogen-bonding network of the type recently observed by high-resolution X-ray diffraction[28].

Although the temperature sensitivity of the attraction is qualitatively consistent with both the hydrophobic effect and van der Waals' attraction, the magnitude of the sensitivity argues for a more specific hydrophilic mechanism. From 5 °C to 35 °C, free energies of transfer for hydrophobic compounds to water from oil change by only about 10–20 % (ref. 7), the expected[35] change in van der Waals' force is at most about 10%, while the attractive force between the collagen molecules changes by a factor of two (Fig. 4). In particular, the free energy of the attraction, G, estimated from the integrated observed attractive force, changes approximately linearly with temperature. The temperature derivative of G gives

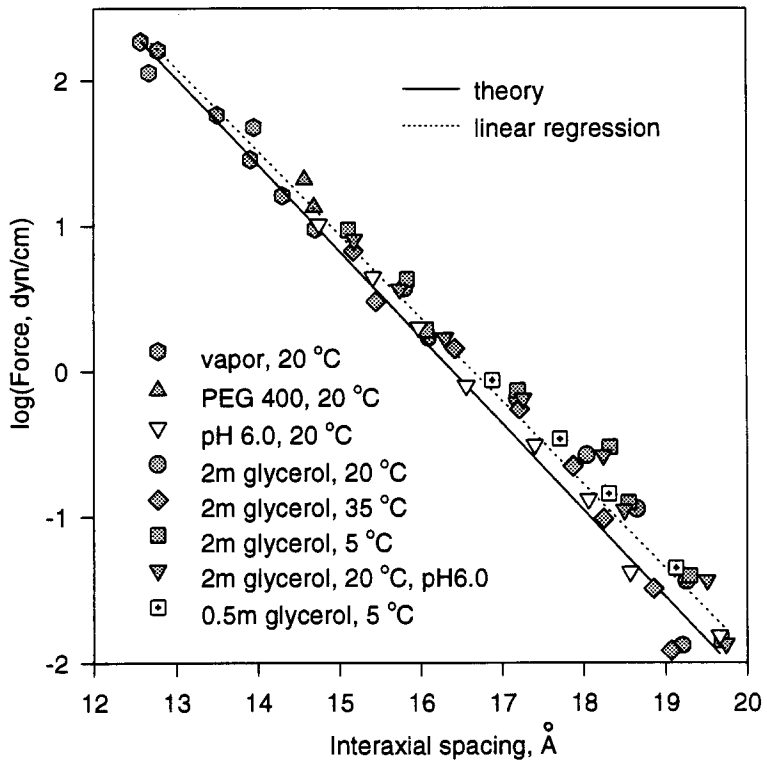

Fig. 3 The forces observed at different glycerol concentrations, temperatures and pH. Glycerol, low pH and low temperature affect the net force by reducing attractive interaction between the helices. Once the attractive force between the collagen helices is removed by one of the treatments illustrated in Fig. 2, the remaining exponential repulsion is negligibly dependent on further changes in temperature, glycerol concentration or pH.

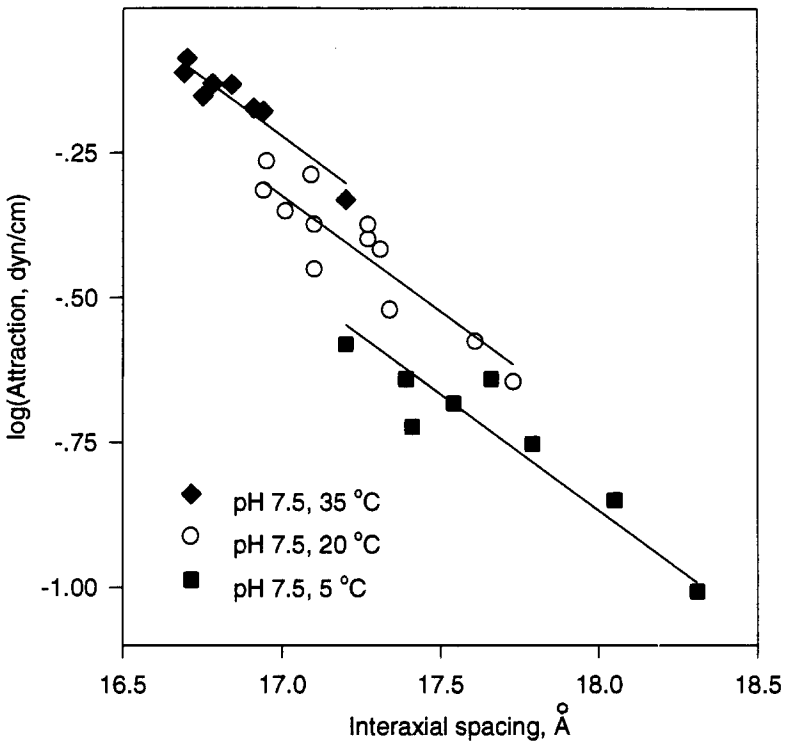

Fig. 4 Temperature-dependent attraction between collagen triple helices obtained by subtraction of the repulsive force shown in Figs 2, 3 from an expanded set of data for the net forces at different temperatures as shown in Fig. 1. Solid lines represent the fit where the force is exponential, the pre-exponential factor depends on the temperature while the decay length is temperature insensitive. This attractive force is canceled by changes in pH, chemical modification of the molecules and by addition of glycerol and glucose (Figs 2, 3), but not by methanol, ethanol, ethylene glycol or sorbitol (Fig. 5). This specificity suggests that the attraction is mediated by hydrogen bonding, probably forming water bridges that connect the molecules, rather than by hydrophobic, van der Waals', or electrostatic interactions.

the entropy, S, of the observed attraction. This calculation gives $-TS/G \sim 10$ at room temperature, that is the entropic contribution to the free energy of the attraction is practically equal to the enthalpy, $H = G + TS$. Such strong compensation has been seen in the assembly of hydrophilic DNA molecules in the presence of Mn^{2+} (ref. 36). It is not consistent with a hydrophobic basis for attraction since typically the enthalpy of hydrophobic hydration at room temperature is small compared to the entropy[7].

An attraction between complementary hydrophilic surfaces that increases with temperature can arise from at least two sources. First, temperature favours the release of structured water from around hydrophilic groups to the bulk solution[10], an effect similar to that usually discussed for hydrophobic associations. A second possibility is that the protein side chain positions in complementary regions are not perfectly aligned, but must adjust to the most favourable attractive configuration. Warming can make such movement easier by releasing the side chains from their preferred positions[37]. This effect can lead to a very strong temperature dependence particularly close to the denaturation temperature of the protein. We note that collagen triple helices in dilute solution melt at about 40 °C.

Molecular origin of the attraction

The titration of the attractive force between pH 6 and 7.5 naturally suggests the involvement of histidine residues. At neutral pH histidine is neutral and forms a hydrogen bond as a proton acceptor. When protonated, histidine becomes a hydrogen-bond donor. If the interhelical attraction is mediated by a hydrogen-bonding network that includes a histidine, then a pH titration will disrupt this attraction. A hydrogen-bonded bridging-network could also be susceptible to competition from small hydroxylic compounds. The strength of competition would then depend on the specific conformation of the network and the size and geometry of the solute, for example, the ability of glycerol and glucose to disrupt attraction and the inability of methanol, ethylene glycol and sorbitol. Although the chemi-

cal basis for the removal of attraction by strong borohydride reduction and collagen aging is unclear, histidine side chains are susceptible to modification by these treatments.

Of the approximately 3,000 amino acids forming the collagen triple helix only ~0.5% are histidines. However, as estimated above, even 1–2 hydrogen bonds per interaction (3–6 hydrogen bonds per molecule) would provide enough strength to account for the observed attraction. The two α1 chains contain only two histidines in the triple helical part, both of which are in regions considered important recognition and crosslinking sites for collagen self-assembly[14]. These two histidine-containing regions have two unusual features. They are the only two sequences containing as many as nine amino acids that are exactly repeated . They are also in the two largest regions of the helical sequence that lack both prolines and hydroxyprolines. The conformation of the triple helix is probably at its most flexible within these segments, sometimes referred to as 'hinge' regions[14]. As mentioned earlier, increased flexibility might be an essential feature of temperature-favoured assembly[37].

The identification of these regions as the focus of the interhelical attraction, although consistent with our data, is highly speculative. Careful investigation of mutated or chemically modified collagen is the logical next step.

At least in this one case where forces responsible for temperature-induced protein self-assembly have been explicitly measured, the hydrophobic paradigm does not appear to operate. No feature characteristic of hydrophobic interactions is apparent in the attraction between collagen triple helices, even though large hydrophobic residues presumably come into direct apposition during assembly. It is possible that this is because the hydrophilic to hydrophobic contacts are energetically very unfavourable rather than because there is some strong, specific, hydrophobic attraction other then the van der Waals' interaction.

In any case, a far more exciting possibility is likely, a vocabulary of forces built on varied and variable polar interactions seems to be required. Taken together with hydrophobic interactions, the strength, specificity and control of interactions that can be expressed through this enlarged vocabulary should allow new ways to think about protein structure and conformation.

Methods

Sample preparation. Reconstituted collagen films (~0.5 mm thick) were prepared as described[23]. Collagen was treated by pepsin (100 mg per g of rat tail tendons in two doses, 24 h at 4 °C each) to remove non-helical terminal peptides responsible for formation of covalent cross-links in native fibers. The film used for most of the samples was air dried and stored at 4 °C . The force-distance curves measured with samples cut from this film were reproducible for over two years. Another film, however, was stored in 40% solution of polyethylene glycol (PEG 8000; United States Biochemical). The force curves measured with this film freshly made and after more than a year of storage were significantly different.

The samples used for force measurements did not contain significant covalent intermolecular cross-links: They readily redissolved at 4 °C in neutral pH buffer (usually 1 m glycerol and 0.1 M sodium phosphate at pH 7.4), and films reduced by sodium borohydride and redissolved in 0.5 M acetic acid showed monomers with SDS gel electrophoresis. In contrast the same sodium borohydride reduction of samples reconstituted from collagen not treated by pepsin resulted in high degree of polymerization, confirmed by SDS gel electrophoresis. Those samples were almost completely insoluble in acetic acid. The effect of covalent cross-links on intermolecular forces will be described in detail in a separate publication.

Collagen used for one of the series of force measurements was dissolved to 0.5 mg ml^{-1} in 1 m glycerol/0.1 M sodium phosphate, pH 7.4/20 mM (ethylenedinitrilo)tetraacetic acid (EDTA) and treated by sodium borohydride (0.1 mg ml^{-1} of solution) for 30 min. The same amount of fresh sodium borohydride was then added for another 30 min. The reaction was stopped by adjusting to pH 4 with acetic acid. The solution was then extensively dialyzed

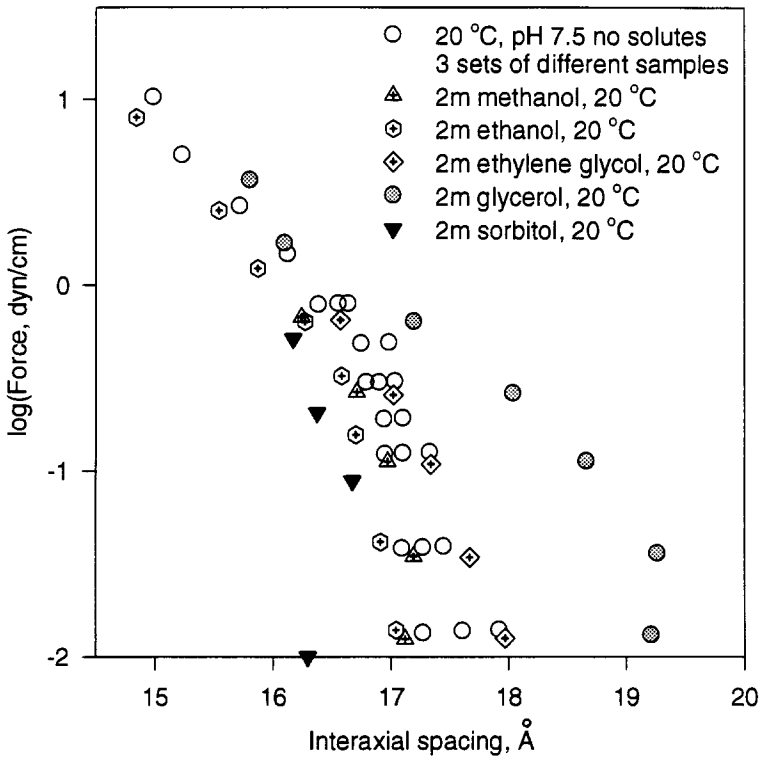

Fig. 5 The effect of methanol, ethanol, ethylene glycol and sorbitol on forces between collagen triple helices. None of these solutes leads to any reduction of attractive forces between the molecules in contrast to the effect of glycerol seen in Figs 3, 4 and shown here as well for comparison. Within the sample-to-sample variation, illustrated here by the results of three different sets of measurements in the absence of solutes, methanol, ethanol and ethylene glycol have no significant effect on the interaction. Sorbitol is probably partially excluded from the space between the triple helices and applies an extra osmotic stress on the helices, an effect similar to the one observed for salt[23]. The sharp, vertical drop of the force curve, observed here at approximately 16.3 Å separation indicates an osmotic effect as discussed in ref. 23.

against 0.5 M acetic acid (pH 2.8) and reassembled into collagen film. The samples cut from this film are referred to as '$NaBH_4$ reduced' collagen.

The films were cut into 2–5 mg samples and equilibrated with solutions of various concentrations of PEG 8000, PEG 400 (Fluka), or dextran 200,000–300,000 M_r (United States Biochemical). In some solutions, different small solutes, including methanol, ethanol, ethylene glycol, glycerol, sorbitol and glucose were added. All solutions contained 2 mM EDTA and 10 mM buffer (Tris chloride or sodium cacodylate depending on the required pH). The final solutions were adjusted to the required pH by small amount of NaOH or HCl.

Force measurement. The force-distance curves were measured by the osmotic-stress technique as described[9,10,12,23]. The effective osmotic stress exerted by PEG in the presence of other solutes was corrected for the volume occupied by those solutes assuming that they did not interact with PEG. The required correction was small enough so that possible errors due to simplifying assumptions were negligible. The force per unit length of collagen, f, was calculated from the applied osmotic stress, Π, assuming pairwise short-range interaction between the molecules[12],

$$f = \frac{\Pi d_{int}}{3^{1/2}} \qquad (2)$$

where the interaxial spacing, d_{int}, was measured by X-ray diffraction as described previously[23].

Forces above 10 dyn cm^{-1} were measured by equilibrating samples in sealed chambers in vapours of saturated solutions of LiCl (relative humidity, RH 15%), KCH_3COO (RH 20%), $CaCl_2$ (RH 32%), NaBr (RH 58%), $Mg(CH_3COO)_2$ (RH 65%), NaCl (RH 77%), KCl (RH 86%), $Na_2C_4H_4O_6$ (RH 92%) so that the sample could exchange water with the salt solution only through the vapour. Relative humidities of these salt solutions at 20 °C were taken from ref. 38. The osmotic stress corresponding to each relative humidity was calculated as described in ref. 9.

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