Metabolic Buffering Exerted by Macromolecular Crowding on DNA−DNA Interactions: Origin and Physiological Significance†

Rivka Goobes, Nava Kahana, Orit Cohen, and Abraham Minsky*

Department of Organic Chemistry, The Weizmann Institute of Science, Rehovot 76100, Israel

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ABSTRACT: Crowding, which characterizes the interior of all living cells, has been shown to dramatically affect biochemical processes, leading to stabilization of compact morphologies, enhanced macromolecular associations, and altered reaction rates. Due to the crowding-mediated shift in binding equilibria toward association, crowding agents were proposed to act as a metabolic buffer, significantly extending the range of intracellular conditions under which interactions occur. Crowding may, however, impose a liability because, by greatly and generally enhancing macromolecular association, it can lead to irreversible interactions. To better understand the physical determinants and physiological consequences of crowding-mediated buffering, we studied the effects of crowding, or excluded volume, on DNA structures. Results obtained from isothermal titration calorimetry (ITC) and UV melting experiments indicate that crowding-induced effects are marginal under conditions that a priori favor association of DNA strands but become progressively larger when conditions deteriorate. As such, crowding exerts "genuine" buffering activity. Unexpectedly, crowding-mediated effects are found to include enthalpy terms that favorably contribute to association processes. We propose that these enthalpy terms and preferential stabilization derive from a reconfiguration of DNA hydration that occurs in dense DNA-rich phases obtained in crowded environments.

The total concentration of proteins and nucleic acids in bacteria is in the range of 300−400 g/L, leading to a highly crowded intracellular environment (*1*, *2*). In eukaryotic cells, crowding results from the collectively large concentration of macromolecules and organelles in the fluid part of the cytoplasm, as well as from the confinement of this fluid within small pores whose boundaries are defined by a dense cytoskeleton lattice (*3*, *4*). This omnipresent crowding means that a large proportion of the intracellular environment is physically occupied and thus unavailable to other macromolecular species. Such levels of excluded volume increase the free energy of the system by substantially attenuating the configurational entropy of the constituent macromolecules. Consequently, processes that reduce excluded volume will be entropically favored and, hence, preferentially promoted in crowded environments (*5*−*9*).

A direct outcome is that processes leading to macromolecular compaction, such as DNA packaging, folding of extended polypeptides into compact functional proteins, association and aggregation of polymers, and formation of tight oligomeric structures (*10*), are significantly enhanced in cellular environments. Several studies have indeed demonstrated that the crowding-mediated tendency for maximizing compaction represents a general and crucial intracellular determinant that acts to dramatically stimulate association between biopolymers and to substantially modulate reaction rates (*7*, *8*, *11*−*15*). Through their effect on binding, crowding agents alter the pathway of biochemical processes such as RecA activities (*16*), or chaperonin-mediated protein folding (*17*), as well as modify recognition and specificity patterns of interactions involving macromolecules (*18*). The premise that volume exclusion represents a crucial factor in cellular processes has recently been highlighted by hypotheses that nucleosomes have evolved to counteract a compulsory crowding-induced DNA collapse (*19*) and that molecular chaperones emerged in order to mitigate extensive crowding-induced intracellular protein aggregation (*20*).

Crowding attenuates the sensitivity of biochemical processes to concentration limits of reactants and to ionic strength, thereby extending the operational range of enzymes (*21*−*26*). Enhanced binding caused by excluded volume was proposed to offset environmental perturbations such as alterations in concentration of salts or in pH values that are regularly occurring in the cellular milieu. These findings, which collectively point to a "metabolic buffering" capacity of macromolecular crowding, provide an interpretation to observations that enzymatic activities can effectively proceed in vivo under conditions that are inhibitory in noncrowded solutions (*11*, *27*).

The profound physiological significance of crowding-mediated metabolic buffering prompted us to examine the extent of this phenomenon, as well as its physical and structural determinants. By analyzing the effects of crowding agents on the formation and thermal stability of double- and triple-stranded DNA motifs, we find that whereas these structures are invariably stabilized by crowding, stabilization is relatively small under environmental conditions that are a

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* To whom correspondence should be addressed. E-mail: avi.minsky@weizmann.ac.il.

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Table 1: List of ODNs Used in the Study

DNA	name	DNA	name
5′-A_{20}CGC-3′	A_{20}	5′-T_5IT_{12}-3′	$T_{17}I$
5′-GCGT_{20}-3′	T_{20}	5′-T_5GT_{12}-3′	$T_{17}G$
5′-T_{18}-3′	T_{18}	5′-$T_5IT_6IT_5$-3′	$T_{16}I_2$
5′-A_{18}-3′	A_{18}	5′-$T_5GT_6GT_5$-3′	$T_{16}G_2$

priori favorable for association of DNA strands but becomes larger when these conditions deteriorate. These observations are consequential, because they imply that the extreme crowding that prevails in cellular environments does not impede cellular physiology by pronoting irreversible macromolecular associations. By using isothermal titration calorimetry, we demonstrate that crowding-mediated effects cannot be interpreted solely in terms of entropy changes, as generally assumed, but also include enthalpy terms that favorably contribute to association processes. We propose that the phenomenon of preferential stabilization, as well as the enthalpic contributions of excluded volume, derives from a differential reconfiguration of DNA hydration that occurs in crowded environments and is related to crowding-promoted formation of dense, DNA-rich phases.

MATERIALS AND METHODS

Oligodeoxynucleotides and Chemicals. Oligodeoxynucleotides (ODNs,[1] specified in Table 1) were purchased from Midland Certified Reagent Co. (Midland, TX) and purified by ion-exchange HPLC. The purity of ODNs was analyzed by HPLC and mass spectroscopy and found to be >95%. The concentration of the ODNs was determined by UV absorption using extinction coefficient parameters from published nearest neighbor parameters (*28*). The precision of this determination was within 10%, as indicated by ITC experiments in which single-stranded species were titrated with their complementary strands (under conditions refractory to triplex formation) to give titration curves with a stoichiometry ratio around 1. The purified ODNs were extensively dialyzed against 20 mM piperazine-*N*,*N*-bis(2-ethanesulfonic acid) (PIPES), pH 7.0.

Stock solutions of double-stranded DNA were prepared by mixing eqimolar amounts of complementary strands, heating to 90 °C, and cooling to room temperature at a rate of 0.5 °C/min. Triple-stranded DNA stock solutions were prepared by mixing the appropriate amounts of stock solutions containing single-stranded and preformed double-stranded DNA species. The resulting solutions were diluted in 20 mM PIPES in the presence of NaCl and cosolutes as required.

Poly(ethylene glycol) (PEG) of different molecular weights and Dextran T-70 were purchased from Sigma. The polymers were dissolved in doubly distilled water, purified by extensive dialysis against water, and lyophilized to dryness. Ethylene glycol was purchased from Aldrich. Cosolute concentrations are reported as weight percent per volume.

Isothermal Titration Calorimetry (ITC). ITC experiments were conducted on an MCS−ITC system (MicroCal Inc.). In titrations where double-stranded DNA formation was probed, single-stranded DNA species (11 μM) were injected 23 times in 5 μL increments at 3 min intervals into the isothermal cell containing the complementary single-stranded DNA target (0.5 μM). In titrations where triple-stranded DNA formation was followed, single-stranded DNA species (60 μM) were injected 23 times in 5 μL increments at 3 min intervals into the isothermal cell containing the double-stranded target (2.25 μM). Titration curves were corrected for heat of dilution of the single strands, which were obtained separately by injecting the oligonucleotide into the buffer (in both the absence and presence of PEG). ITC experiments conducted in the presence of PEG were performed with the neutral polymer present in both the injectant solution and the isothermal cell at identical concentration. Corrected heats were divided by the mole number of the injectant and analyzed with Origin software supplied by the manufacturer (*29*).

Circular Dichroism (CD). CD spectra were recorded in the spectral range of 210−300 nm on an Aviv spectropolarimeter, Model 202. DNA samples, 30 μM of the single-stranded form and 2.5 μM of the duplex and triplex forms, were equilibrated for 15 min in a 0.1 and 1.0 cm path length quartz cell, respectively, at the desired solution composition before scanning. Spectra of buffer solutions were subtracted to correct for baseline artifacts. CD spectra were normalized in units of molar ellipticity (deg cm^{-1} M^{-1}).

UV Spectrophotometry. Thermal transitions were obtained by measuring UV absorption at 260 nm on a Cary 5E spectrophotometer (Varian) equipped with a Peltier temperature controller. The temperature was measured at an accuracy of ±0.3 °C by a probe that was placed in a buffer-containing cell. Melting profiles were recorded at heating and cooling rates of 0.5 °C/min and were reversible. Reported melting temperatures (T_m) are those at which the derivative of absorbance versus temperature is maximal.

Osmotic Pressure Measurements. Osmotic pressures were measured by freezing point depression on an Advanced Micro Osmometer, Model 3300. The logarithm of water activities (a_w) was calculated from the measured osmolality (millimoles per kilogram) as

$$\psi = (RT/M_w) \ln a_w$$

where ψ and M_w represent the water potential and the molecular weight of water (0.018 kg/mol), respectively, R is the gas constant [8.314 J/(mol·K)], and T is the temperature in kelvin. The relation between water potential and osmolality, assuming independence of the water potential on temperature at room temperature, is given by

$$\text{water potential (in MPa)} = \text{osmolality (in mmol/kg)}/-400$$

RESULTS

Effects of Neutral Polymers on Duplex DNA. The thermal stability of long double-stranded DNA structures has previously been shown to increase in the presence of inert polymers such as PEG or dextran (*30*). We find that short DNA species are similarly affected by the neutral polymers. Derivative melting curves obtained from UV melting experiments of the duplex A_{18}·T_{18} (Table 1; "·" designates Watson−Crick base pairing and "*" represents third-strand

[1] Abbreviations: a_w, water activity; CD, circular dichroism; EG, ethylene glycol; I, inosine; ITC, isothermal titration calorimetry; K_a, equilibrium association constant; ODNs, oligodeoxynucleotides; PEG, poly(ethylene glycol) (MW 8000, unless otherwise indicated); TFO, triplex-forming oligonucleotide; T_m, melting temperature.

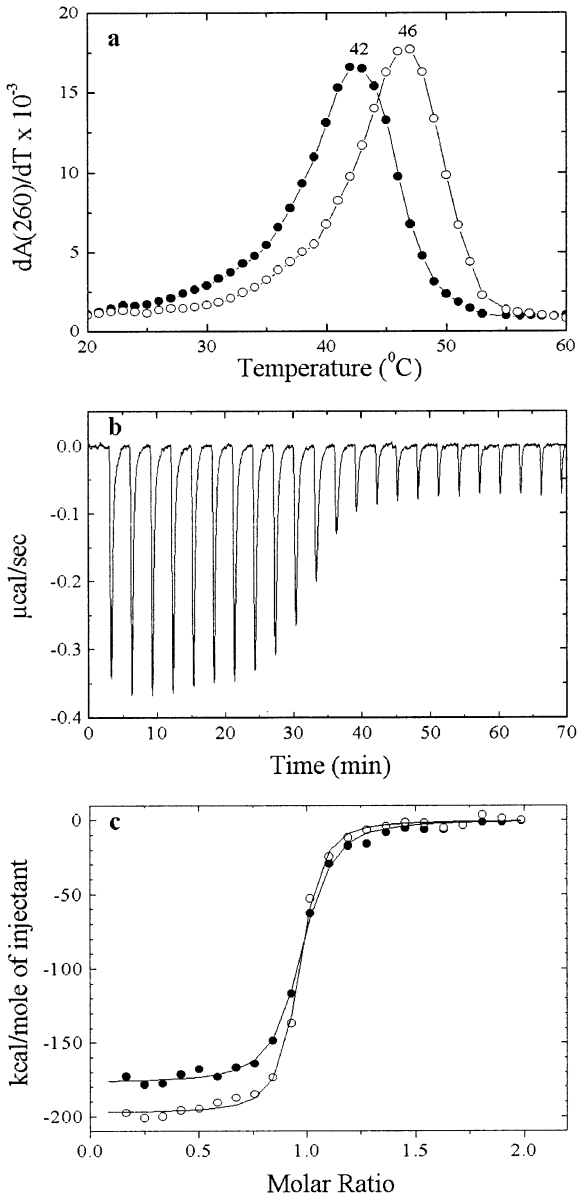

FIGURE 1: (a) Derivative UV (A_{260}) melting curves of the duplex A_{18}·T_{18} (1.6 μM) in a solution composed of 20 mM PIPES and 0.1 M NaCl in the absence (●) and presence (○) of 15% PEG. (b) ITC profile of the titration of A_{18} (0.5 μM) with T_{18} (11 μM) performed at 25 °C in a solution composed of 20 mM PIPES and 0.1 M NaCl. (c) Titration curves, obtained in the absence (●) and presence (○) of 15% PEG, corrected for heat of dilution and shown as a function of the molar ratio between T_{18} and A_{18}. Data were fitted by a nonlinear least-squares method (*29*).

binding), in the absence and presence of PEG, demonstrate that the thermal stability of the double-stranded DNA segment is increased by 4 °C in the crowded solution (Figure 1a, Table 2). Similar PEG-mediated stabilization is observed for double-stranded DNA molecules that contain one or two mismatched base pairs. As indicated in Table 2, the extent of stabilization (4−5 °C) is independent of the number and nature of the mismatched base. The neutral polymer Dextran T-70 also increases the melting point of the double-stranded forms, albeit less effectively than PEG (2 °C; results not shown). The finding that neutral polymers enhance the thermal stability of relatively short double-stranded DNA segments renders the stabilization process amenable to calorimetric studies because, in contrast to long DNA molecules that tend to undergo massive collapse and aggregation in the presence of PEG, short segments remain completely soluble.

Table 2: T_m Values (°C) of Duplex Structures (1.6 μM) in a Solution Composed of 20 mM PIPES and 0.1 M NaCl,[a] in the Absence and Presence of 15% PEG, Obtained from UV Melting Measurements

duplex	A_{18}·T_{18}	A_{18}·T_{17}I	A_{18}·T_{17}G	A_{18}·$T_{16}I_2$	A_{18}·$T_{16}G_2$
−PEG	42	37	35	30	25
+PEG	46	41	40	35	30

[a] Similar experiments were performed at 1 M NaCl, where 15% PEG was found to increase the T_m of all species by 1 °C.

The thermodynamic parameters characterizing the formation of A_{18}·T_{18} and of A_{18}·T_{17}I (Table 3) were derived from ITC measurements in which A_{18} was titrated with the oligonucleotides T_{18} and T_{17}I in the absence and presence of PEG. To prevent the formation of triple-stranded motifs that would hamper quantification of the calorimetric experiments, ITC measurements were conducted under relatively low-salt conditions under which triplex structures are highly unstable, as indicated by UV analysis (see below). Figure 1b depicts a typical isothermal profile. The heat evolved in each injection was corrected for the heat of dilution and divided by the number of moles injected. The resulting values were plotted as a function of the molar ratio between the titrant oligonucleotide T_{18} and A_{18} and fitted to a sigmoidal curve by using a nonlinear least-squares method (*29*) (Figure 1c). The equilibrium association constant (K_a) and the enthalpy change (ΔH) that characterize strand association are directly obtained from the titration curve. The Gibbs free energy change (ΔG) and the entropy change (ΔS) of the process are calculated from the equation $\Delta G = -RT \ln K_a = \Delta H - T\Delta S$. The titration curves obtained from the interaction of T_{18} and T_{17}I with A_{18} in the absence and presence of 15% PEG reach half-saturation near the equimolar ratio, indicating a one-to-one binding.

The results presented in Figure 1c and in Table 3 indicate that formation of the native as well as the mismatched duplex DNA forms is characterized by higher association constants in the presence of PEG. Notably, in both cases, PEG-mediated enhanced binding derives from a favorable enthalpy change that slightly but consistently exceeds the unfavorable change in entropy that is affected by the neutral polymer.

Effects of Neutral Polymers on Triplex DNA. Previous studies have demonstrated that, in the presence of inert polymers, the thermal stability of triple-stranded DNA motifs is enhanced to an extent that substantially exceeds that exhibited by double-stranded DNA structures of comparable length (*31*, *32*). As shown above to be the case for duplex motifs, triplex stabilization was found to be affected by PEG as well as by Dextran-70, but the stabilization produced by PEG is significantly more pronounced. Here, we have extended these observations by showing that triplex motifs containing mismatched bases are also effectively stabilized by PEG. Notably, the magnitude of the PEG-mediated increase of the triplex melting temperature is found to be constant and independent of the nature and number of the mismatched bases. Thus, native triplex, as well as triple-stranded DNA motifs in which one or two thymines in the TFO were substituted with inosines or guanines, is stabilized

Table 3: Thermodynamic Parameters for Duplex Formation between A_{18} and the ss-DNA Species T_{18} and $T_{17}I$ at 25 °C in a Solution Composed of 20 mM PIPES and 0.1 M NaCl, in the Absence and Presence of 15% PEG, Obtained from ITC Measurements[a]

ss-DNA	PEG	K_a (M^{-1})	ΔG (kcal mol^{-1})	ΔH (kcal mol^{-1})	$-T\Delta S$ (kcal mol^{-1})
T_{18}	−	$(4.32 \pm 0.69) \times 10^8$	-11.78 ± 0.09	-177.1 ± 2.1	165.4 ± 2.0
	+	$(10.40 \pm 2.73) \times 10^8$	-12.31 ± 0.15	-194.6 ± 2.4	182.3 ± 2.3
$T_{17}I$	−	$(1.41 \pm 0.18) \times 10^8$	-11.12 ± 0.08	-154.8 ± 1.9	143.7 ± 1.8
	+	$(3.48 \pm 0.35) \times 10^8$	-11.66 ± 0.06	-170.0 ± 1.2	158.3 ± 1.2

[a] The concentration of ss-DNA in the syringe is 11 μM. The A_{18} concentration in the ITC cell is 0.5 μM. The reported values are the average of at least three experiments.

Table 4: T_m Values (°C) of Triplex Structures (1.6 μM) Formed between the Duplex $A_{20}{\cdot}T_{20}$ and the Specified TFOs in a Solution Composed of 20 mM PIPES and 1 M NaCl, in the Absence and Presence of 15% PEG, Obtained from UV Melting Measurements

TFO	T_{18}	$T_{17}I$	$T_{17}G$	$T_{16}I_2$	$T_{16}G_2$
−PEG	42	25	21	12	∼1
+PEG	57	41	35	27	15

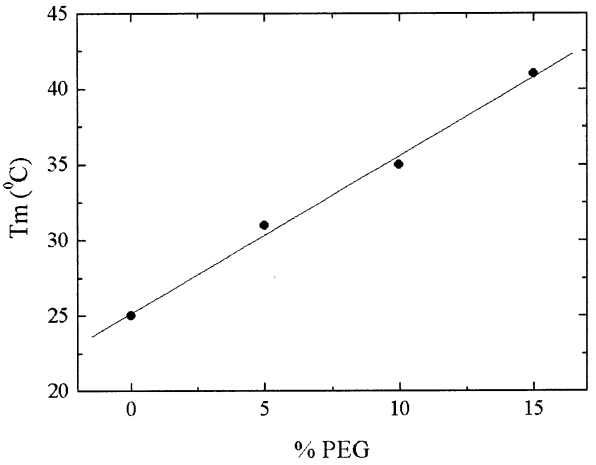

FIGURE 2: Dependence of the triplex $T_{17}I{*}A_{20}{\cdot}T_{20}$ stability on PEG concentration. The experiments were conducted in 20 mM PIPES and 1.0 M NaCl. DNA concentration was 1.6 μM.

by 14−16 °C in the presence of 15% PEG (Table 4). Dextran T-70 at the same concentration enhances the thermal stability of the various triplex species by 4−5 °C (results not shown).

Effects of PEG Concentration and Size. The stabilization of DNA molecules affected by neutral polymers was examined as a function of PEG concentration. We find that increasing the concentration of PEG at the range of 0−15% is accompanied by a linear increase of the melting temperature of both double- and triple-stranded DNA motifs. A characteristic example of this linear correlation, depicting the T_m values of the triplex $T_{17}I{*}A_{20}{\cdot}T_{20}$ as a function of PEG concentration, is presented in Figure 2. Due to their large viscosity, we could not examine samples at PEG concentration higher than 15%.

We proceeded to examine the correlation between the size of PEG polymers of variable length and the thermal stability of DNA species. The results, presented in Table 5, indicate that the size-dependent effects exerted by these cosolutes on double-stranded and triple-stranded DNA structures significantly differ. Whereas the presence of the monomer EG has no effect on the T_m of the triplex motif, a significant increase in the thermal stability of this structure is already observed when PEG 200 is included, and this stabilizing effect is augmented as PEG molecular weight is increased

Table 5: T_m Values (°C) Obtained from UV Measurements of the Triplex $T_{17}I{*}A_{20}{\cdot}T_{20}$ and the Duplex $A_{20}{\cdot}T_{20}$ Forms (1.6 μM) Studied in 20 mMM PIPES and 1.0 M NaCl, in the Absence and Presence of 15% Cosolutes

	a	EG 4.5%	EG 15%	PEG 200 15%	PEG 1000 15%	PEG 3350 15%	PEG 6000 15%	PEG 8000 15%
triplex	25	25	25	33	39	40	41	41
duplex	66	66	61	61	65	66	67	67

[a] No cosolutes.

to 1000. Further increase of the neutral polymer size is accompanied by a negligible effect. In contrast, the thermal stability of the duplex is substantially attenuated in the presence of EG, as indeed previously shown (*32*). A pronounced destabilization is also observed in the presence of PEG 200, whereas larger sizes of the neutral polymer reveal only a marginal effect.

Neutral cosolutes affect macromolecular interactions through their influence upon the volume excluded for other macromolecules and by their ability to modify water activity (*12*, *32*, *33*). To assess the relative contribution of the various cosolutes to these two factors, we conducted osmotic pressure measurements, from which the values of water activity of the aqueous solutions containing the various cosolutes were derived as specified in Materials and Methods. We find that, at the ionic strength used for the UV melting experiments, PEG species of high molecular weight (PEG 3350, 6000, and 8000) reduce water activity to an identical extent (Table 6), whereas a larger decrease is produced by the smaller cosolutes (PEG 200 and 1000). A decrease of water activity to the same value obtained in the presence of 15% PEG 8000 is produced by the monomer EG at a concentration of 4.5%. At 15%, the decrease of water activity affected by EG is too large to be measured.

Temperature-Dependent Effects of Neutral Polymers on Triplex DNA. To get deeper insight into the mechanism responsible for triple-stranded DNA stabilization by neutral polymers, we have obtained the thermodynamic parameters of triplex formation in the absence and presence of PEG as a function of temperature (Table 7, Figure 3). Over the whole temperature range examined, the equilibrium binding constants characterizing the formation of the triplex $T_{18}{*}A_{20}{\cdot}T_{20}$ (obtained by titration of the preformed duplex $A_{20}{\cdot}T_{20}$ with T_{18}) are larger in the presence of PEG than those detected in the absence of the neutral polymer. Significantly, whereas the K_a values decrease as the temperature is increased, this temperature-dependent decrease is smaller in the presence of PEG than in noncrowded solutions (Table 7). Thus, PEG-mediated stabilization of the triplex motif becomes more pronounced at higher temperatures.

Table 6: Osmolality (mmol/kg) and Calculated Logarithm of Water Activity of a Solution Composed of 20 mM PIPES and 1.0 M NaCl, in the Absence and Presence of Cosolutes, Obtained from Osmotic Pressure Measurements

	a	PEG 200 15%	PEG 1000 15%	PEG 3350 15%	PEG 6000 15%	PEG 8000 15%	EG 4.5%[b]
osmolality	1990 ± 10	3690 ± 40	3220 ± 50	2900 ± 120	2900 ± 100	2900 ± 100	2900 ± 20
ln a_w	−0.037	−0.067	−0.058	−0.053	−0.053	−0.053	−0.053

[a] No cosolute. [b] 15% EG increases the osmolality to a nonmeasurable value.

Table 7: Temperature Dependence of the Thermodynamic Parameters of Triplex Formation between T_{18} and the Duplex A_{20}·T_{20} in a Solution Composed of 20 mM PIPES and 1 M NaCl, in the Absence and Presence of 15% PEG, Obtained from ITC Measurements[a]

T (°C)	PEG	K_a (M^{-1})	ΔG (kcal mol^{-1})	ΔH (kcal mol^{-1})	$-T\Delta S$ (kcal mol^{-1})
20	−	$(2.62 \pm 0.26) \times 10^7$	−9.95 ± 0.06	−59.8 ± 0.6	49.7 ± 0.5
	+	$(1.18 \pm 0.21) \times 10^8$	−10.83 ± 0.10	−70.9 ± 0.9	59.9 ± 0.8
25	−	$(1.67 \pm 0.17) \times 10^7$	−9.86 ± 0.06	−62.1 ± 0.7	52.2 ± 0.6
	+	$(6.27 \pm 1.42) \times 10^7$	−10.64 ± 0.13	−76.9 ± 1.1	66.2 ± 1.1
30	−	$(7.20 \pm 0.38) \times 10^6$	−9.51 ± 0.03	−68.4 ± 0.6	58.8 ± 0.6
	+	$(5.34 \pm 0.64) \times 10^7$	−10.72 ± 0.07	−79.7 ± 0.7	68.9 ± 0.6
35	−	$(3.17 \pm 0.39) \times 10^6$	−9.17 ± 0.08	−71.3 ± 2.2	62.0 ± 2.1
	+	$(3.64 \pm 0.46) \times 10^7$	−10.67 ± 0.08	−87.1 ± 0.8	76.4 ± 0.8

[a] The concentration of TFO is 60 μM in the syringe. The duplex concentration is 2.25 μM in the cell. Values are an average of three experiments.

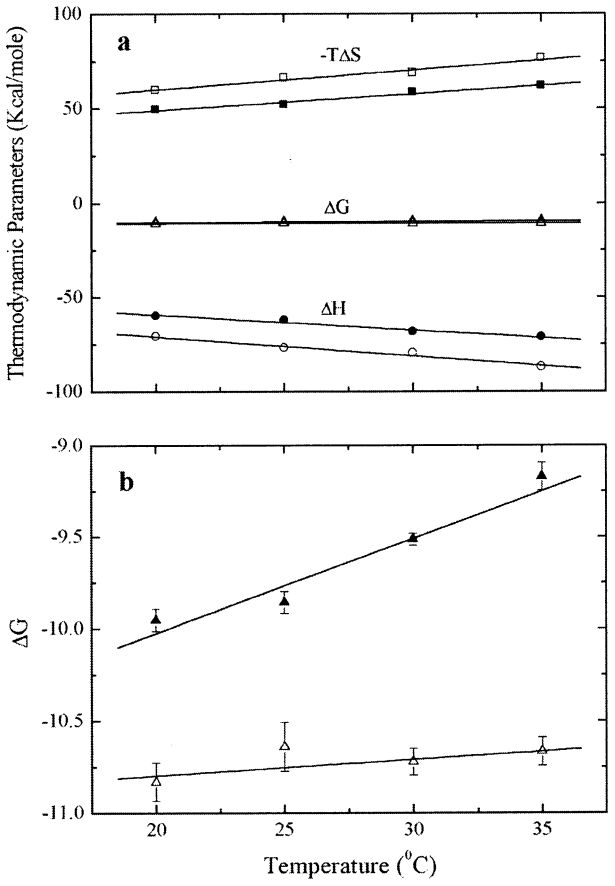

FIGURE 3: (a) Temperature dependence of ΔH, ΔG, and $-T\Delta S$ for the T_{18}*A_{20}·T_{20} triplex formation in the absence (filled symbols) and presence (empty symbols) of 15% PEG. (b) Magnification of the ΔG scale. Conditions are the same as in Table 7. In the absence of PEG the slopes of ΔH, $-T\Delta S$, and ΔG are −0.81 ± 0.10, 0.87 ± 0.10, and 0.052 ± 0.005, respectively. In the presence of PEG, the corresponding values are −1.03 ± 0.10, 1.04 ± 0.12, and 0.008 ± 0.008.

The results presented in Table 7 and in Figure 3 indicate that triplex formation is driven by a large negative enthalpy change that exceeds the unfavorable entropy change, confirming earlier results (*34*−*38*). As the temperature is increased, both the favorable contribution of enthalpy and unfavorable contribution of entropy to the free energy change increase, thus resulting in apparent enthalpy−entropy compensation. The PEG-mediated enhanced stabilization of the triple-stranded DNA motif at elevated temperature derives from a difference in the extent of this compensation. Specifically, in the absence of PEG, temperature increase is accompanied by a larger increment of unfavorable entropy change than of favorable enthalpy change, leading to a temperature-dependent decrease in the free energy change of triplex formation. In the presence of PEG, ΔH and ΔS are affected by temperature to a similar extent, resulting in an almost complete enthalpy−entropy compensation that, in turn, leads to the observed PEG-mediated stabilization (Figure 3b).

Reliance of Isothermal Titration Measurements. The accuracy of the ITC measurements critically depends on a precise identification of all species involved, as well as of all processes that may occur as titration proceeds. The evaluation of thermodynamic parameters exhibited by triplex formation might be hampered by the presence of single-stranded DNA species in the ITC cell due to duplex dissociation or disproportionation (*34*, *39*). That such species are not present to a detectable extent is implied by the observation that titration of the TFOs into the ITC cell containing the duplex results in a monophasic titration curve under all studied conditions. The presence of single-stranded molecules would have led to the initial formation of double-stranded molecules. As a result, and because ΔH of duplex formation is significantly larger than that accompanying the formation of triple-stranded motifs (*34*), a biphasic ITC curve would have been obtained.

To further examine the process occurring during the ITC experiment, we have used circular dichroism (CD) methodology, because the CD spectra exhibited by single-, double-, and triple-stranded DNA motifs are highly characteristic and conspicuously different (*34*). The ellipticities exhibited by the reactants T_{18} and A_{20}·T_{20}, as well as by T_{18}*A_{20}·T_{20}, in solutions identical to those used for the ITC experiments are characteristic of single-, double-, and triple-stranded short

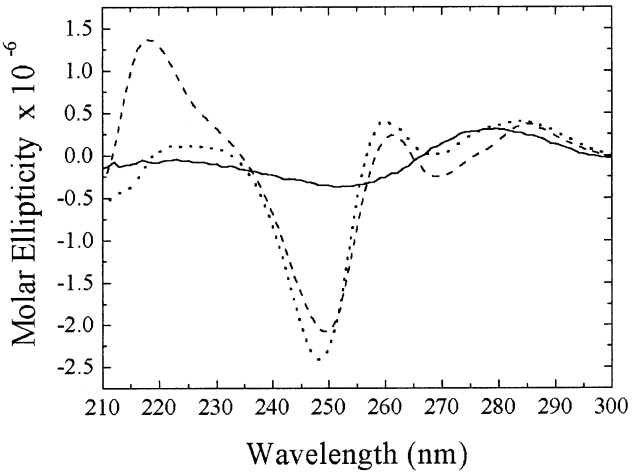

FIGURE 4: Circular dichroism spectra presented as molar ellipticity (deg cm^{-1} M^{-1}) versus wavelength (nm) of the following DNA forms: single strand T_{18} (30 μM) (solid line), duplex A_{20}·T_{20} (2.5 μM) (dashed line), and triplex T_{18}*A_{20}·T_{20} (2.5 μM) (dotted line). The spectra were taken in solutions that were identical to those used for the ITC experiments (20 mM PIPES and 1.0 M NaCl, in the absence and presence of 15% PEG). Identical CD spectra were observed over the whole range of temperature in which ITC experiments were conducted (20−35 °C).

DNA segments, respectively. The ellipticities remain unaltered in the absence and presence of PEG over the whole range of temperature in which the calorimetric studies were conducted (Figure 4). These observations indicate that the presence of PEG and the increased temperature do not markedly affect the secondary conformation of the various DNA species and that under these experimental conditions an equimolar mixture of T_{18} and A_{20}·T_{20} contains T_{18}*A_{20}·T_{20} as the predominant product. Moreover, the CD results imply that the DNA segments do not form aggregates, as such aggregation would have altered the shape of the CD spectra. This finding is significant because PEG-induced aggregation of short triple-stranded DNA segments, which might hamper the analysis of the ITC data, has been shown to occur, albeit under different experimental conditions, and to result in anomalous ellipticities (*40*).

Salt-Dependent Effects of Neutral Polymers on DNA Thermal Stability. Living systems adapt to large changes in the amounts of intracellular cations (*27*). The finding that nucleic acid−protein interactions are extremely sensitive to changes in the ionic strength when conducted in vitro, but almost unaffected by such changes when occurring in the cytoplasm, has been interpreted in terms of crowding-mediated compensation (*11*, *27*). A large sensitivity to ionic strength effects is well-known to characterize in vitro DNA−DNA interactions. Due to their large axial charge density, the stability of duplexes, and even more so of triple-stranded DNA molecules, substantially decreases as the salt concentration is reduced (*39*, *41*). The effects exerted by changes in the ionic strength on the thermal stability of both triplex and duplex motifs, as well as the larger sensitivity of the triple-stranded structure to salt concentrations, are clearly demonstrated in Figure 5.

To deepen our understanding of the nature, extent, and significance of the effects exerted by neutral polymers on the thermal stability of DNA structures, we have conducted UV melting experiments over a broad range of salt concentration in the absence and presence of crowding agents. A close inspection of the results presented in Figure 5 reveals several intriguing features. First, whereas both the duplex and the triplex structures are stabilized in the presence of PEG over the entire range of salt concentration studied, the effect of the neutral polymer on the triple-stranded DNA conformation is substantially larger, consistent with earlier reports (*31*, *32*). Second, for both double- and triple-stranded DNA segments, the stabilizing influence of PEG becomes significantly more pronounced as the ionic strength is decreased. Indeed, the stabilizing effect of PEG on the duplex motif at the two largest salt concentrations is negligible and constant (Figure 5c). Thus, PEG-mediated stabilization is found to be more effective under salt conditions that are less favorable for the formation of double- and triple-stranded DNA structures.

UV melting experiments were conducted also in the presence of 15% Dextran T-70. The effects exerted by this neutral polymer at various salt concentrations are qualitatively similar to those exerted by PEG but significantly smaller. Specifically, the increase in the melting temperature of the DNA molecules induced by Dextran T-70 is approximately one-third of the value revealed by PEG for a given ionic strength (results not shown).

DISCUSSION

The thermal stability of long double- and triple-stranded DNA molecules was previously shown to be enhanced in the presence of neutral polymeric cosolutes (*30*, *31*). The observations reported here, which indicate that neutral polymers substantially affect the formation and thermal stability of short DNA motifs of defined length and composition, allow for deeper insight into the nature of the effects exerted by these cosolutes.

While our results confirm theoretical considerations and previous findings according to which chemically inert volume-excluding polymers promote association between macromolecules and enhance the stability of the resulting complexes, they highlight the nonuniform nature of these effects. Specifically, triple-stranded DNA structures were shown to be stabilized in the presence of PEG to a higher extent than double-stranded molecules (*31*, *32*). We find that while this PEG-mediated preferential stabilization also characterizes short DNA segments, its magnitude depends on environmental conditions. Thus, whereas the binding constants characterizing triplex formation are larger in the presence of PEG over the whole range of studied temperatures, the polymer-mediated increase in K_a becomes larger as the temperature is increased, with a $K_{a,PEG}/K_a$ ratio of 4.5 at 20 °C and 11.5 at 35 °C (Table 7). This differential effect is clearly demonstrated in Figure 3b, where the free energy change of triplex formation is plotted as a function of temperature in the absence and presence of PEG. The extent of polymer-induced stabilization also depends on the ionic strength. As shown in Figure 5, the increase in the thermal stability of both triplex and duplex motifs in the presence of PEG becomes more pronounced as the ionic strength is reduced.

The effects exerted by neutral polymers thus appear to be specifically pronounced when the intrinsic stability of the resulting complex is low. Thus, the less stable triple-stranded DNA motifs are stabilized by the inert polymers to a higher

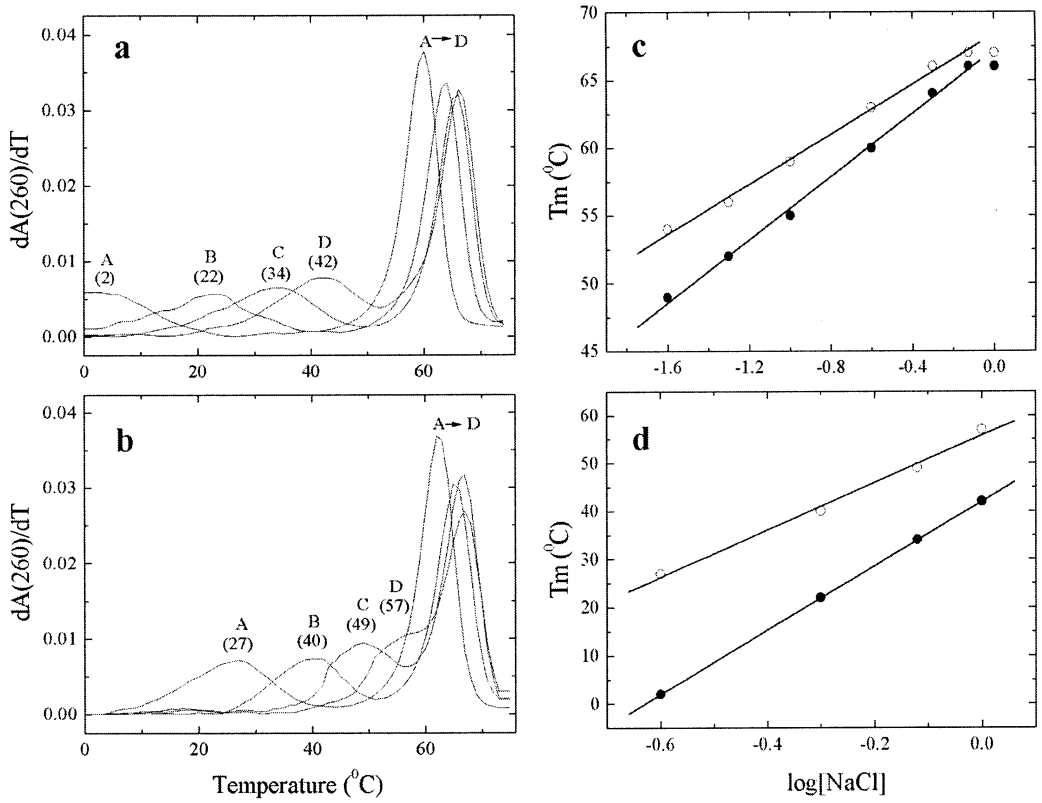

FIGURE 5: Derivative UV (A_{260}) melting curves of the triple-stranded DNA T_{18}*A_{20}·T_{20} in a solution composed of 20 mM PIPES, in the absence (a) and presence (b) of 15% PEG, at the following NaCl concentrations: (A) 0.25 M, (B) 0.5 M, (C) 0.75 M, and (D) 1.0 M. (c) T_m dependence of the duplex and (d) of the triplex motifs on the logarithm of salt concentration with (○) and without (●) PEG. (The measurements of the duplex T_m were extended to 0.025 M.) The slopes for duplex melting in the absence and presence of PEG are 11.7 ± 0.3 and 9.2 ± 0.3, respectively. For triplex melting, the slopes in the absence and presence of PEG are 67 ± 1 and 49 ± 3, respectively. DNA concentration was 1.6 μM.

extent than double-stranded structures. Similarly, the formation and stability of the various DNA structures are selectively enhanced by PEG at unfavorable salt and temperature conditions. Notably, under conditions that result in optimal binding, crowding-mediated effects are relatively small or negligible. This is clearly indicated by the observation that when the ionic strength is raised to a value above which no further stabilization of duplex DNA is achieved, no PEG-induced stabilization is discernible (Figure 5c). Close inspection of previous reports reveals that neutral polymers exert preferential effects not only on DNA−DNA interactions but also on DNA−protein binding. For example, PEG-mediated effects on nick-translation activity of *Escherichia coli* DNA polymerase I (Pol I), ascribed to polymer-induced stabilization of the DNA−Pol I complex, are small at salt concentrations that are optimal for the formation of the complex but become considerable at nonoptimal ionic strength (*22*, *23*).

We therefore conclude that metabolic buffering exerted by macromolecular crowding is not a consequence of a nonspecific shift of equilibria toward binding but rather a differential phenomenon. This distinction is of profound physiological significance because, as previously noted, a large and general increase of macromolecular association brought about by intracellular crowding may lead to irreversible binding between biopolymers (*42*).

While solving one physiological problem, crowding-mediated effects, which appear to preferentially enhance the stability of a priori unstable complexes, could lead to another predicament. DNA structures containing mismatched bases are significantly less stable than native structures. A preferential crowding-induced stabilization of these motifs may hamper repair processes because it would decrease the energy gap between native and mismatched structures. The results presented in Tables 2−4 indicate that whereas crowding agents promote binding and enhance thermal stability of both native and mismatched DNA structures, the effect is not differential. Thus, melting temperatures of double- and triple-stranded DNA structures containing mismatched bases are raised by crowding to the same extent as the corresponding native motifs. Similarly, the ratio $K_{a,PEG}/K_a$ for the formation of the duplex A_{18}·T_{17}I that contains the mismatched base pair A·I is identical to that characterizing the formation of the native A_{18}·T_{18} motif (Table 3). Consequently, the energy gap between native and mismatched structures is not affected in crowded environments.

To assess the origin of the observed preferential effects, we performed calorimetric studies of the processes leading to the formation of the various DNA structures in the absence and presence of PEG. As indicated in Figures 1c and 3, and in Tables 3 and 7, formation of these species in the presence of PEG is promoted by an increase of favorable enthalpic contribution that slightly but consistently exceeds an unfavorable entropy change. The data presented in Figure 3 and Table 7 indicate that the enhanced effect of PEG at higher temperatures derives from a more complete enthalpy−entropy compensation than that occurring in the absence of the neutral polymer. Specifically, the increase in unfavorable entropy contributions to triplex formation that accompanies

the increase in temperature is more effectively compensated in the presence of PEG by favorable enthalpy contributions.

The predominance of enthalpic contributions of PEG-mediated influence on the formation of DNA structures, as well as the preferential effects exerted by the polymer, may imply the presence of direct, chemical interactions between PEG and DNA. The existence of an attractive interaction between this polymer and proteins has indeed been proposed (*5*). We claim that while a completely nonspecific approach to the analysis of PEG−protein interactions may be inadequate, this is unlikely to be the case for DNA molecules. First, we find that although the effects exerted by Dextran T-70 on the formation and thermal stability of DNA species are smaller than those of PEG, the effects of these two neutral polymers are qualitatively similar (see also ref *38*). Second, the monomer EG does not alter the stability of triplex motifs, in contrast to the substantial effects exerted by the polymeric derivatives of EG (Table 5). This finding indicates that PEG-mediated effects do not result from specific, chemical interactions between DNA and the neutral polymer, because such interactions would be exerted by EG as well. Third, high-resolution X-ray studies of DNA crystals obtained in the presence of PEG demonstrate that the polymer is always completely excluded from the crystalline DNA phase. Finally, direct DNA−PEG contacts are thermodynamically unfavorable (*43*), resulting in DNA phase separation from PEG solutions (*44*). Thus, if the effects exerted by PEG and by other chemically inert polymers on DNA are nonspecific as indeed recently demonstrated (*45*), how can the preferential influence of these polymers, as well as the presence of enthalpic contributions, be accounted for?

Neutral, chemically inert cosolutes can exert their effects by excluding volume as well as by decreasing water activity (*12*, *32*). When the effects of the various cosolutes on the thermal stability of DNA structures (Table 5) are examined in the context of water activity (Table 6), a clear correlation is detected. Small cosolutes that substantially reduce water activity destabilize double-stranded DNA structures and either do not affect (EG) or moderately stabilize (PEG 200) triple-stranded DNA motifs. These findings, which are consistent with earlier observations (*12*, *32*), indicate that small cosolutes, whose effects on excluded volume are negligible, act by decreasing water activity. In contrast, the effect of large cosolutes on water activity is small, implying that these cosolutes promote the formation and thermal stability of DNA motifs predominantly by excluding volume. This conclusion is supported by the observation that the presence of 4.5% EG does not affect the melting temperature of either the duplex or the triplex motifs (Table 5). Because the attenuation of water activity produced by 4.5% EG is identical to that produced by 15% PEG (Table 6), this observation indicates that the polymers act as crowding agents.

It is generally accepted that the effects of excluded volume are strictly steric, promoting processes that lead to compact morphologies (*4*−*6*, *9*, *18*). Yet, simple geometric models imply that melting of double helix DNA involves dissociation of a thin cylinder into two bulky single-stranded coils, whereas melting of a triple helix results in a double helix of comparable diameter and one bulky coil. Following these models, crowding should have larger promoting effects on the formation and thermal stability of double-stranded than of triple-stranded structures, in contrast to findings presented here and in previous reports (*31*, *32*). We claim that this apparent discrepancy is solved when the hydrated diameters of the various DNA components involved in the interactions are considered.

Single-, double-, and triple-stranded DNA species are differentially hydrated (*46*). Formation of double-stranded DNA motifs is accompanied by a significant decrease of the hydrated volume (*47*). This decrease is assigned to base stacking which reduces the nonpolar regions around which water molecules form structured networks, as well as to a larger charge density that leads to enhanced electrostriction of water molecules and hence smaller volume. The same factors lead to a further decrease in the hydrated volume upon formation of the triple-stranded poly(dT)*poly(dA)•poly(dT) from the corresponding duplex (*48*). We thus propose that the more compact *hydrated* volume of the triplex motif is responsible for the observed larger effects of crowding on the formation and stabilization of this structure.

We further suggest that the favorable enthalpic contribution of PEG to macromolecular interactions and the preferential buffering effects exerted by the neutral polymer on these interactions are consistent with the crucial role of structured water molecules in these interactions. PEG has been shown to effectively induce DNA phase separation (*44*). Within the crowded DNA-rich phase obtained in the presence of PEG, a reconfiguration of DNA hydration is likely to occur due to the close approach imposed upon DNA molecules (*49*, *50*). Such a close approach has been demonstrated for long DNA molecules (*49*) and was recently shown to characterize short DNA segments as well (*40*). Moreover, a significant difference in PEG-induced DNA assembly and ordering between short double- and triple-stranded DNA motifs has been reported and ascribed to the different charge densities of these two DNA structures (*40*). These observations, which indicate a more condensed and ordered packaging of triple-stranded motifs than of double-stranded structures in the presence of PEG, imply that PEG may *differentially* affect the hydration of DNA structures. A PEG-mediated restructuring of bound water molecules that accompanies DNA association is likely to contribute to the observed enthalpy change of the process.

The proposed correlation between polymer-induced DNA phase separation into dense DNA-rich phases and DNA hydration properties allows for an interpretation of the buffering effects exerted by neutral polymers on DNA−DNA interactions. It has been previously shown that, due to the release of structured water, DNA molecules within DNA-rich phases move closer together as the temperature is raised (*51*, *52*). Our observations, which indicate a progressively larger stabilizing effect of PEG as the temperature is increased (Figure 3, Table 7), may be interpreted in terms of an enhanced restructuring of bound water molecules that occurs in crowded DNA phases at elevated temperatures and results in smaller hydrated DNA volume. A direct link between alteration in DNA hydration and PEG-mediated buffering effect at high temperature is thus pointed out.

An additional point that can be interpreted by the effects of PEG on DNA hydration through phase separation concerns the finding that whereas the polymer compensates for unfavorable environmental conditions that destabilize DNA complexes, no preferential influence occurs when these

complexes are destabilized by the presence of base pair mismatches. Formation of dense DNA-rich phases alters the outer hydration layers of DNA motifs but negligibly affects the inner water layers within DNA grooves. While substitution of a thymine residue with inosine or guanine bases modulates the structure of water layers in the grooves, it is unlikely to significantly affect the outer hydration layers. Since PEG-mediated differential effects are assigned to alterations of the outer hydration layers, base substitution, and therefore mismatches, should not be *preferentially* stabilized by crowding agents, as indeed observed. Thus, the energy gap between native base pairs and mismatches is not altered by crowding, and hence repair processes should not be affected. Finally, the particular effectiveness of PEG as a crowding agent can be directly assigned to the larger ability of globular PEG molecules (*6*, *12*) to promote DNA phase separation than that of rodlike polymers such as dextran (*10*, *53*). Thus, while PEG and dextran exert qualitatively similar effects on macromolecular associations, the influence of PEG is more pronounced.

CONCLUSIONS AND PERSPECTIVES

Volume exclusion is an omnipresent determinant in cellular environments that, as a general rule, acts to substantially promote processes which result in compaction. This study highlights three physiologically significant issues concerned with crowded environments: the preferential buffering capacity of excluded volume effects, the crucial role of hydration in dictating these effects, and the causal correlation between crowding and phase separation.

The notion that alterations in environmental conditions can be effectively compensated by crowding agents, which thus provide a mechanism for adaptation, has been previously raised (*6*, *27*). Such buffering against unfavorable conditions, e.g., in ionic strength or temperature, was ascribed to a general crowding-mediated increase in association constants of macromolecules. It has, however, been pointed out that such a large and general increase may lead to irreversible macromolecular association (*42*). The observations presented here indicate that crowding exerts small or negligible effects on macromolecular interactions occurring under salt and temperature conditions that are optimal for the particular interaction but become progressively larger as these conditions deteriorate. As such, crowding represents a "genuine" buffering factor.

It has been proposed that the extreme crowding encountered in cellular environments necessarily results in intracellular phase separation and microcompartmentation (*54*). The notion presented here, that preferential effects of crowding on macromolecular associations and stability are mediated by phase separation that leads to DNA-rich phases in which the structure of bound water molecules is altered due to close approach, is therefore physiologically relevant. Indeed, highly condensed and ordered DNA phases were detected in living cells (*55*, *56*), and their features were shown to be dictated by hydration (*57*). In this aspect, PEG, which promotes phase separation to a higher extent than other inert polymers due to its spherical conformation, represents a particularly appropriate agent for mimicking physiological crowding. Moreover, our premise that highlights the role of structured hydration provides an interpretation to the finding that intrinsic alterations such as base mismatches, which destabilize DNA assemblies but do not affect the properties of outer hydration layers, are not preferentially affected by crowding.

Finally, the results presented in this study highlight the assertion (*9*, *20*) that the effects of excluded volume should not be neglected when interactions between biomacromolecules are investigated.

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