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Direct measurement of forces between self-assembled proteins: Temperature-dependent exponential forces between collagen triple helices

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ABSTRACT **We report direct measurements of force vs. separation between self-assembled proteins. These forces are observed between collagen triple helices in native and reconstituted fibers. They are a combination of a short-range repulsion, which varies exponentially over at least five decay lengths, and an inferred, longer-ranged attraction responsible for spontaneous assembly. From 5°C to 35°C the relative contribution of the attraction to the net force increases with temperature. These forces are strikingly similar to the "hydration" forces measured between several other linear macromolecules (DNA, polysaccharides) and between lipid bilayer membranes. The decay length of the repulsive force agrees well with a theoretical estimate based on axial periodicity of the triple helix, suggesting another connection between molecular architecture and protein–protein interaction.**

To predict protein structure, to gauge the strength and specificity of protein contact, and to target rationally designed drugs to intended sites all require knowledge of the forces acting between molecules in aqueous solution. Until recently, the lack of direct measurements has made it necessary to assume properties of intermolecular forces either inferred from model systems or chosen for mathematical convenience.

Beginning with direct osmotic-stress measurements on lipid bilayers (1, 2), followed by similar measurements on DNA double helices (3) and stiff polysaccharides (4), it has become clear that over the last few nanometers before molecular contact one encounters powerful, exponentially varying repulsive forces of a kind that have never been incorporated into theories of molecular assembly. Occasionally (5) these measurements have also revealed temperature-strengthened attractive forces at 5- to 15-Å separations. We now report the appearance of similar attractive and repulsive forces among proteins, specifically between collagen triple helices.

Collagen is a particularly apt system for measuring forces between protein surfaces. Triple helices will spontaneously assemble at neutral pH to form well-ordered fibers. In excess-water solutions the water content and corresponding helical separation are determined by a balance of attractive and repulsive forces between helices. Helical separation can be measured by x-ray diffraction and can be varied by fiber exposure to a vapor of controlled humidity (6) or to the osmotic stress of large excluded neutral polymers (7). In this way, one can measure the forces between the helical surfaces with their intricate pattern of hydrophilic and hydrophobic amino acid side chains exposed to water (8).

In addition to short-range exponential repulsion, the forces between collagen helices, measured from 5° to 35°C, show evidence of a strong longer-ranged temperature-enhanced attraction responsible for collagen self-assembly into fibers from dilute solution. As with many biologically important assembly and recognition reactions (9), the assembly of collagen is favored by increased temperature. Similar to forces measured in other systems, exponentially varying repulsion combined with a temperature-enhanced attraction suggests an energetically and entropically important release of water organized around surface groups.

Having now been measured among all classes of biological materials, these exponential forces compel us to enlarge traditional ideas of "hydrophobic interactions" due to ordered water around nonpolar residues. There is at least as strong a possibility of forces from ordering solvent around polar groups. "Hydration" interactions among polar groups open the possibility for a far more intricate connection between the structure of the molecular surface and the specificity of protein association. Here, for example, we show how the measured exponential force decay rate can be related to axial periodicity of the collagen triple helix.

METHODS AND MATERIALS

Collagen Preparation. Type I collagen was extracted from rat tail tendon. Native collagen fibers were exhaustively washed with 4 M NaCl/10 mM Tris, pH 7.5/20 mM (ethylenedinitrilo)tetraacetic acid (EDTA)/2 mM *N*-ethylmaleimide/1 mM phenylmethylsulfonyl fluoride and stored at 5°C in the same buffer solution. Acid-soluble collagen used in fiber reconstitution was prepared and purified by rounds of alternate solubilization in 0.5 M acetic acid (pH 2.8) and high-salt precipitation (10). Native collagen was initially solubilized in the presence of pepsin (100 mg/g of collagen) to remove the C- and N-terminal telopeptides (10) responsible for covalent crosslinks between triple helices. Collagen thus prepared contained only monomers, as determined by SDS/gel electrophoresis.

Dilute, acid-soluble collagen solutions were dialyzed to remove all residual salt, concentrated to 10–20% (wt/wt) protein in an Amicon 8050 high-pressure cell, and further dried by air exposure at 5°C. The resulting 0.2- to 0.5-mm-thick, transparent collagen film was then cut into 2- to 5-mg pieces and stored in 40–50% polyethylene glycol (PEG; United States Biochemical, 8000 average M_r)/10 mM Tris, pH 7.5/2 mM EDTA solution at 5°C.

The inability of reconstituted collagen to form intermolecular, acid-labile, covalent crosslinks was confirmed by sodium borohydride reduction (11). After reduced reconstituted collagen was dissolved in 0.5 M acetic acid (pH 2.8), only monomers were seen by SDS/gel electrophoresis. In contrast, native collagen fibers reduced by the same procedure became insoluble in acid.

The purification and sample preparation of chicken erythrocyte DNA for osmotic-stress measurements in 150 mM $Mn(ClO_4)_2$/10 mM Tris (pH ≈7.0) solution has been described (5).

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Force Measurements. Application of the osmotic-stress technique coupled with x-ray scattering for direct force measurement has been described in detail (2, 8, 12–14). Immersion of collagen fibers in solutions of nonpenetrating neutral solutes, such as PEG or dextran, allows one simultaneously to measure the work of removing water and to follow the consequent change in molecular separation. The external force from the osmotic pressure of the excluded polymers applies a force on the collagen fibers that is balanced by a net repulsive stress between helices, a repulsion that resists fiber dehydration. This technique has been used for force measurements in several other macromolecular systems (2–4, 13).

Native and reconstituted collagen fibers were equilibrated with solutions of various concentrations of different polymers [PEG 8000 M_r (United States Biochemical), PEG 400 M_r (Fluka), or dextran 200,000–300,000 M_r (United States Biochemical)] in 10 mM Tris/2 mM EDTA, pH 7.5. Preequilibration of reconstituted collagen samples with 40–50% PEG in 10 mM Tris/2 mM EDTA solution for at least 7 days was required to prevent excessive swelling of the sample upon transfer into the final polymer solution.

The average distance between collagen triple helices (equatorial Bragg spacing) was measured by x-ray diffraction as a function of the applied polymer osmotic stress, Π (13). Unlike the well-defined, quasi-hexagonal structure of native fibers (15), the structure of reconstituted collagen is less ordered with only one diffuse equatorial maximum (16). To estimate interaxial spacing, d_{int}, for reconstituted collagen from the measured average equatorial Bragg spacing, d_{Br}, we assume hexagonal packing of the triple helices, such that

$$d_{int} = 2d_{Br}/\sqrt{3}. \quad [1]$$

Although the osmotic stress here is applied to a lattice, for the rapidly decaying forces observed, the pressure exerted by the lattice can be interpreted as the sum of interactions between neighboring molecules (3) and even as the repulsive pressure between apposing molecular surfaces (13).

RESULTS

Fig. 1 compares force–distance curves measured for native and reconstituted collagen fibers at 20°C in 10 mM Tris, pH 7.5/2 mM EDTA. Force–distance curves are seen independent of the chemical nature of the polymer applying osmotic stress, ruling out the possibility that results reflect direct polymer–protein interactions. Qualitatively similar, exponentially varying repulsive forces are seen for both native and reconstituted collagen at high pressures. The finite swelling of collagen fibers with decreased osmotic stress indicates a longer-ranged attractive force responsible for the downward turn of the force–distance curve. An equilibrium separation between the collagen helices at zero applied stress is determined by the balance of repulsive and attractive forces. The observed stress vs. distance is the net of the two.

The larger separation between the reconstituted helices both at equilibrium (16) and under applied osmotic stress might be related to the difference in the covalent crosslinking of triple helices or to the loss during purification of some component regulating the assembly of native collagen (17). Reconstituted fibers were reassembled from collagen helices after enzymatically removing the nonhelical telopeptides to prevent specifically the formation of intermolecular covalent crosslinks.

The 67-nm longitudinal repeat period associated with the stagger of triple helices in the fiber (measured from the positions of meridional x-ray reflections) is the same within experimental error (≤2%) for native and reconstituted collagens. There appears to be no substantial difference in longitudinal packing of collagen between the two (16). No change in the longitudinal repeat period with applied osmotic

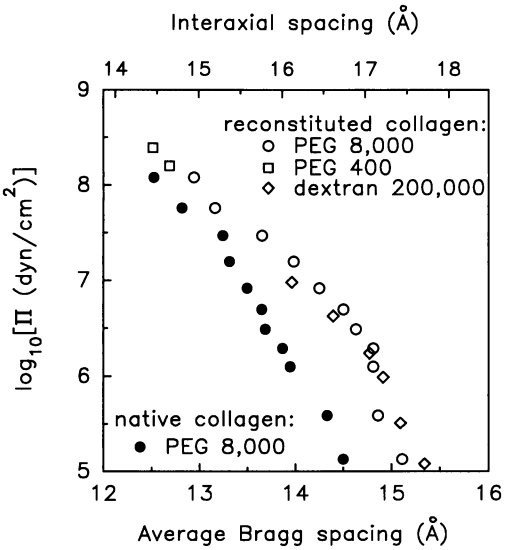

FIG. 1. Exponentially varying force vs. separation between collagen triple helices in native (solid symbols) and reconstituted (open symbols) fibers at 20°C in 10 mM Tris/2 mM EDTA, pH 7.5. Reconstituted fibers were made from pepsin-treated, acid-soluble collagen. Forces are plotted as the logarithm of the measured osmotic stress (dyn, dynes; 1 dyne = 10 μN) required to keep the molecules packed at a measured average spacing. The equivalence of stress from three different polymers (PEG, average M_r 400 and 8000, and dextran, average M_r 200,000–300,000) is a control to rule out contributions from direct polymer–protein interactions. The transformation from Bragg (bottom axis) to interaxial (top axis) spacing uses the hexagonal packing of the triple helices.

pressure is observed over the range of stresses shown in Fig. 1 (data not shown). Osmotic stress causes neither an appreciable elastic deformation of the helices nor an apparent change in their longitudinal packing.

The observed salt dependence of intermolecular forces is shown in Fig. 2*a*. At low osmotic pressures, interaxial spacings decrease with increased ionic strength. A direct effect of salt through electrostatic double-layer repulsion is unlikely, given the insensitivity of both force-decay lengths and force magnitudes to ionic strength at high osmotic stress. Rather, salt either is mediating the attractive force or is preferentially excluded from the space between collagen helices, so as to apply an extra osmotic pressure in addition to the PEG or dextran stress.

Figs. 2*b* and 3 demonstrate that the effect of salt is consistent with a simple exclusion and an additional osmotic stress. In Fig. 2*b*, the stress–distance data with PEG in 0.2 and 1.0 M NaCl are replotted, taking the total osmotic pressure acting on the collagen as the sum of the PEG stress and an effective salt stress, assuming the salt concentration between helices is 30 ± 10% of the bulk. With this fitted level of exclusion, the salt-adjusted data superimpose with the stress–distance curve with PEG in residual NaCl (from pH adjustment), a salt concentration too low (≤10 mM) to contribute significantly to the PEG pressure. Alternatively, the same pressure vs. interaxial spacing curve is seen in Fig. 3 for PEG applied stress in the low-salt buffer and for effective NaCl pressures in the absence of PEG.

Fig. 4 shows the temperature dependence of the equilibrium spacing of reconstituted collagen in 10 mM Tris/2 mM EDTA in the absence of applied polymer stress. The salt concentration is low enough (≤10 mM) that spacings are negligibly affected by the salt partition. As temperature increases, the interaxial distance between helices decreases.

The corresponding force–distance curves for reconstituted collagen are shown in Fig. 5*a* for three temperatures. These

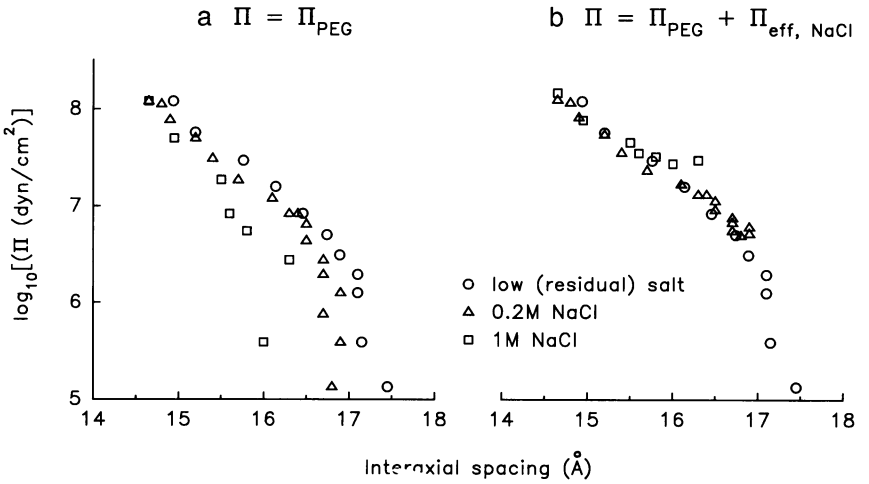

FIG. 2. Forces observed in solutions of three different salt concentrations. (*a*) Applied stress is taken as the PEG (8000 M_r) osmotic pressure alone. (*b*) Total stress is taken as the sum of PEG osmotic pressure and an effective (eff) NaCl pressure, assuming the salt concentration between helices is ≈30% that of the bulk [$\Pi_{eff,NaCl} = 2\varphi_{NaCl}\, 0.7c_{NaCl}$, where φ_{NaCl} is the osmotic coefficient of NaCl (18) and c_{NaCl} is the molal bulk concentration of NaCl]. Differences among different salt concentrations seen in *a* disappear when one recognizes the additional osmotic stress of salt excluded from between the protein helices.

data exhibit several close similarities with the osmotic-stress curves of spontaneously assembled DNA helices in the presence of Mn^{2+} shown in Fig. 5*b*. Both materials show an exponentially increasing force as helices are dehydrated from an equilibrium spacing determined by a balance of attraction and repulsion. As temperature increases, the equilibrium spacing and the observed repulsive force *decrease*. At high stress, the force curves at different temperatures seem to converge to a common curve. This convergence suggests that the attractive component of the net observed force increases with higher temperature.

DISCUSSION

These direct measurements of force vs. distance between protein surfaces have focused primarily on reconstituted-collagen interactions. Pepsin-treated collagen is a simpler system for measuring protein–protein interactions than is native collagen with its complications from flexible terminal peptides, from covalent crosslinks between helices, and from other nonhelical components possibly present in native fibers but lost during reconstitution. The comparatively small difference between the stress–distance curves for native and reconstituted collagen seen in Fig. 1, however, does not reflect a qualitative change in the character of forces.

With amino acid side chains able to extend out from the triple helical backbone, defining the diameter of collagen and, therefore, the distance between the protein surfaces is somewhat problematic. Apparent, hard surface contact can be achieved only at low water contents and very low vapor pressures, corresponding to osmotic stresses at least an order of magnitude higher than the pressures used in this study. The stress-independent interaxial spacing for very low water content fibers (<10 wt % of water) is ≈12 Å (an ≈10.5-Å Bragg spacing) (7, 16, 19). We take this 12-Å spacing as the "dry" diameter of the collagen helix. In the absence of salt or of polymer-applied osmotic stress, then, Fig. 4 indicates that reconstituted collagen helices are separated by a 5- to 6.5-Å layer of water.

The observed change in interaxial spacing caused by osmotic stress is due to a change in this water layer rather than to elastic deformation of the helices. Indeed, the ab-

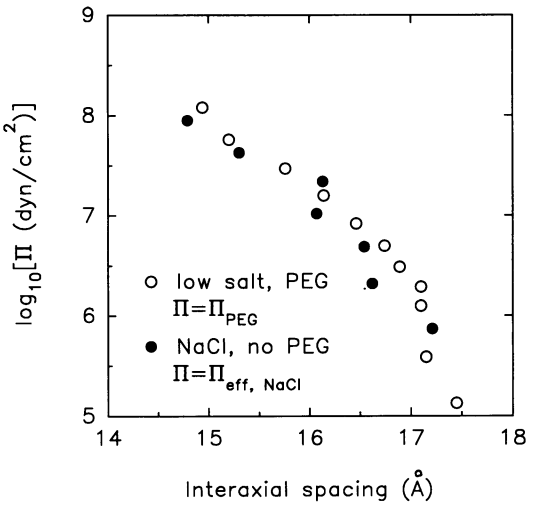

FIG. 3. Forces measured as a function either of PEG (8000 M_r) osmotic stress in low-salt-concentration solutions (○, data from Fig. 1) or of the effective stress from excluded NaCl in the complete absence of PEG (assuming the same partition coefficient of NaCl as in Fig. 2). As in Fig. 2, the effect of salt on forces appears not due to electrostatic double-layer interaction but to a partitioning of salt. All samples contain 10 mM Tris/2 mM EDTA, pH 7.5, in addition to PEG or NaCl.

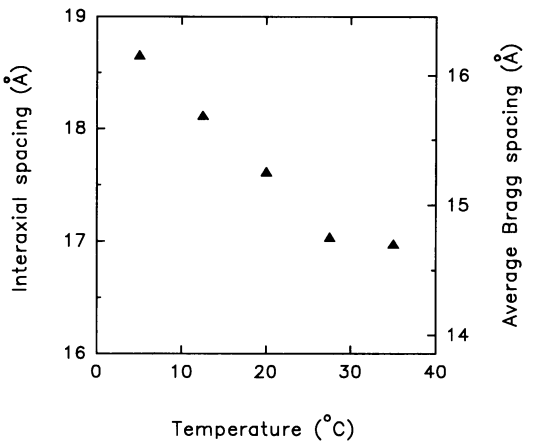

FIG. 4. Decrease in interhelical spacing of reconstituted collagen with increased temperature, in 10 mM Tris, pH 7.5/2 mM EDTA buffer without stressing polymer.

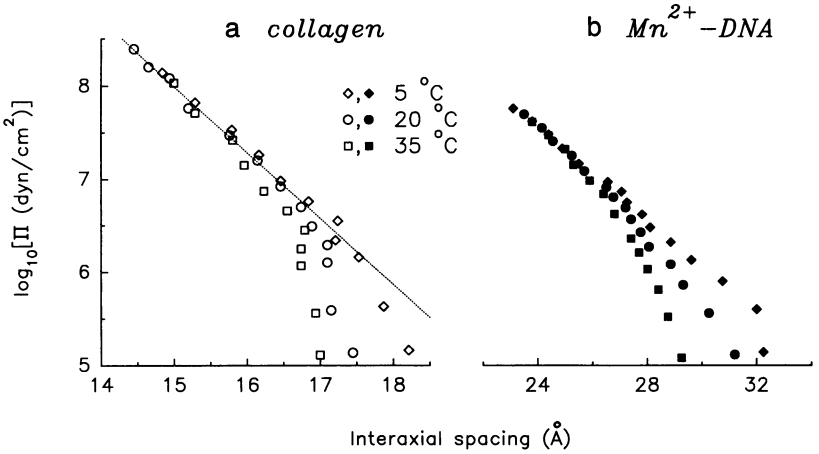

FIG. 5. Force–distance curves for collagen and DNA measured at three different temperatures: collagen fibers in 10 mM Tris/2 mM EDTA, pH 7.5 buffer (*a*) and, for comparison, DNA double helices in 150 mM $Mn(ClO_4)_2$/25 mM Tris, pH 7.0 solutions (*b*). Consistently, lattices expand upon being cooled; forces gradually approach purely exponential repulsion. This exponentiality extends over two decades of applied pressure at 5°C. The dotted line, with a 0.6-Å exponential decay rate, is the best-fit curve to the 5°C data at $\log_{10}\Pi > 6$. At all temperatures, forces seem to converge to this exponential line at high pressures.

sence of detectable changes in axial periodicity and longitudinal packing of the helices, even at the highest osmotic pressures used here, argues against significant contribution from elastic resistance of the triple helices themselves. The measured stress–distance curves give direct intermolecular forces.

The direct measurements of forces between these protein surfaces reveal features different from those anticipated from conventional views of protein–protein interaction (20, 21). The prominent feature of the forces between collagen triple helices is their exponentiality at higher pressures and smaller spacings. An exponential repulsion at 5°C extends over at least two decades of applied pressure or five exponential decay lengths, even though the interhelical spacing varies only slightly over 3 Å. Power-law forces expected for unscreened charge interactions or for direct molecular collisions due to thermal motions of the collagen backbone, of amino acid side chains, or of small ions between helices will not fit the data.

The insensitivity to salt concentration of force amplitude and decay length at high stress and the very short decay length of ≈0.6 Å are not consistent with electrostatic double-layer repulsion. Rather than the inclusion of salt implicit in double-layer interactions, there appears to be a partial exclusion of NaCl that effectively exerts an osmotic stress in addition to the PEG or dextran.

A striking feature of the assembly reaction of collagen triple helices into fibrils is that it is favored with increased temperatures (9). This decreased solubility of collagen upon increased temperature is reflected in the force curves of the assembled, ordered phase (Figs. 4 and 5*a*). Both the interhelical spacing and the net repulsive force decrease with increased temperature. Any decrease in fiber water volume with increased temperature but fixed stress implies an *increase* in entropy as molecules are pushed together (5, 22, 23). Any increase in entropy with decreased spacing between the helices also argues against a dominant contribution here from the "steric repulsion" or motional entropy loss of flexible helices or side chains that is often invoked to explain repulsion between rod-like polymers in liquid crystals (24, 25).

What can be said about the nature of these temperature-dependent exponential forces?

The entropy increase can be due to greater mobility of surface groups (26) (side chains, surface water, and ions) as molecules come closer or to the release of ordered water into the reference phase. "Hydration forces" between polar surfaces (5, 22, 23) and the traditional "hydrophobic effect" of nonpolar moieties (27) both recognize an ordering of water near a surface to create a difference in the entropy with the bathing medium.

The close similarity between collagen-stress curves and Mn^{2+}-DNA curves (Fig. 5) implies a common underlying interaction. Qualitatively similar exponential forces have been seen in materials as different as DNA (3, 13), lipid bilayers (2), and polysaccharides (4). In these cases, the exponential nature of the repulsion and the salt-concentration insensitivity of the decay length led us to postulate hydration forces. As polar molecules are brought together, they repel exponentially with a work of approach that appears to involve removal of water organized around the structures of hydrophilic groups on the apposing surfaces (12, 23, 28, 29). The apparent exponential perturbation in water structure surrounding polar or charged groups could be caused either by electrostatic polarization or by hydrogen bonding.

The only major difference between the closely similar Mn^{2+}-DNA and collagen force data (Fig. 4) is in the decay length of the exponential. Whereas the Mn^{2+}-DNA shows an ≈1.3- to 1.5-Å decay length, the exponential decay length for reconstituted collagen fibers is only ≈0.6 Å. This difference, however, is consistent with other data showing dependence of the hydration-force-decay length on the structure of interacting surfaces (30) and can be rationalized by considering the difference in helical repeat spacings between collagen and DNA.

Indeed, both from experimental results on DNA and some lipid systems (5, 13, 23, 30, 31) and from theoretical analysis (S.L. and V.A.P., unpublished work; refs. 29 and 30), it appears that complementary ordering of polar groups with unlike charges on apposing surfaces of net neutral molecules will produce temperature-favored attractive hydration forces. One can think of a competition between repulsive hydration forces and attractive hydration that can create an energy minimum at a point of balance (5, 13, 30, 31). Neglecting the effect of surface group spacing when they are disordered, the decay length of a residual repulsion at close separations is expected to be $\lambda_W/2$, where λ_W is the natural correlation length of water (13, 29, 30). When the spacing between periodically ordered surface groups is comparable to λ_W, the observed decay length is determined by both. For a surface repeat spacing a, the calculated planar decay length (29) is

$$\lambda = \frac{1}{2\sqrt{(1/\lambda_W)^2 + (2\pi/a)^2}}. \qquad [2]$$

The expected λ for interacting cylinders will be slightly smaller than for planar surfaces due to surface curvature. The wide variation in force-decay lengths observed between different materials can be accounted for by this coupling between surface structure and correlation lengths with λ_W

(for more discussion and rigorous derivation of Eq. 2 see refs. 29 and 30).

We can now compare decay lengths measured between Mn^{2+}-DNA and between collagen helices with those predicted for hydration forces. The limiting water correlation length, λ_W, has been estimated as 3–5 Å (30) from the pure-repulsion decay length between DNA helices (3, 13), between two polysaccharides (4), and between didodecylphosphate bilayers in tetramethylammonium chloride (32). This planar 3- to 5-Å decay length from force measurements agrees well with other estimates (33) based on different kinds of experiments. Decay lengths predicted by Eq. 2 for Mn^{2+}-DNA and collagen (net neutral, periodically ordered molecules) are not very sensitive to the assumed value of λ_W between 3 and 5 Å.

The helical pitch gives a periodicity a of groups facing one another on apposing helical surfaces. Taking the average 9.6-Å helical pitch observed for collagen (15) and $\lambda_W \simeq 3$–5 Å, we find $\lambda \simeq 0.7$ Å (0.68–0.73 Å), in good agreement with the measured value of ≈0.6 Å. The predicted decay length is dominated by the surface periodicity. The decay length similarly calculated for spontaneously assembled Mn^{2+}-DNA with a much larger 34-Å helical repeat is 1.3–1.8 Å, compared with the observed 1.3–1.5 Å. Although we do not exclude other interpretations, the forces observed here between collagen triple helices are fully consistent with hydration force expectations.

While hydration is generally regarded as essential to protein function and structure (34), the lack of direct force measurements has, until now, allowed the omission of hydration forces in theories of protein association and folding (20, 21). We can now argue that water structuring around exposed polar residues and concomitant hydration forces play an active role in protein folding and assembly reactions.

The expanding set of direct force measurements revealing the intricate features of exponential hydration forces is allowing us to consider in more detail the energetic consequences of molecular complementarity. Specifically, the correlation of positions on the neighboring molecules, both of complementary charged or polar residues and of large hydrophobic residues, has been suggested as the underlying mechanism of assembly in molecular collagen models (35). The realized possibility of measuring forces between such surfaces now provides a systematic strategy to connect molecular architecture with molecular recognition and assembly through directly observed interactions.

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