# Thermodynamic Aspects of Triplex DNA Formation in Crowded Environments

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*Received July 10, 2001*

Biochemical studies are regularly conducted in highly dilute solutions, whereas the intracellular milieu is extremely crowded.[1] This difference is significant since thermodynamic activities of biopolymers are substantially larger in crowded media than in thermodynamically "ideal" solutions,[2] resulting in enhanced rates and binding constants of macromolecular interactions.[3] Here, we present the first calorimetric study of crowding-mediated effects on the thermodynamic parameters of biopolymer interactions. Spink and Chaires have shown that the stability of triple-stranded DNA structures is enhanced in crowded media.[4] We find that the crowding agent PEG[5] compensates for triplex-destabilization caused by non-native bases. Isothermal titration calorimetry (ITC) experiments indicate that triplex stabilization is effected through a favorable contribution of the crowding agent to the enthalpy of triplex formation. This observation, which apparently contradicts the notion that crowding effects are predominantly entropic, provides new insight into the thermodynamics of macromolecular interactions in crowded environments.

Derivative melting curves obtained from UV melting experiments of the preformed triplexes $T_{18}$*$(AT)_{20}$, $T_{17}I$*$(AT)_{20}$, and $T_{16}I_2$*$(AT)_{20}$ (Table 1) reveal low- and high-temperature transitions that are assigned to triplex and duplex melting, respectively (Figure 1a). As reported previously, substitution of a single thymine for inosine markedly decreases triplex melting temperature, and replacement of two thymines with inosines results in a highly unstable triple-stranded structure.[6] On the basis of these effects, it was concluded that inosines represent a poor substitute for native pyrimidines in TFOs. Results presented in Figure 1b indicate, however, that in the presence of 15% (w/v) PEG, triplex motifs composed of native bases, as well as those containing inosines, are stabilized by 15−16 °C. Melting experiments in which 5% and 10% PEG were included indicate that triplex stability increases linearly with PEG concentration. A smaller stabilizing effect, amounting to 5−6 °C for all triplex structures, is obtained in the presence of 15% Dextran T-70 (results not shown). Notably, whereas PEG substantially enhances triplex stability, its effect on the thermal properties of the duplex form is negligible, as indeed was previously shown.[4]

The thermodynamic parameters characterizing triplex formation were derived from ITC measurements in which $(AT)_{20}$ was titrated with the oligonucleotides $T_{18}$, $T_{17}I$, and $T_{16}I_2$ in the absence and presence of PEG. Titration curves (Figure 2) reach half-saturation near the equimolar ratio, indicating a one-to-one binding in all cases. The equilibrium association constant for the interaction of $(AT)_{20}$ with a TFO in which a single thymine was replaced with inosine ($T_{17}I$) is 33-fold smaller than $K_a$ exhibited by the interaction between $(AT)_{20}$ and $T_{18}$ (Table 2). This drop is assigned to inosine-related nonoptimal Hoogsteen interactions between the TFO and the duplex.[6] In the presence of 15% PEG, $K_a$ for triplex formation are significantly larger than the corresponding values obtained in noncrowded solutions, or in the presence of Dextran (Table 2). PEG's effects are particularly evident for the interaction of $T_{16}I_2$ with $(AT)_{20}$, allowing for triplex formation under conditions that are refractory to the process in a noncrowded environment.

These results raise three intriguing questions: The first is why is the thermal stability of duplex DNA practically unaffected by neutral polymers, in contrast to the triplex motifs? The second issue involves the different effects exerted by the two neutral polymers, and the third concerns the thermodynamic origin of

**Table 1.** List of DNA Species

| duplex DNA | name |
|---|---|
| 5′-$A_{20}$CGC-3′<br>3′-$T_{20}$GCG-5′ | $(AT)_{20}$ |
| **TFOs** | **name** |
| 5′-$T_{18}$-3′ | $T_{18}$ |
| 5′-$T_5IT_{12}$-3′ | $T_{17}I$ |
| 5′-$T_5IT_6IT_5$-3′ | $T_{16}I_2$ |

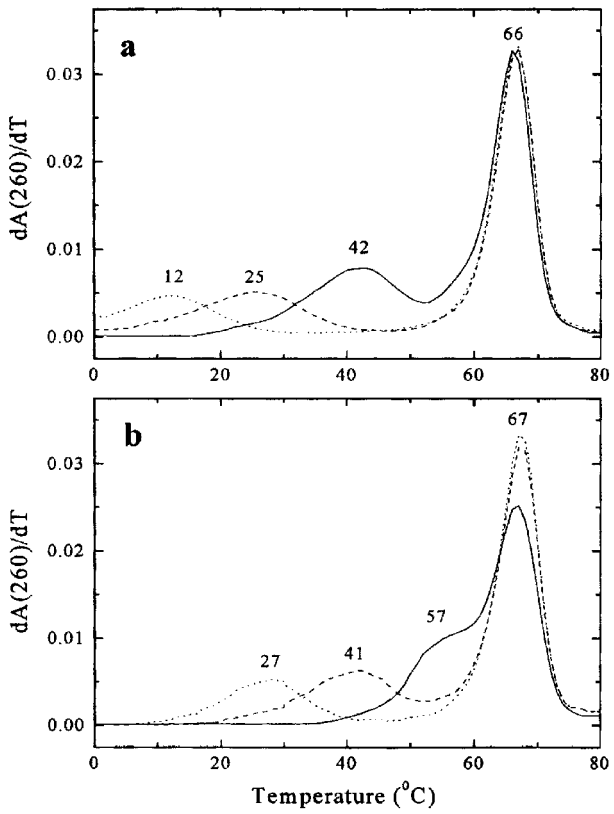


**Figure 1.** Derivative UV ($A_{260}$) melting curves of triple-stranded structures formed between $(AT)_{20}$ and $T_{18}$ (solid line), $T_{17}I$ (dashed line), and $T_{16}I_2$ (dotted line) in a solution composed of 20 mM PIPES and 1.0 M NaCl in the absence and presence of 15% PEG (panels a and b, respectively). The DNA concentration was 1.6 $\mu$M.

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(3) (a) Zimmerman, S. B.; Harrison, B. *Proc. Natl. Acad. Sci. U.S.A.* **1987**, *84*, 1871−1875. (b) Jarvis, T. C.; Ring, D. M.; Daube, S. S.; von Hippel. P. H. *J. Biol. Chem.* **1990**, *265*, 15160−15167. (c) Cayley, S.; Lewis, B. A.; Guttman, H. J.; Record, M. T. *J. Mol. Biol.* **1991**, *222*, 281−300. (d) Record, M. T.; Courtenay, E. S.; Cayley, S.; Guttman, H. J. *Trends Biochem. Sci.* **1998**, *23*, 190−194.
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(5) Abbreviations: I, inosine; ITC, isothermal titration calorimetry; TFO, triplex forming oligonucleotide; $K_a$, equilibrium association constant; PEG, poly(ethylene glycol) (8000).
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10.1021/ja016577d CCC: $20.00 
Published on Web 11/20/2001

**Table 2.** ITC Data for $(AT)_{20}$ Interaction with TFOs in the Absence and Presence of 15% Co-solutes[a]

| TFO | co-solute | $K_a$ ($M^{-1}$) | $\Delta G$ (kcal/mol) | $\Delta H$ (kcal/mol) | $T\Delta S$ (kcal/mol) |
|---|---|---|---|---|---|
| $T_{18}$ | | $(5.82 \pm 0.81) \times 10^7$ | $-10.42 \pm 0.09$ | $-58.2 \pm 0.6$ | $-47.8 \pm 0.6$ |
| | PEG | $(3.50 \pm 0.73) \times 10^8$ | $-11.45 \pm 0.12$ | $-72.6 \pm 0.7$ | $-61.1 \pm 0.6$ |
| | Dextran T-70 | $(8.90 \pm 1.50) \times 10^7$ | $-10.67 \pm 0.10$ | $-59.6 \pm 0.6$ | $-48.9 \pm 0.5$ |
| $T_{17}I$ | | $(1.76 \pm 0.36) \times 10^6$ | $-8.38 \pm 0.12$ | $-56.0 \pm 2.7$ | $-47.6 \pm 2.5$ |
| | PEG | $(3.87 \pm 0.35) \times 10^7$ | $-10.18 \pm 0.05$ | $-72.2 \pm 0.6$ | $-62.0 \pm 0.6$ |
| $T_{16}I_2$ | | n.d. | n.d. | n.d. | n.d. |
| | PEG | $(3.35 \pm 0.31) \times 10^6$ | $-8.76 \pm 0.05$ | $-71.2 \pm 1.5$ | $-62.4 \pm 1.4$ |

[a] n.d.: not determined. Conditions are as specified in the caption of Figure 2.

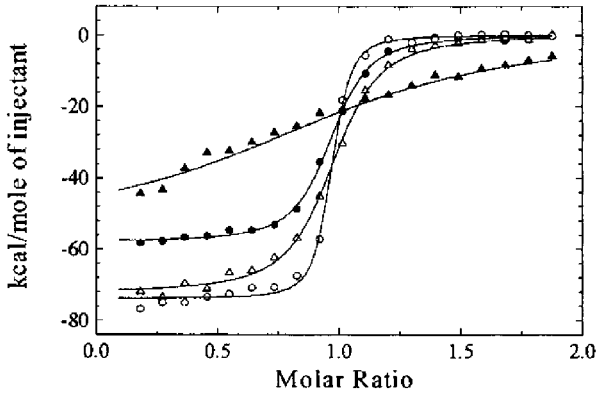


**Figure 2.** ITC profile of the titration of $(AT)_{20}$ with either $T_{18}$ (circles) or $T_{17}I$ (triangle) performed on a MCS-ITC system (MicroCal Inc.) at 20 °C in a solution composed of 20 mM PIPES and 1.0 M NaCl in the absence (●, ▲) and presence (○, △) of 15% PEG (purified by extensive dialysis against water) both in the ITC syringe and cell. Each point corresponds to a 5-$\mu$L injection of 60 $\mu$M TFO solution into the cell containing 2.5 $\mu$M $(AT)_{20}$. Titration curves were corrected for heat of dilution (obtained separately by injecting the oligonucleotide into the buffer in the absence and presence of PEG) and presented as a function of TFO-to-duplex molar ratio. Data analysis was as described in ref 7.

these effects. The concept of macromolecular crowding is based on the notion that high-volume occupancy of polymers reduces the space available for macromolecules. Association processes in crowded media are promoted because they increase the available volume and hence the entropy of the system.[2] The results presented in Table 2 appear to be inconsistent with this notion, as they indicate that PEG decreases the entropy of triplex formation, and that polymer-dependent triplex stabilization is entirely enthalpic. A simple explanation, according to which PEG exerts its effect through direct interactions with DNA, is, however, at odds with the observations that the DNA phase separates from PEG solutions,[8a] and that direct DNA-PEG interactions are thermodynamically unfavorable.[8b]

The issue concerning the predominance of the enthalpic contribution to triplex formation in aqueous-PEG solutions is being currently investigated, and only a tentative interpretation can be provided at this stage. PEG has been shown to act as a potent osmotic stressing agent,[9] to effectively induce DNA phase separation,[8a] and to exert volume exclusion.[2] Indeed, these very properties are exploited for PEG-mediated DNA purification and precipitation purposes. Within the crowded and osmotically stressed DNA-rich phase obtained in the presence of PEG, a reconfiguration of DNA water layers is likely to occur due to alteration of water activity and the close approach imposed upon DNA molecules.[4b,9a,10] This reconfigutration is significant, since the duplex and triplex DNA motifs are hydrated to a different extent; specifically, the triple-stranded structure appears to contain a smaller number of uniquely bound water molecules than the double-stranded motif.[4b] Thus, agents that modulate DNA hydration will *differentially* affect the thermal stability, as well as the reactivity and hence the enthalpy of formation, of the duplex and triplex structures. Notably, the rodlike conformation of Dextran is significantly less effective in producing DNA phase separation and exerting volume exclusion than the globular PEG molecules,[8a,2c] thus providing an interpretation to the different effects exerted by the two polymers.

The current study, which represents a preliminary attempt to dissect the thermodynamic consequences of volume exclusion, highlights the significance of alteration of macromolecular hydration in crowded environments, and implies that the consideration of excluded volume as a wholly entropic phenomenon might be an oversimplification. While the premises discussed here are being further investigated, the results clearly demonstrate that crowding conditions akin to those encountered in cells effectively mitigate triplex destabilization associated with non-native bases, thus extending the range of TFOs that can be used for gene therapy.

**Acknowledgment.** This work was supported by the Israel Science Foundation and by the Minerva Foundation, Germany.

JA016577D

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