Solvent Hydrogen-Bond Network in Protein Self-Assembly: Solvation of Collagen Triple Helices in Nonaqueous Solvents

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ABSTRACT Forces between type I collagen triple helices are studied in solvents of varying hydrogen-bonding ability. The swelling of collagen fibers in reconstituted films is controlled by the concentration of soluble polymers that are excluded from the fibers and that compete osmotically with collagen for available solvent. The interaxial spacing between the triple helices as a function of the polymer concentration is measured by x-ray diffraction. Exponential-like changes in the spacing with increasing osmotic stress, qualitatively similar to the forces previously found in aqueous solution, are also seen in formamide and ethylene glycol. These are solvents that, like water, are capable of forming three-dimensional hydrogen-bond networks. In solvents that either cannot form a network or have a greatly impaired ability to form a hydrogen-bonded network, strikingly different behavior is observed. A hard-wall repulsion is seen with collagen solvated by ethanol, 2-propanol, and *N,N*-dimethylformamide. The spacing between helices hardly changes with increasing polymer concentration until the stress exceeds some threshold where removal of the solvent becomes energetically favorable. No solvation of collagen is observed in dimethoxyethane. In solvents with an intermediate ability to form hydrogen-bonded networks, methanol, 2-methoxyethanol, or *N*-methylformamide, the change in spacing with polymer concentration is intermediate between exponential-like and hard-wall. These results provide direct evidence that the exponential repulsion observed between collagen helices at 0–8-Å surface separations in water is due to the energetic cost associated with perturbing the hydrogen-bonded network of solvent molecules between the collagen surfaces.

INTRODUCTION

It is widely recognized that water plays an important role in protein structure, interaction, and function (Rupley and Careri, 1991; Westhof, 1993). Significant progress has been made in structural (Karplus and Faerman, 1994) and thermodynamic (Makhatadze and Privalov, 1993; Privalov and Makhatadze, 1993) studies of protein solvation. Still, our understanding of these phenomena is far from complete. In particular, it is well known that hydrogen-bond networks play a critical role in the properties of bulk water (Bellissent-Funel and Dore, 1994; Stillinger, 1980), but it is much less clear how much the reorganization of this network in contact with protein surfaces affects protein interactions, structure, and stability. In this work we examine the role of water hydrogen-bond networks in protein-protein interactions.

Forces between macromolecular surfaces in water have been measured for several years now by several experimental techniques (for a recent review see (Parsegian and Evans, 1996)). Unexpectedly strong exponential forces have been observed over the last 10 to 20 Å of separation between many biological macromolecules. It has been suggested that these forces result from the energetic cost of reorganizing the hydrogen-bonding network of water near macromolecular surfaces. These measured forces are indeed often referred to as "hydration" forces, reflecting the fact that they are associated with removal of the last few water layers. A direct link between the measured forces and water structuring, however, has not been established. Other interpretations of the data have been proposed. They include direct and image-charge electrostatic interactions, nonlocal electrostatic effects, osmotic pressure of trapped counterions, and entropic repulsion due to steric restriction of various modes of molecular motions. Considering the difficulty in separating various energetic contributions, it is not surprising that different authors still favor different theories. [For discussion of various measurements and theories see recent reviews (Israelachvili and Wennerstrom, 1996; Leikin et al., 1993; McIntosh and Simon, 1994) which present the subject from different points of view.]

Previous measurements of force versus separation between proteins (type I collagen triple helices) in water have shown that the net force results from a balance between a short-range exponential repulsion and a longer-range, temperature-dependent attraction, both qualitatively similar to hydration forces measured between other macromolecules (Leikin et al., 1994, 1995a). Comparison of the results with different theoretical predictions has indicated that both interactions are consistent with the estimated energetic cost of reorganizing the interstitial hydrogen-bond network of water (Leikin et al., 1994, 1995a). This conjecture is also consistent with the recent high-resolution crystal structure of a collagen-like peptide that shows highly structured, hydrogen-bonded clusters of water surrounding the triple helices. In particular, the interhelical water bridges, spanning peptide groups on apposing helices, may be responsi-

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ble for maintaining finite spacing between the peptide molecules (Bella et al., 1994, 1995). Still, without direct evidence linking water structuring and interaction, the physical basis of the measured forces remains controversial.

One obvious way to assess the importance of a hydrogen-bonded network for the observed interaction is by comparing forces between collagen helices in water and in various nonaqueous solvents with different hydrogen bonding capabilities. This approach has been previously used to elucidate the role of water in forces between lipid bilayers. Exponential forces very similar to those measured in water were seen in studies of lipid bilayers solvated by ethylene glycol (Persson and Bergenstahl, 1985), 1,3-propane diol, and formamide (McIntosh et al., 1989). Except for one common feature—the ability to form hydrogen-bond networks—these solvents and water have very different physical and chemical properties. Correlation of the measured force decay length (McIntosh et al., 1989) with molecular size of the solvent also suggested that the solvent plays a direct role in mediating the interaction. Nevertheless, very different interpretations of these results are still possible (Israelachvili, 1991; Leikin et al., 1993; McIntosh et al., 1989; McIntosh and Simon, 1994).

Reconstituted fibers of collagen surprisingly solvate and apparently retain their triple helical structure in many nonaqueous solvents, not only those capable of forming hydrogen bond networks (ethylene glycol, formamide), but also in solvents with greatly impaired hydrogen bonding ability (*N,N*-dimethylformamide, dimethoxyethane, ethanol and 2-propanol), as well as in solvents with intermediate hydrogen bonding strength (methanol, methoxyethanol, and *N*-methylformamide). Qualitatively very different behaviors of the interhelical forces are observed in these solvents. From an analysis of similarities and differences in the observed osmotic sensitivity of collagen swelling within and between these groups of solvents, we now provide further evidence that the exponential repulsive force between collagen molecules in water is directly related to the energetic cost of reorganizing the interstitial water-hydrogen bond network.

MATERIALS AND METHODS

Sample preparation

Type I collagen was extracted from rat tail tendons as described previously (Leikin et al., 1994, 1995a). Solubilized collagen was extensively digested with pepsin (≈100 mg/g of tendons in two doses, 24 h at 4°C each) to remove nonhelical terminal peptides responsible for formation of covalent cross-links in native fibers. The protein was purified by three cycles of salt precipitation and acetic acid solubilization. The collagen was reassembled into a highly ordered, transparent film (≈0.5 mm thick and 40 mm in diameter) by ultrafiltration in an Amicon 8050 high-pressure cell. The film was air dried and stored at 4°C. The pepsin treatment was sufficient for preventing spontaneous reformation of intermolecular covalent cross-links in the film. The absence of the cross-links was confirmed by SDS gel electrophoresis after sodium borohydride reduction as described in Leikin et al. (1994, 1995a).

The solvents used were all reagent grade quality and were used without further purification: double-distilled water (buffered by 10 mM TrisCl and 2 mM EDTA, pH 7.5, or by 10 mM sodium cacodylate and 2 mM EDTA, pH 6.0); methanol (Mallinckrodt, Paris, KY); ethanol (Pharmco, Brookfield, CT); 2-propanol and ethylene glycol (J. T. Baker, Phillipsburg, NJ); 2-methoxyethanol, dimethoxyethane, formamide, *N*-methylformamide, and *N,N*-dimethylformamide (all from Fluka, Buchs, Switzerland). Solutions of polyethylene glycol (400 MW, Spectrum, Gardena, CA; and 8,000 MW, United States Biochemical, Cleveland, OH) and polyvinyl pyrrolidone (10,000 MW, Aldrich, Milwaukee, WI) were used to apply osmotic stress on the condensed arrays of collagen (MW indicates average molecular weight). Throughout the paper we refer to these polymers as PEG 400, PEG 8,000, and PVP 10,000.

Polymer solutions of different concentrations were prepared gravimetrically, using a Mettler AT261 electronic balance (Mettler-Toledo, Greifensee, Switzerland). Polymer concentrations were further determined from the refractive indices of polymer solutions using an Abbe refractometer. A linear increase in the refractive index with volume fraction of the polymer was observed in all preparations and solvents. No phase separation of polymer solutions was detected.

Equilibration

Small, dry (Bragg spacing ≤ 11.5 Å, surface separation ≤ 1 Å) samples (~1 × 1 mm) cut from reconstituted collagen films were most frequently solvated directly in solutions of PEG or PVP. The samples were equilibrated in volume excess (~1000 ×) of PEG or PVP solutions at room temperature for at least 1 week, with one change of bathing solution after several days. Equilibration was essentially complete after 1–2 days, as judged from the stability of measured interhelical spacings.

Some dry samples were presolvated in a 60 wt % water:30% solvent: 10% PEG 400 mixture for 1–2 days, then transferred into a 90% solvent: 10% PEG 400 mixture, and only then into the final solution. The 10% PEG 400 was used as a precaution to prevent overswelling of the fibers. This second procedure was required for 2-propanol and dimethylformamide. For these solvents, direct equilibration against solvent/PEG solutions did not result in the absorption of solvent by the dry collagen film, as judged by the interaxial spacing. The indirect solvation procedure, replacing water by solvent, is presumably effective by being able to reduce the energy barrier to introduce the first solvent molecules into the condensed collagen array.

To reduce absorption of water from air we have used freshly opened solvents and minimized exposure of the solvents and PEG solutions to air. All equilibrations were performed in tightly sealed 1.5-ml microtubes fitted with o-rings. Water content in all nonaqueous solvents, except methanol, and in PEG solutions, was below 1% as estimated from refractive index measurements. The closeness of water and methanol refractive indices precluded an accurate measurement of aqueous content in these solutions. Contamination by <1% water likely does not significantly affect the interpretation of the observed qualitative difference in collagen swelling in the different nonaqueous solvents.

We have confirmed by Raman spectroscopy that the exchangeable water initially hydrating these samples was replaced by solvent in the subsequent equilibrations. Specifically, using fully deuterated dimethylformamide (-d_7), we have estimated the relative amounts of water and dimethylformamide in the sample as ≤0.1 g/g of collagen and ~1 g/g of collagen, respectively, from the ratio of the integrated intensities of the stretching C–H (collagen), O–H (water), and C–D (dimethylformamide) vibrational modes. The residual O–H part of the spectrum observed in this sample is equivalent to collagen hydrated in a vapor of ~10% relative humidity under which condition it has an interaxial Bragg spacing of ~10.5 Å. We presume this tightly bound and apparently nonexchangeable water is essential for the structure and stability of collagen triple helices (Fraser et al., 1979) and can be removed only by extensive high-vacuum drying. We consider it an intrinsic part of the collagen structure. Because the x-ray spacing does not change after its removal (Nomura et al., 1977), we use the definition of zero separation between collagen molecules that corresponds to 10.5 Å equatorial Bragg spacing and includes this water. No water was detected in the Raman spectrum of the final equilibrating solution. Details of the Raman measurements will be reported elsewhere.

X-ray diffraction measurements

After equilibration, samples were sealed in custom designed x-ray cells so that they remained in contact with a small amount of the equilibrating solution. Occasionally, refractive indices of bathing solutions in the cells were determined after x-ray measurements to ensure that samples were sealed sufficiently tightly to prevent solvent loss. The cells were placed into temperature-controlled holders (maintained at 20 ± 0.1°C) mounted in two point-focus x-ray cameras with 0.01 $Å^{-1}$ resolution in reciprocal space. This resolution was sufficient for measurement of interaxial spacings in the range from 12 to 20 Å with corresponding accuracy from 0.2 to 0.5 Å.

Descriptions of the x-ray cells, temperature controllers, and x-ray cameras were given in Mudd et al. (1987). The only modification from previous work was that large-diameter acrylic tubes, sealed with Mylar windows and filled with helium (a continuous, low-pressure flow), were placed in the beam path between the samples and x-ray films to reduce background scattering of x-rays.

The x-ray source was an Enraf-Nonius FR590 (AL Delft, the Netherlands) generator equipped with a fine-focus, fixed copper anode x-ray tube. Diffraction patterns from collagen arrays were recorded by direct exposure of Kodak DEF5 x-ray film. The x-ray film images were digitized using a Panasonic WV-BD400 video camera connected to Macintosh II fx through Video Image 1200 board (Scion Corp., Frederick, MD) and stored as 8-bit TIFF files (256 gray scale levels). The images were captured and analyzed using National Institutes of Health Image software customized for analysis of x-ray diffraction patterns.

The average separation between surfaces of collagen triple helices, d, was estimated from the measured Bragg spacing, d_{Bragg}, as

$$d = \frac{2}{\sqrt{3}}(d_{Bragg} - d^0_{Bragg}), \quad (1)$$

where $d^0_{Bragg} \approx 10.5$ Å is the Bragg spacing of completely dehydrated samples. Simple hexagonal packing of the molecules is assumed. The x-ray patterns shown in Fig. 1 indicate the significant amount of disorder in packing of collagen molecules that is characteristic of reconstituted (Eikenberry and Brodsky, 1980) and, to a smaller degree, of native (Fraser et al., 1987) collagen fibers. Although different models for the packing geometry of collagen have been proposed (Hulmes et al., 1995), no precise structure (especially in reconstituted fibers) is as yet known. Inasmuch as we are interested only in the qualitative behavior of the swelling of collagen films as dependent on polymer concentrations in different solvents, we will neglect this complication and still use the average surface separation defined by Eq. 1 as a meaningful parameter rather than the directly measured average Bragg spacing.

Sample stability

The collagen samples were surprisingly stable in all polymer and solvent solutions except for low concentrations of PEG or PVP in formamide. At PEG 400 concentrations below 20 wt %, the samples in formamide slowly dissolved and the collagen denatured. Denaturation of collagen in formamide solutions was confirmed by the loss of optical signal in electric birefringence measurements.

Prolonged (more than a month) exposure of samples to nonaqueous solvent/PEG 400 mixtures resulted in development of covalent cross-links (the samples became insoluble in 0.5 M acetic acid at pH 2.8) and concomitant discoloration. The cross-linking, however, did not affect x-ray spacings. Collagen samples cross-linked in nonaqueous PEG solutions (insoluble in acetic acid) had the same spacings after reequilibration in aqueous PEG or PVP solutions (pH 6.0) as freshly made, aqueous samples that had no cross-links. We suspect that the cross-linking occurred mainly on sample surfaces as a result of PEG degradation and was due to reaction of collagen with oxidation products of PEG 400 ether linkages. No discoloration or cross-linking was observed in samples that were not exposed to PEG directly but were equilibrated in the vapor of 50% PEG solutions.

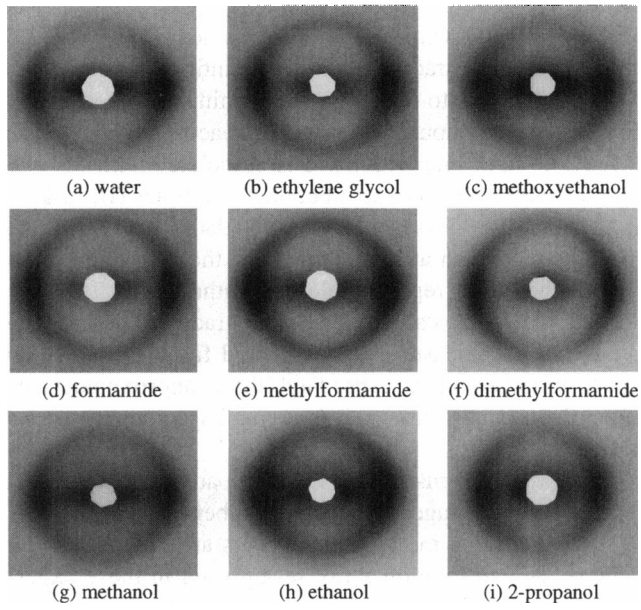

FIGURE 1 X-ray diffraction patterns for reconstituted collagen films equilibrated in PEG 400 solutions of water (8% PEG, d_{Br} = 14.7 Å), ethylene glycol (41% PEG, d_{Br} = 14.5 Å), 2-methoxyethanol (50% PEG, d_{Br} = 15.6 Å), formamide (20% PEG, d_{Br} = 13.8 Å), methylformamide (20% PEG, d_{Br} = 14.1 Å), dimethylformamide (35% PEG, d_{Br} = 14.1 Å), methanol (20% PEG, d_{Br} = 14.7 Å), ethanol (56% PEG, d_{Br} = 15.7 Å), and 2-propanol (5% PEG, d_{Br} = 16.2 Å). All patterns are similar except for Bragg spacings. No meridional patterns are observed with the 0.01 $Å^{-1}$ resolution x-ray cameras used in these experiments.

Wherever possible we tried to minimize this problem by exposing the collagen samples to PEG solutions for as little time as possible (consistent with equilibration) in those solvents that discolored fastest.

Vapor phase solvation measurements

Several control samples were equilibrated at room temperature against a vapor of 50% (w/w) PEG 400 solutions in methanol, ethanol, 2-propanol, *N*-methylformamide, *N,N*-dimethylformamide, and 2-methoxyethanol. The samples were first equilibrated against the pure solvents before the vapor equilibration. The collagen films were then mounted in quartz x-ray capillary tubes that were joined to and sealed with specially modified 1.5-ml microtubes containing the PEG solutions. This set-up insured there was no direct contact between the collagen samples and the PEG solutions; the solvent exchanged only through the vapor phase. The tubes were directly mounted into the x-ray cameras. X-ray spacings were measured after 12 days and again after a month of the equilibration.

RESULTS

X-ray diffraction patterns

Fig. 1 compares x-ray diffraction patterns obtained in the solvent/PEG 400 mixtures. The dark equatorial spots indicate a preferred orientation of the triple helices parallel to the face of the reconstituted collagen film (the samples were mounted with this face parallel to the x-ray beam). With sample faces oriented perpendicular to the beam, homogeneous rings, characteristic of a powder pattern with no preferred orientation, are observed. The relative angular

spread and intensity of the oriented, equatorial scattering peaks compared with the residual homogeneous ring depends on the accuracy of sample mounting and on sample deformations due to handling. Broadening of the equatorial peaks, more pronounced at larger spacings, significantly exceeds system line width and is associated with disorder characteristic of reconstituted collagen samples (Brodsky et al., 1988; Eikenberry and Brodsky, 1980). Average Bragg spacings measured at the positions of the maximum intensity are, however, reproducible within the 0.01 $Å^{-1}$ resolution of the x-ray cameras. The accuracy of the average Bragg spacing measurement estimated from the resolution and from the empirical reproducibility is approximately the same, decreasing from ≈0.2 Å at small spacings to ≈0.5 Å at largest spacings.

The x-ray patterns shown in Fig. 1 are characteristic of reconstituted collagen fibers (Eikenberry and Brodsky, 1980), except that meridional spacings are not visible with the 0.01 $Å^{-1}$ resolution x-ray set-up used in these experiments (because sequential orders of the 67 nm spacing, separated in the reciprocal space by $2\pi/670 \approx 0.01$ $Å^{-1}$, cannot be resolved). High-resolution, low-angle x-ray data for the same reconstituted samples solvated in water do contain multiple orders of the 67 nm meridional spacing from the staggered overlap of collagen helices in the assembly, in addition to the equatorial pattern. [For detailed discussion of typical fiber diffraction patterns from collagen see, e.g., Brodsky et al., (1988); Eikenberry and Brodsky, (1980).]

With only one exception, no qualitatively significant difference in the equatorial scattering was observed among the solvents shown in Fig. 1. The value of the Bragg spacing, of course, depended both on the solvent and on the polymer concentration, but always remained in the range from 10.5 to 19 Å. Only in collagen samples directly equilibrated with 60–80% (w/w) solutions of PEG 400 in ethanol was the observed equatorial x-ray scattering qualitatively different. The complex pattern was consistent with an overlap of two poorly resolved diffraction maxima. The outer maximum corresponded to a ≈10.5 Å Bragg spacing, characteristic of completely dehydrated collagen. A presolvation procedure (first in pure ethanol, and only then in ethanol/PEG 400 mixture) eliminated the outer diffraction peak resulting in the normal equatorial diffraction pattern shown in Fig. 1. The dry phase apparently solvates only very slowly in ethanol/PEG solutions; this is due either to a high energy barrier for initial solvation or to local packing defects in the film that stabilize this metastable phase.

Force curves and collagen swelling in water

Forces between macromolecules have been measured by combining x-ray diffraction with polymer osmotic stress or vapor pressure. An ordered condensed array of macromolecules is equilibrated against a solution (or vapor) containing a polymer too large to enter the macromolecular phase. The osmotic pressure of the polymer in solution (or vapor relative humidity) exerts a force on the condensed phase. The separation between macromolecules as a function of the applied polymer stress can be measured by x-ray scattering. By using this osmotic stress technique, we have previously measured the forces between collagen triple helices in water (Leikin et al., 1995a). At pH 6.0 the interaction is only repulsive, while at pH 7.5 an attractive contribution is seen. The repulsive force increases exponentially from ~0.01 to >100 dyn/(cm of molecular length) as the surface separation decreases from ~8 Å to molecular contact. Part of this force-versus-distance curve is shown in Fig. 2 as log(Π) versus surface separation between the triple helices, where Π is osmotic pressure of the bathing PEG solution. The osmotic pressure, Π, is linearly related to the intermolecular force per unit length, see, e.g., Rau et al. (1984) [for more detailed explanation of the osmotic stress method of force measurement see also Parsegian et al. (1986)].

In this work we mainly use PEG 400 as the stressing polymer rather than the larger polymers used previously, as this smaller PEG is miscible in all proportions with the solvents investigated here. The larger PEGs are much less soluble. The equivalent sphere diameter for PEG 400 in water is ~11 Å (Kuga, 1981). This polymer is large enough to be excluded from the tight spaces between collagen

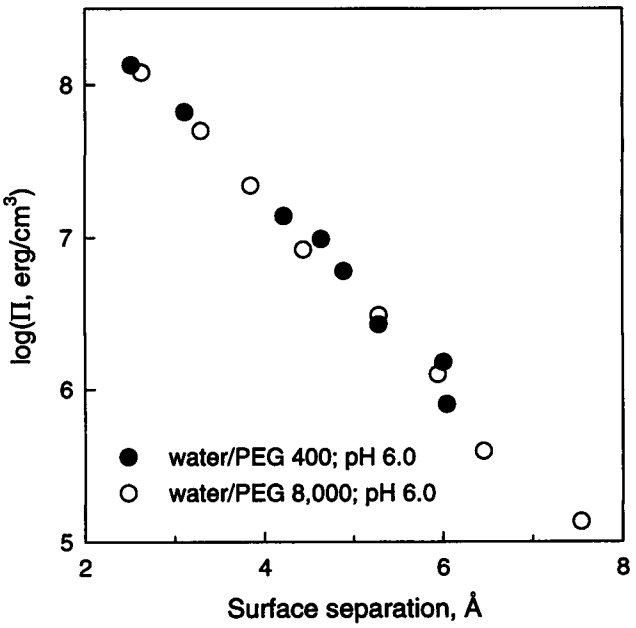

FIGURE 2 Osmotic stress (Π) versus surface separation curves measured with PEG 8000 and PEG 400 at pH 6. The data are plotted assuming complete exclusion of both polymers from collagen fibers. Good agreement between the curves confirms this assumption. In this osmotic stress experiment, the equilibrium separation is determined by a balance of an intermolecular repulsion pushing the helices apart and osmotic action of the excluded polymer pushing the helices together. The osmotic pressure versus separation plots shown here correspond to the lower pressure part of a force-versus-distance curve we have presented in Leikin et al. (1995a). For more detailed discussion of this force measurement technique and a conversion factor between the force and the osmotic pressure see, e.g., Parsegian et al. (1986) or Rau et al. (1984).

helices in the condensed array (which in our measurements never exceed 9–10 Å). It is expected to act simply osmotically, as is confirmed in Fig. 2 showing the close correspondence of log(Π) versus surface separation curves for PEG 400 and 8,000 in water. The equivalent sphere diameter of PEG 8,000 is ~55 Å (Kuga, 1981). We have shown previously that the effect of this much larger polymer on collagen spacings is purely osmotic. In water, PEG 400 then also acts purely osmotically. Additional control experiments confirming the osmotic action of PEG 400 in nonaqueous solvents are described later in this paper.

Even when polymer osmotic pressures are not known (as in many nonaqueous solvents), the data can still be usefully and productively represented as the qualitative "swelling" behavior of collagen by plotting wt % PEG 400 in the bathing solution (g PEG/100 g total), rather than Π, versus surface separation between the triple helices as shown in Fig. 3. At low PEG concentrations, the exponential behavior of the force curve in water is qualitatively reproduced. In fact, the deviation from the approximate linear relationship between the osmotic pressure and wt % PEG 400, expected with increasing PEG concentration, does not significantly affect the qualitative shape of the swelling curve until the concentration exceeds ~60-80%. We will use this exponential-like shape as characteristic of a water-like, soft potential for swelling of collagen in mixtures of PEG 400 with nonaqueous solvents.

Except at low polymer concentrations, the force and swelling curves shown in Figs. 2 and 3 are insensitive to solution conditions. Only when the PEG 400 concentration is lowered below 1 wt % ($\Pi < 10^6$ dyn/cm^2 or 1 atm) does further swelling of the condensed collagen array in water become dependent on pH, temperature, and the presence of different solutes (Leikin et al., 1994, 1995a).

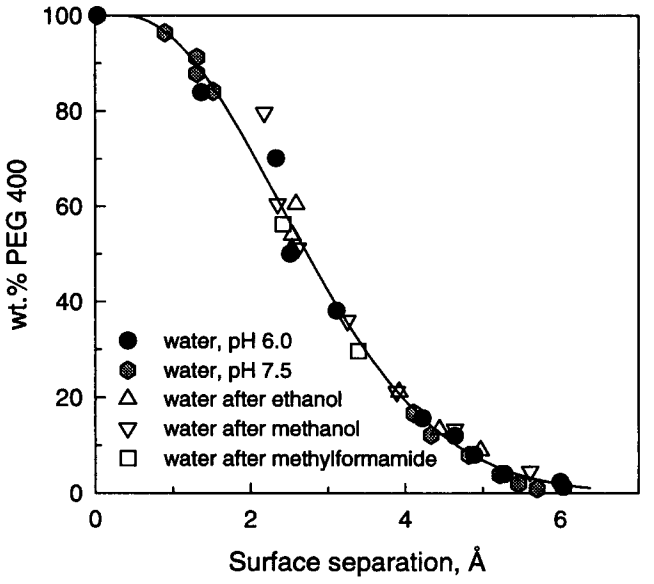

FIGURE 3 Collagen swelling curves in water-PEG 400 solutions (either 10 mM Tris, 2 mM EDTA, pH 7.5 or 10 mM sodium cacodylate, 2 mM EDTA, pH 6.0). Also included are data points for collagen films that were first equilibrated against nonaqueous solvents and only then against water-PEG 400 solutions (pH 7.5). As we have demonstrated previously (Leikin et al., 1995a), the force-distance curves of collagen are different at pH 7.5 and 6.0 due to an attractive interaction, responsible for self-assembly of collagen fibers at pH 7.5, but absent at pH 6.0. This attraction, however, has very little effect on the appearance of the swelling curve in PEG 400. The attractive contribution at pH 7.5 is small compared to the repulsion at PEG 400 concentrations > ~1-2 wt %. The swelling curve has an exponential-like appearance at low PEG concentration due to the exponential force-distance relationship, as shown in the previous figure and explained in the text. Even though the osmotic pressure of PEG 400 is strictly proportional to the concentration only in dilute solutions, the qualitative exponential-like shape is preserved for very high concentrations up to ~60-80 wt % PEG, i.e., as long as the PEG/water molar ratio remains small.

Collagen swelling in nonaqueous solvents

A water-like swelling curve is also observed in ethylene glycol and formamide (Fig. 4). Similar to water, both solvents have both the hydrogen-bond donor and acceptor groups necessary to form a network. The ethylene glycol swelling curve is exponential-like, but is shifted to larger separations between triple helices compared to water. The swelling is limited by some weak, residual attraction which prevents complete dissolution of collagen molecules in the absence of PEG. The x-ray scattering profile without added PEG is, however, too diffuse to determine the spacing accurately.

Within experimental error, the formamide swelling curve >20 wt % PEG 400 coincides with that in water. At lower PEG concentrations the condensed phase in formamide abruptly dissolves and the collagen denatures. At higher PEG concentrations, however, the samples and x-ray scattering patterns are stable for months. Normal Bragg spacings are observed after transfer of these samples back into water-PEG solutions.

Substituting methyl groups for hydroxyl or amide protons of water, ethylene glycol, and formamide will impair the hydrogen bonding ability of these solvents by removing the hydrogen-bond donors as well as by sterically blocking access to the molecule. The structures and basic physical properties of methylated derivatives of ethylene glycol and formamide, as well as several alcohols, that are "methylated derivatives of water," are summarized in Table 1.

The dimethyl derivatives, dimethoxyethane (dimethyl ethylene glycol) and *N,N*-dimethylformamide, have the most drastically affected hydrogen bonding abilities. The complete absence of hydrogen bond donors on either solvent precludes formation of any solvent-solvent hydrogen-bonded network. No collagen swelling is detected in dimethoxyethane, which appears to be completely excluded from the fibers.

N,N-Dimethylformamide solvates collagen, the measured Bragg spacing increases by ~4 Å compared to collagen at very low humidity (Table 2). By Raman spectroscopy we have found (as described in Methods) that in the absence of

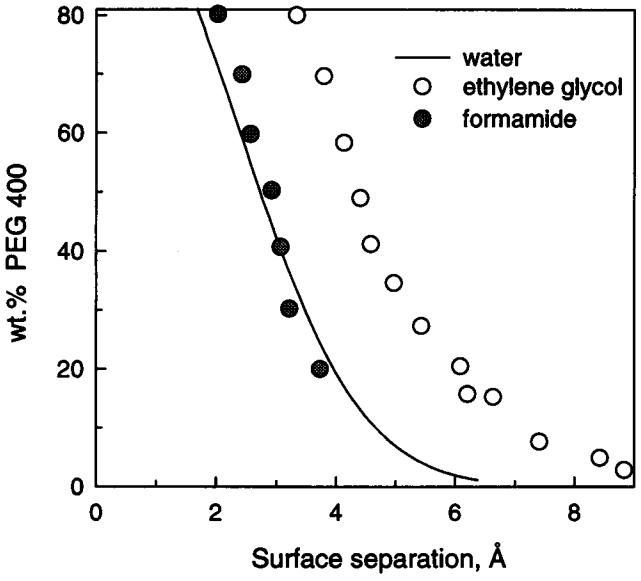

FIGURE 4 Collagen swelling curves in formamide and ethylene glycol. Here and in the following figures we show only the data < 80 wt% PEG 400. In this range of PEG concentrations the PEG/solvent molar ratio is always lower than 1. The swelling curve in water is shown by the solid line as a reference. Note that the qualitative shape of the swelling curves in these solvents is similar to that in water.

PEG the samples contain approximately equal amounts of dimethylformamide and collagen with only a small, residual amount of water. This is the volume fraction of solvent expected at the measured Bragg spacing (~14.4 Å) if the diameter of the collagen helix is the same as in water and the surface separation between the helices is ~4 Å. In other words, assuming that collagen is a solid cylinder and that solvent fills the remaining space in the film, we estimate that the helix diameter does not change by more than ~1 Å between water and dimethylformamide solutions. Furthermore, the amount of water observed in the same Raman experiment (~0.1 g/g protein or ~5% volume fraction) suggests that collagen retains the native aqueous helical parameters by keeping the necessary "structural" waters tightly bound to the helices.

The surface separation between the collagen helices in dimethylformamide practically does not change with increasing osmotic stress. This is seen in the near-vertical swelling curve plotted in Fig. 5 (calculated with the collagen helix diameter taken to be the same as in water). Similar swelling behavior is observed for collagen in both ethanol and 2-propanol as also shown in Fig. 5. The hydroxyl group on each of these alcohols has both a hydrogen bond donor and an acceptor, but the bulky alkyl groups attached to the hydroxyl make difficult the formation of hydrogen bonded clusters larger than dimers. The collagen arrays swell to a limited extent in the absence of PEG 400 and do not respond to the osmotic stress with increasing PEG concentration up to ~50 wt %.

The insensitivity of spacing to PEG 400 concentration is not due to the penetration of PEG 400 between the triple helices in these solvents and a consequent overestimation of the actual difference in polymer concentration between bulk solution and the collagen phase (a reduced osmotic pressure acting on the triple helices). Collagen equilibrated against the vapor phase of 50% PEG 400 solutions in *N,N*-dimethylformamide and 2-propanol shows the same spacing as samples in direct contact with the solutions (data shown in Fig. 5). Additionally, the same behavior is seen with a much larger polymer, PVP 10,000 (Fig. 6). Only in ethanol is a small difference in the spacing observed between direct solution equilibration in PEG 400 and the vapor phase or PVP 10,000 measurements, perhaps indicating only partial exclusion of PEG 400 in this particular solvent (Figs. 5 and 6). However, because the difference is small, the interpretation of the data is not substantively affected.

The loss of osmotic sensitivity in dimethylformamide, ethanol, and 2-propanol indicates a "hard-wall"-like potential between the helices, which is neither an experimental artifact from PEG penetration nor a trivial consequence of a direct, hard-wall molecular contact between the collagen surfaces. The hard-walls are apparently formed by adsorbed solvent molecules.

The swelling curves for solvents with an intermediate ability to form hydrogen-bonded networks are shown in Fig. 7. These monomethylated derivatives of water (methanol), formamide (*N*-methylformamide), and ethylene glycol (2-methoxyethanol) show a swelling behavior that is intermediate between the hard-wall repulsion seen with the solvents that are unable to form hydrogen-bonded networks (Figs. 5 and 6), and the "soft potential," exponential-like force seen with good hydrogen bonding solvents. Collagen swells to a limited extent in the absence of PEG. The intermolecular spacing linearly decreases with increasing PEG concentration, but the change in the spacing is smaller than in the unmethylated parent solvents. Spacings of samples equilibrated against the vapor of 50% PEG 400 solutions in methylformamide and methanol are within experimental error of the spacings for samples that were in direct contact with the solutions, again ensuring that PEG 400 is excluded from the condensed collagen phase in these solvents. In the case of 2-methoxyethanol, as for ethanol, the exclusion of PEG 400 may be only partial as indicated by a larger difference between the vapor and solution equilibration spacings.

DISCUSSION

The swelling curve measurements in the different solvent/PEG 400 mixtures are strictly analogous to our previous osmotic stress force measurements between collagen triple helices in water (Leikin et al., 1994, 1995a). As we have demonstrated, PEG 400 is mostly excluded from the very tight spaces between helices and, therefore, acts osmotically in all the solvents examined. Polymer concentrations, however, are not simply and directly translated into osmotic pressures. Only in dilute solutions does the osmotic pressure

TABLE 1 Properties of pure solvents

Solvent	Chemical formula	MW* (g/mol)	MV* (Å^3)	ε* (25°C)	n* (20°C)
Water	H_2O	18	30	79	1.333
Ethylene glycol	HO—CH_2—CH_2—OH	62	92	37	1.427
2-Methoxyethanol	HO—CH_2—CH_2—O—CH_3	76	131	17	1.402
Dimethoxyethane	CH_3—O—CH_2—CH_2—O—CH_3	90	173	7	1.380
Formamide	O=CH—NH_2	45	66	111	1.445
N-Methylformamide	O=CH—$NHCH_3$	59	97	189	1.432
N,N-Dimethylformamide	O=CH—$N(CH_3)_2$	73	129	38	1.431
Methanol	CH_3—OH	32	67	33	1.331
Ethanol	CH_3—CH_2—OH	46	97	24	1.361
2-Propanol	CH_3—CHOH—CH_3	60	128	18	1.378

Hydrogen-bonding ability: Water, ethylene glycol, and formamide each have at least two donor and two acceptor sites and are capable of forming complex hydrogen-bonded networks (Pimentel and McClellan, 1960). Methanol, ethanol, 2-propanol, 2-methoxyethanol, and *N*-methylformamide have only one donor site and are believed to form linear hydrogen-bonded chains decreasing in average size with increasing alkyl group size (Neuefeind et al., 1996; Pimentel and McClellan, 1960). *N,N*-dimethylformamide and dimethoxyethane do not have donor sites and cannot form solvent-solvent hydrogen bonds. Abbreviations: MW, molecular weight; MV, molecular volume; ε, dielectric constant; n, refractive index.

increase linearly with the weight fraction of PEG 400 so that swelling and force curves are straightforwardly related. In aqueous solutions, however, the qualitative similarity between exponential forces and swelling curves holds up to about 60–80 wt % PEG 400 as illustrated in Figs. 2 and 3. In nonaqueous solvents, unless there are very strong solvent-polymer interactions, the chemical potentials of the solvents in PEG 400 solutions will primarily depend on the polymer size and solution volume occupied. Osmotic pressures of PVP 40,000 MW in water, 1,3-propanediol, and formamide (McIntosh et al., 1989) demonstrate that the effect of solvent identity is second-order compared with polymer weight concentration. Still, the inference of forces from swelling curves in the different solvents must for now be qualitative, as the osmotic pressures of PEG 400 solutions in these solvents have not been directly measured.

Remarkably, collagen seems to retain its triple helical structure in all nonaqueous solvents used for this study, except in formamide at low PEG concentrations. Indeed, major changes in the protein structure, such as loss of the triple helix, are expected to be irreversible and to have a qualitative effect on the integrity of the reconstituted film and on the fiber x-ray diffraction pattern. For example, collagen denaturation in formamide without PEG resulted in complete dissolution of the film. However, in all other cases there was no visible change in the films. The films remained transparent, indicating long-range molecular ordering with minimal defects on the scale of the visible light wavelength. The observed diffraction patterns (Fig. 1) were closely similar to those in water, including a preferred molecular orientation, the range of Bragg spacings (11–19 Å), and the

TABLE 2 Maximum swelling spacings for collagen in nonaqueous solvents without PEG (where measurable)

Solvent	Bragg spacing (Å)	Surface separation (Å) (from Eq. 1)
2-Methoxyethanol	17.3	7.9
Dimethoxyethane	10.5	0
N-Methylformamide	14.4	4.5
N,N-Dimethylformamide	14.2	4.3
Methanol	15.0	5.2
Ethanol	16.6	7.0
2-Propanol	16.5	6.9

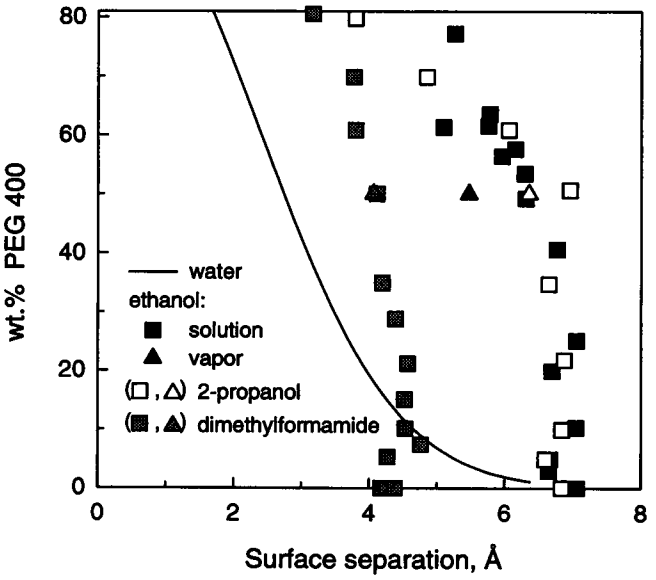

FIGURE 5 Collagen swelling curves in *N,N*-dimethylformamide, ethanol, and 2-propanol (*squares*) and the spacings measured after vapor phase equilibration of the samples with 50 wt % PEG/solvent mixtures (*triangles*). The agreement between the liquid and vapor phase data confirms that PEG 400 is mostly excluded from collagen fibers. Unlike the previous two figures, collagen swelling in these solvents with no or greatly reduced ability to form hydrogen-bonded networks is dominated by a "hard-wall" repulsion. The intermolecular spacing does not respond to osmotic stress until samples are exposed to high PEG 400 concentrations (~50%). The small difference between the vapor and solution spacings in ethanol can be due to a number of reasons, including the effect of the collagen/vapor interface, incomplete equilibration in the vapor phase due to temperature fluctuations (which may play an important role at high relative humidity), etc. It is also possible that this difference is due to partial penetration of PEG 400 into the fibers in the presence of ethanol.

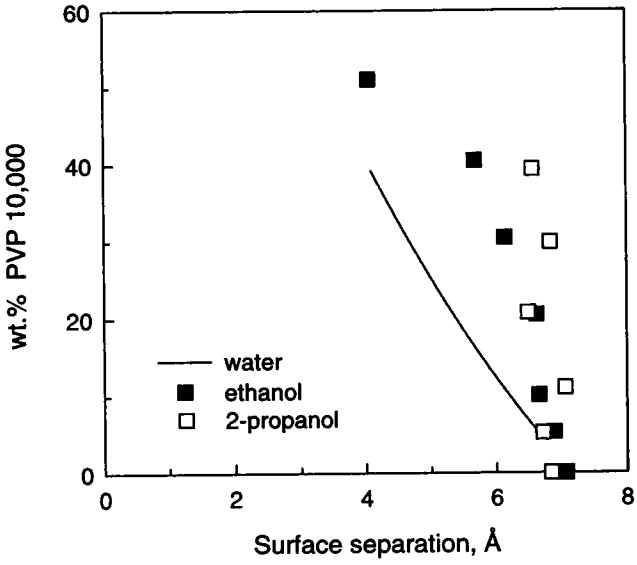

FIGURE 6 Collagen swelling curves in water, ethanol, and 2-propanol solutions of PVP 10,000. These curves are qualitatively similar to those in PEG 400/solvent mixtures. However, the spacing between collagen helices in ethanol decreases with increasing PVP concentration even at low wt % PVP, while it remains constant in PEG 400/ethanol mixtures at least up to 40 wt % PEG. This is consistent with the penetration of a small fraction of PEG 400 inside collagen fibers in the presence of ethanol noted in Fig. 5.

width and characteristic shape of the peaks. The absence of any irreversible changes was confirmed by the reproducibility of the swelling curves in water, measured directly and after exposure to the nonaqueous solvents.

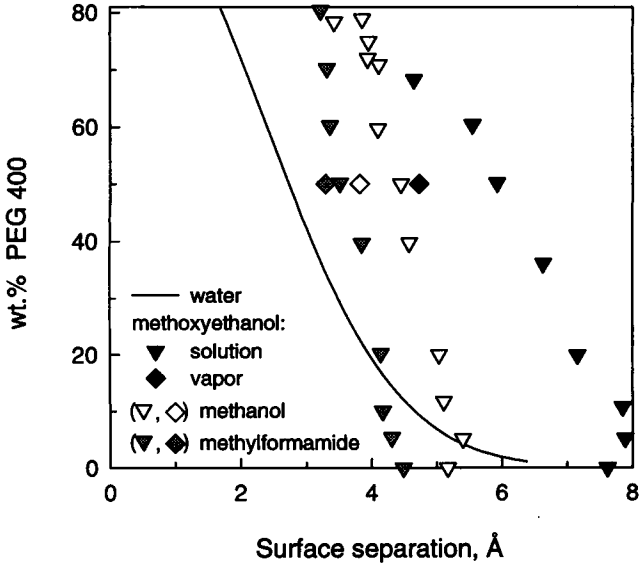

FIGURE 7 Collagen swelling curves in methanol, *N*-methylformamide, and 2-methoxyethanol. Similar to Fig. 5, the diamonds show the spacings after vapor phase equilibration. Swelling curves in these solvents are intermediate between the exponential-like shape of Figs. 3 and 4 and the "hard-wall" repulsion of Fig. 5.

Still, the available information is not sufficient to exclude less drastic nonaqueous solvent-induced structural changes in helical parameters, e.g., changes in helical winding, pitch, diameter, etc. Of course, side-chain conformations may also be solvent-dependent.

In particular, an increase in the helix diameter could change the surface separation resulting in direct, molecular contact between the helices and in an apparent hard-wall repulsion. However, in probably the only case where this could realistically happen (dimethylformamide solvation), we have shown that the helix diameter remains approximately the same as in water.

Although changes in the helical pitch or side-chain conformation are expected to affect interhelical force amplitude, they would not be expected to account for the qualitatively distinct difference in character of the swelling curves seen between hydrogen-bonding solvents (Fig. 4) and non-hydrogen-bonding solvents (Fig. 5).

Regardless of the restrictions and qualifications that must necessarily limit the interpretation of the data, the swelling curves of collagen arrays in the different solvents show two distinctly different limiting behaviors. An exponential-like variation of the swelling curves with spacing is seen for those solvents that can form hydrogen-bonded networks, such as water, ethylene glycol, and formamide (Fig. 4). This soft potential behavior is in marked contrast to the hard-wall repulsion observed with solvents that do not form extensive hydrogen-bonded networks, such as *N*,*N*-dimethylformamide, ethanol, and 2-propanol (Fig. 5).

The dimethylformamide, ethanol, and 2-propanol swelling curves (Fig. 5) seem to indicate that the solvent molecules adsorbed on the collagen surfaces behave as "hard spheres." Such hard spheres cannot be removed incrementally with small changes in surface separation, but rather must be removed as intact layers at large energetic expense. The incompressible layers of adsorbed solvent effectively form hard walls, giving the swelling curve a hard-wall appearance; the spacing begins to change significantly only at very high osmotic stress. This is quite different from the behavior observed in water, ethylene glycol, or formamide, which can be rearranged into different complex hydrogen-bonded structures upon incremental removal, so that the surface separation is strongly affected by low osmotic pressures.

Solvents with intermediate hydrogen-bonding properties, the monomethylated derivatives of the network-forming solvents, methanol, 2-methoxyethanol (methyl ethylene glycol), and *N*-methylformamide, show intermediate swelling behavior. The spacing between the collagen molecules responds to increasing polymer concentration more than in non-self-hydrogen-bonding solvents, but less than in water, ethylene glycol, or formamide (Fig. 7).

Physical properties of these solvents, including molecular weights and volumes, static dielectric constants, refractive indices (directly related to high-frequency dielectric constants), and hydrogen bonding abilities are listed in Table 1. The marked difference in the swelling behavior in ethylene

glycol and in *N,N*-dimethylformamide, in spite of their very similar dielectric constants and refractive indices, indicates that a force other than electrostatic or van der Waals dominates the close interactions between collagen helices. Even though the hydrogen bonding ability is difficult to quantify, its qualitative correlation with the changes in the swelling curves is clear.

These results with the different solvents show that short-range repulsion between collagen triple helices in water is consistent with a force mediated by solvent-solvent hydrogen bonds. This is in agreement with the inferences drawn from the high-resolution x-ray structure of an uncharged, collagen-like triple helical peptide. There are no direct molecular contacts between these triple helical peptides that have no flexible side chains. All interhelical contacts are mediated by hydrogen-bonded bridges of water (Bella et al., 1994, 1995). Numerous other studies using a variety of techniques have also demonstrated "non-bulk" properties of water in collagen fibers (Berendsen and Migchelsen, 1965; Chapman et al., 1971; Grigera and Berendsen, 1979; Nomura et al., 1977; Peto et al., 1990; Sasaki et al., 1983; Shinyashiki et al., 1990). Recent combined Raman and osmotic stress study of reconstituted collagen films has shown that changes in the relative intensities of different vibrational stretching modes of hydrogen-bonded water molecules correlate with the work required for fiber dehydration (Leikin et al., 1995b). All available evidence shows that there is a reorganization of the water hydrogen-bond network as collagen helices move closer. The results here with the different solvents furthermore strongly suggest that it is this reorganization that underlies the repulsive interhelical force and the energetic cost of removing water from collagen fibers.

Interpretation of the limiting spacing (Table 2) for the swelling of collagen in the absence of PEG is significantly more difficult. We have previously argued that the attractive force that limits the swelling at pH 7.5 in water is associated with hydrogen-bonded water bridges connecting specific recognition sites (Leikin et al., 1995a). However, we presently do not have any evidence suggesting the possible nature of attractive interactions that limit collagen swelling in nonaqueous solvents. Other, nonspecific forces, such as van der Waals attraction may be important, particularly in the non-self-hydrogen-bonding solvents.

CONCLUDING REMARKS

Exponentially varying repulsive forces at close spacing (the last ~10 Å between surfaces) have been now observed for several different kinds of macromolecules and lipid bilayers in water, from highly charged surfaces [DNA (Rau et al., 1984), four-stranded guanosine helices (Mariani and Saturni, 1996), xanthan (Rau and Parsegian, 1990), and some lipid bilayers (Parsegian and Rand, 1995)], to low-charge-density collagen (Leikin et al., 1995a) or net neutral, but zwitterionic lipid bilayers (Parsegian and Rand, 1995), (McIntosh and Simon, 1994), to even totally uncharged hydroxypropylcellulose (Bonnet-Gonnet et al., 1995) and schizophyllan (Rau and Parsegian, 1990). The basic similarity of the forces curves, the insensitivity of the repulsion between highly charged molecules to salt concentration, and the unexpectedly large magnitude of the force all suggested that the origin of the repulsion was the structuring of water between the two surfaces rather than electro-static, double-layer forces or steric interactions, commonly considered dominating. In spite of the overwhelming evidence that the observed forces between polar surfaces are much larger and qualitatively very different from conventional expectations, the postulation of hydration forces was by a process of elimination, rather than a direct observation of changes in water structuring. A major difficulty is that at the osmotic pressures typically reached by polymer solutions only a small energy perturbation per water molecule is necessary when summed over all waters to give the observed energy change in pushing two macromolecular surfaces together. Only by having very sensitive measures of energetic and structural perturbations of water or, as we have done here, by examining solvents with different properties can direct evidence be obtained linking the forces observed at close spacings with water structuring. The strikingly different qualitative forces between collagen triple helices in the different solvents that correlates with hydrogen bonding ability strongly support the hydration force interpretation of the data.

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